FDA Briefing Document

Pharmacy Compounding Advisory Committee (PCAC) Meeting

June 8, 2022

The attached package contains background information prepared by the Food and Drug Administration (FDA) for the panel members of the Pharmacy Compounding Advisory Committee (advisory committee). We are bringing certain compounding issues to this advisory committee to obtain the committee's advice. The background package may not include all issues relevant to the final regulatory recommendation and instead is intended to focus on issues identified by the Agency for discussion by the advisory committee. The FDA background package often contains assessments and/or conclusions and recommendations written by individual FDA reviewers. Such conclusions and recommendations do not necessarily represent the final position of the individual reviewers, nor do they necessarily represent the final position of the Review Division, Office, or Agency.

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I. Introduction

Section 503A of the Federal Food, Drug, and Cosmetic Act (FD&C Act) describes the conditions that must be satisfied for human drug products compounded by a licensed pharmacist in a State-licensed pharmacy or Federal facility, or by a licensed physician, to be exempt from the following three sections of the FD&C Act: section 505 (concerning the approval of drugs under new drug applications (NDAs) or abbreviated new drug applications (ANDAs)); section 502(f)(1) (concerning the labeling of drugs with adequate directions for use); and section 501(a)(2)(B) (concerning current good manufacturing practice (CGMP) requirements).

A. Bulk Drug Substances That Can Be Used by Compounders under Section 503A

One of the conditions that must be met for a compounded drug product to qualify for the exemptions in section 503A of the FD&C Act is that a licensed pharmacist or licensed physician compounds the drug product using bulk drug substances that meet one of the following criteria:

> (1) Comply with the standards of an applicable United States Pharmacopeia (USP) or National Formulary (NF) monograph, if a monograph exists, and the USP chapter on pharmacy compounding;
> (2) If such a monograph does not exist, are drug substances that are components of drugs approved by the Secretary; or
> (3) If such a monograph does not exist and the drug substances are not components of drugs approved by the Secretary, appear on a list developed by the Secretary through regulations issued by the Secretary under subsection (c) of section 503A.

(*See* section 503A(b)(1)(A)(i) of the FD&C Act.)

FDA is considering those substances nominated for inclusion on the list of bulk drug substances that can be used to compound drug products under section 503A of the FD&C Act (503A Bulks List). In the *Federal Register* of February 2019, FDA published notice of a final rule establishing the criteria for evaluation of bulk drug substances for inclusion on the 503A Bulks List (84 FR 4696):

(1) The physical and chemical characterization of the substance;

(2) Any safety issues raised by the use of the substance in compounded drug products;

(3) The available evidence of effectiveness or lack of effectiveness of a drug product compounded with the substance, if any such evidence exists; and

(4) Historical use of the substance in compounded drug products, including information about the medical condition(s) the substance has been used to treat and any references in peer-reviewed medical literature.

In evaluating the candidates for the 503A Bulks List under these criteria, the Agency will use a balancing test. Specifically, the Agency will consider each criterion in the context of the others and to balance them, on a substance-by-substance basis, to decide whether a particular substance is appropriate for inclusion on the list.

B. Withdrawn or Removed List

1. Process for Identifying Candidates for or Amendments to the Withdrawn or Removed List

Under sections 503A and 503B of the FD&C Act, FDA is to develop a list of drugs that have been withdrawn or removed from the market because they have been found to be unsafe or not effective (the Withdrawn or Removed List (codified at 21 CFR § 216.24)).

The following outlines the process that has been and will be used in the future to identify new candidates for this list, or to identify proposed amendments to the list, such as removing an entry or amending an entry on the list to qualify it in some way because the drug has been shown to be safe and effective for some use.

FDA stated in a final rule published in the October 7, 2016, *Federal Register* that FDA intends to continue updating the Withdrawn or Removed List through notice and rulemaking (see 81 FR 69668).

Process for Identifying Candidates For or Amendments to the List:

- FDA periodically reviews available information to identify and compile a list of possible new candidate drugs that have been withdrawn or removed from the market because they have been found to be unsafe or not effective. The information may include, for example, *Federal Register* notices announcing withdrawal of approval of a drug application for safety or effectiveness reasons, *Federal Register* notices

announcing an Agency determination that a drug product was removed from sale for reasons of safety or effectiveness, relevant FDA Alerts, FDA Drug Safety Communications, FDA News Releases, Public Health Advisories, Dear Healthcare Practitioner Letters, Citizen Petitions, and Sponsor letters.

- In addition, periodically, FDA reviews available information to determine whether any new drug applications have been approved for a drug product containing as an active ingredient any of the drugs on the list to determine whether any of the drug entries on this list should be modified to account for this new safety and effectiveness determination and approval. For example, if a drug has been approved in a new formulation, indication, route of administration or dosage form since the list was last revised, FDA might consider proposing a modification to the list to remove the drug from the list or to exclude the particular formulation, indication, route of administration or dosage form.

- Appropriate divisions within the Office of New Drugs (OND) then evaluate each identified candidate or proposed modification using the available information about the drug and prepare a review of the information that documents their recommendations as to whether to include the drugs on the Withdrawn or Removed List or remove a drug or modify an entry.

- We intend to propose regulations to revise the Withdrawn or Removed List periodically, as appropriate, as we identify drugs that we tentatively determine should be listed. We would also propose regulations when we tentatively determine that changes to the status of drug products already on the list should result in a revision to their listing, for example, if some version of a drug on the list has been approved for marketing.

2. Drugs Under Evaluation for the Withdrawn or Removed List

The Agency is considering “Lorcaserin hydrochloride: All drug products containing lorcaserin hydrochloride” for inclusion on the Withdrawn or Removed List. See Tab 5 for the background material that forms the basis for FDA’s proposal to include this drug on the list.

II. Substances Nominated for Inclusion on the 503A Bulks List (in order of discussion at the meeting)

A. Enclomiphene Citrate (Tab 1)

1. Nominations (**Tab 1a**)
   a. Empower Pharmacy

2. FDA Evaluation (**Tab 1b**)

B. Glutathione (Tab 2)

1. Nominations (**Tab 2a**)
   a. Alliance for Natural Health USA
   b. American Association of Naturopathic Physicians
   c. American College for Advancement in Medicine
   d. Integrative Medical Consortium
   e. McGuff Compounding Services, Inc.
   f. National Community Pharmacists Association
   g. Professional Compounding Centers of America

2. Nomination Clarification (**Tab 2b**)
   a. Alliance for Natural Health USA
   b. American Association of Naturopathic Physicians
   c. Integrative Medical Consortium
   d. McGuff Compounding Services, Inc.
   e. National Community Pharmacists Association
   f. Professional Compounding Centers of America

3. FDA Evaluation (**Tab 2c**)

C. Ammonium Tetrathiomolybdate (Tab 3)

1. Nominations (**Tab 3a**)
   a. Pharmacy Solutions

2. FDA Evaluation (**Tab 3b**)

D. Ferric Subsulfate (Tab 4)

1. Nominations (**Tab 4a**)
   a. Fagron
   b. International Academy of Compounding Pharmacists

2. FDA Evaluation (**Tab 4b**)

III. Lorcaserin Hydrochloride - Drug Considered for the Withdrawn or Removed List

A. Lorcaserin Hydrochloride (Tab 5)

1. FDA Evaluation (**Tab 5a**)

IV. Points to Consider

A. June 8, 2021, a.m. session

Points for the PCAC to Consider Regarding Whether to Include Certain Bulk Drug Substances on the 503A Bulks List

1. FDA is proposing that enclomiphene citrate NOT be included on the 503A Bulks List. Should enclomiphene citrate be placed on the list?

2. FDA is proposing that glutathione NOT be included on the 503A Bulks List. Should glutathione be placed on the list?

B. June 8, 2022, p.m. session

Points for the PCAC to Consider Regarding Whether to Include Certain Bulk Drug Substances on the 503A Bulks List

1. FDA is proposing that ammonium tetrathiomolybdate NOT be included on the 503A Bulks List. Should ammonium tetrathiomolybdate be placed on the list?

2. FDA is proposing that ferric subsulfate solid or powder NOT be included on the 503A Bulks List. Should ferric subsulfate solid or powder be placed on the list?

Points for the PCAC to Consider Regarding Whether to Include Certain Bulk Drug Substances on the Withdrawn and Removed List

3. FDA is proposing that "Lorcaserin hydrochloride: All drug products containing lorcaserin hydrochloride" be ADDED to the Withdrawn or Removed List under sections 503A and 503B of the FD&C Act. Do you agree?

Tab 1

Enclomiphene Citrate

Tab 1a

Enclomiphene Citrate Nominations

Division of Dockets Management (HFA-305)
Food and Drug Administration
Department of Health and Human Services
5630 Fishers Lane
Rm. 1061
Rockville, MD 20852

Re: Docket FDA-2015-N-3534

"Bulk Drug Substances That Can Be Used To Compound Drug Products in Accordance With Section 503A of the Federal Food, Drug, and Cosmetic Act"

Dear Sir or Madam,

Empower Pharmacy appreciates the opportunity to address the FDA's request for nominations of bulk drug substances that may be used in accordance with section 503A.

We hereby nominate enclomiphine citrate for FDA's consideration as a bulk drug substances that may be used under Section 503A.

This active ingredient does not appear on an FDA-published list of drugs that present demonstrable difficulties for compounding. In addition, it is not a component of a drug product that has been withdrawn or removed from the market because the drug or components of the drug have been found to be unsafe or not effective.

Thank you for your consideration.

Respectfully submitted,

Shaun Noorian
CEO, Empower Pharmacy

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What is the name of the nominated ingredient?	Enclomiphene Citrate
Is the ingredient an active ingredient that meets the definition of "bulk drug substance" in § 207.3(a)(4)?	Yes
Is the ingredient listed in any of the three sections of the Orange Book?	No
Were any monographs for the ingredient found in the USP or NF monographs?	No
What is the chemical name of the substance?	Enclomiphene Citrate
What is the common name of the substance?	Enclomiphene Citrate
Does the substance have a UNII Code?	J303A6U9Y6
What is the chemical grade of the substance?	Non-graded
What is the strength, quality, stability, and purity of the ingredient?	Purity NLT 98% Water content NMT 1% Impurities NMT 2% Z-isomer NMT 0.5% Controlled room temperature storage 2 year expiry
How is the ingredient supplied?	Bulk powder
Is the substance recognized in foreign pharmacopeias or registered in other countries?	No
Has information been submitted about the substance to the USP for consideration of monograph development?	No
What dosage form(s) will be compounded using the bulk drug substance?	Capsules or Tablets
What strength(s) will be compounded from the nominated substance?	12.5 mg, 25 mg, 50 mg
What are the anticipated route(s) of administration of the compounded drug product(s)?	Oral
Are there safety and efficacy data on compounded drugs using the nominated substance?	Kim ED, McCullough A, Kaminetsky J. Oral enclomiphene citrate raises testosterone and preserves sperm counts in obese hypogonadal men, unlike topical testosterone: restoration instead of replacement. BJU Int. 2016 Apr;117(4):677-85. Hill S, Arutchelvam V, Quinton R. Enclomiphene, an estrogen receptor antagonist for the treatment of testosterone deficiency in men. IDrugs. 2009 Feb;12(2):109-19. Wiehle RD, Fontenot GK, Wike J, Hsu K, Nydell J, Lipshultz L; ZA-203 Clinical Study Group. Enclomiphene citrate stimulates testosterone production while preventing oligospermia: a randomized phase II clinical trial comparing topical testosterone. Fertil Steril. 2014 Sep;102(3):720-7. ClinicalTrials.gov. National Library of Medicine (U.S.). (2012, February). Safety Study of Enclomiphene Citrate in the Treatment of Men With Secondary Hypogonadism. Identifier NCT01534208. Retrieved November 19, 2007 from: https://clinicaltrials.gov/ct2/show/NCT01534208

Has the bulk drug substance been used previously to compound drug product(s)?	Yes
What is the proposed use for the drug product(s) to be compounded with the nominated substance?	Enclomiphene Citrate is used to increase serum testosterone, LH and FS to normal levels and maintain sperm concentrations within the normal range. Wiehle R, Cunningham GR, Pitteloud N, et al. Testosterone Restoration by Enclomiphene Citrate in Men with Secondary Hypogonadism: Pharmacodynamics and Pharmacokinetics [published online ahead of print, 2013 Jul 12]. BJU Int. 2013;112(8):1188-1200.
What is the reason for use of a compounded drug product rather than an FDA-approved product?	Enclomiphene has been identified as the isomer in the non-racemic mixture found in Clomiphene Citrate, that raises LH and FSH levels, with a shorter half-life. This suggests that treatment with enclomiphene should have more favorable outcomes in treating androgen deficient men with the goal of maintaining fertility, potentially without the side effects associated with clomiphene citrate use and with a more favorable impact on hypogonadal symptoms. Rodriguez KM, Pastuszak AW, Lipshultz LI. Enclomiphene citrate for the treatment of secondary male hypogonadism. Expert Opin Pharmacother. 2016;17(11):1561-1567. Earl JA, Kim ED. Enclomiphene citrate: A treatment that maintains fertility in men with secondary hypogonadism. Expert Rev Endocrinol Metab. 2019 May;14(3):157-165. Helo S, Mahon J, Ellen J, Wiehle R, Fontenot G, Hsu K, Feustel P, Welliver C, McCullough A. Serum levels of enclomiphene and zuclomiphene in men with hypogonadism on long-term clomiphene citrate treatment. BJU Int. 2017 Jan;119(1):171-176.
Is there any other relevant information?	

Tab 1b

FDA Evaluation of Enclomiphene Citrate

DATE: May 2, 2022

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TO: Pharmacy Compounding Advisory Committee

SUBJECT: Evaluation of Enclomiphene Citrate for Inclusion on the 503A Bulk Drug Substances List

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I. INTRODUCTION

Enclomiphene citrate was nominated for inclusion on the list of bulk drug substances that can be used in compounding under section 503A of the Federal Food, Drug, and Cosmetic Act (FD&C Act).[1] Enclomiphene citrate was evaluated for the following use: to increase serum testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) to normal levels in the treatment of secondary hypogonadism.[2]

Enclomiphene citrate products proposed in the nomination are capsules or tablets for oral administration in 12.5 mg, 25 mg, and 50 mg dosage strengths.

We have evaluated publicly available data on the physicochemical characteristics, safety, effectiveness, and historical use in compounding of this substance. For the reasons discussed below, we believe the evaluation criteria ***weigh against*** placing enclomiphene citrate on the list of bulk drug substances that can be used to compound drug products in accordance with section 503A of the FD&C Act (503A Bulks List).

II. EVALUATION CRITERIA

A. Is the substance well-characterized, physically and chemically, such that it is appropriate for use in compounding?[3]

Databases such as FDA Mercado, PubMed, SciFinder, Google, the European Pharmacopoeia, and USP/NF were searched for information on enclomiphene citrate. The information below summarizes what FDA found in these databases.

Enclomiphene citrate is also called (E)-clomiphene citrate, trans-clomiphene citrate, Androxal, Clomiphene B citrate, Enclomid, and EnCyzix. It has CAS number 7599-79-3, and its chemical name is (E)-2-(4-(2-chloro-1,2-diphenylvinyl)phenoxy)-N,N-diethylethan-1-amine, 2-hydroxypropane-1,2,3-tricarboxylate (1:1). It has the chemical structure as shown in Figure 1 below:

[1] Nomination from Empower Pharmacy (Document ID: FDA-2015-N-3534-0281) can be accessed at https://www.regulations.gov/document/FDA-2015-N-3534-0281.

[2] Enclomiphene citrate was nominated for the use, "…to increase serum testosterone, LH and FS [follicle-stimulating hormone, FSH] to normal levels and maintain sperm concentrations within the normal range." For reasons detailed in Section II.C.1., FDA evaluated it for the use listed above.

[3] Among the conditions that must be met for a drug compounded using bulk drug substances to be eligible for the exemptions in section 503A of the FD&C Act is that the bulk drug substances are manufactured by an establishment that is registered under section 510 of the FD&C Act and that each bulk drug substance is accompanied by a valid certificate of analysis. Sections 503A(b)(1)(A)(ii) and (iii). A bulk drug substance is deemed to be adulterated if the methods used in, or the facilities or controls used for, its manufacture, processing, packing, or holding do not conform to or are not operated or administered in conformity with current good manufacturing practice. Section 501(a)(2)(B).

Figure 1: Chemical Structure of Enclomiphene Citrate[4]

Enclomiphene citrate is a white to off-white or pale-yellow powder and is slightly hygroscopic. It has melting range of about 146-150°C. Enclomiphene citrate is slightly soluble in water and chloroform, freely soluble in methanol, sparingly soluble in alcohol, and practically insoluble in ether.

Currently, there is no compendial monograph for enclomiphene citrate. However, a USP compendial monograph for clomiphene citrate, which is a mixture of trans-clomiphene citrate (enclomiphene citrate) and cis-clomiphene citrate (i.e. zuclomiphene citrate or zuclomifene citrate), is available.[5] Based on the USP monograph, the content of enclomiphene citrate in clomiphene citrate is in the range of about 50% to 70%. Clomiphene citrate is an active ingredient of an approved drug product.[6]

1. *Stability of the API and likely dosage forms*

As per recommended storage conditions in the USP monograph, clomiphene citrate is stable when stored at controlled room temperature and protected from light. Since enclomiphene citrate is one constituent of clomiphene citrate geometric isomeric mixture, it is expected to be stable when stored under the same or similar storage conditions.

2. *Probable routes of API synthesis*

Enclomiphene citrate can be made by separation from the mixture of geometric isomers trans-clomiphene citrate and cis-clomiphene citrate using binaphthyl-phosphoric acid, which is well documented in the US patent US 1,036,212 and is depicted in Figure 2 below. The mixture of two isomers, clomiphene citrate, has a USP monograph and is the active ingredient in an approved drug product.

[4] NIH U.S. National Library of Medicine PubChem. https://pubchem.ncbi.nlm.nih.gov/compound/Enclomiphene-citrate. Accessed Nov 24, 2021.
[5] USP-NF Website. https://www.uspnf.com/. Accessed Nov 24, 2021.
[6] See Orange Book: Clomiphene Citrate, 50 mg tablets, Par Pharmaceutical Inc, approved on August 30, 1999. https://www.accessdata.fda.gov/scripts/cder/ob/index.cfm. Accessed Nov 24, 2021.

Figure 2: Enclomiphene Citrate Chemical Synthesis

Clomiphene Citrate

Enclomiphene Citrate

3. Likely impurities[7]

The impurity profile of enclomiphene citrate is expected to be similar to that of clomiphene citrate as outlined in USP monograph. In addition, residual cis-clomiphene citrate, binaphthyl-phosphoric acid and solvents used in the manufacturing process would be likely impurities in the drug substance enclomiphene citrate.

4. Toxicity of those likely impurities

Impurities are unlikely to be toxic if they are controlled according to relevant ICH guidelines and the USP monograph for clomiphene citrate.

5. Physicochemical characteristics pertinent to product performance, such as particle size and polymorphism

Enclomiphene citrate can be characterized by common analytical tools and techniques. Enclomiphene citrate is slightly soluble in water. Therefore, particle size and polymorphism of the drug substance may be critical for solid oral dosage forms.

6. Any other information about the substance that may be relevant, such as whether the API is poorly characterized or difficult to characterize

None.

[7] This evaluation contains a non-exhaustive list of potential impurities in the bulk drug substance and does not address fully the potential safety concerns associated with those impurities. The compounder should use the information about the impurities identified in the certificate of analysis accompanying the bulk drug substance to evaluate any potential safety and quality issues associated with impurities in a drug product compounded using that bulk drug substance taking into account the amount of the impurity, dose, route of administration, and chronicity of dosing.

Conclusions: In summary, enclomiphene citrate is a well-characterized small molecule, and is expected to be stable under ordinary storage conditions when protected from light in the proposed dosage form.

B. Are there concerns about the safety of the substance for use in compounding?

1. Nonclinical Assessment

The following databases were consulted in preparation of this section: PubMed, PubChem, Embase, SCOPUS Web of Science, ToxNet, GRAS Notices database, NIH dietary supplement database, LactMed, LiverTox, Google, and Google Scholar. The majority of the data described in this section are captured from the publicly available European Medicines Agency (EMA) 2018 report on enclomiphene citrate (referred to in that document as enclomifene) (the EMA 2018 report).[8] The EMA 2018 report reviewed clinical and nonclinical data in support of the "treatment of hypogonadotrophic hypogonadism (secondary hypogonadism) in adult men with a body mass index (BMI) ≥25 kg/m^2 wishing to preserve testicular function and spermatogenesis." The EMA 2018 report did not recommend approval of enclomiphene citrate for the sought treatment. Only data not found in the EMA 2018 report will be referenced.

a. General pharmacology of the drug substance

Enclomiphene citrate is a selective estrogen receptor modulator (SERM) which acts by blocking the estrogenic suppression of the hypothalamic-pituitary-gonadal (HPG) axis. As a result, the pituitary secretes more LH and FSH, and this stimulates the testes to produce more testosterone. In the baboon, administration of enclomiphene resulted in a significant increase in circulating testosterone, suggesting potential utility of enclomiphene citrate for the treatment of secondary hypogonadism (Rodriguez et al. 2016).

The secondary pharmacodynamic (PD) effects of enclomiphene and its isomer, zuclomifene, were evaluated in an ovariectomized mouse model. The uterotrophic response of the treated mice was limited to minor macroscopic effects in both isomer treatment groups (dilation of uterine glands and increase in mean uterine weights).

b. Pharmacokinetics (PK)/Toxicokinetics (TK)

In a single dose PK study in mice, absorption of enclomiphene was rapid. The time to reach maximum levels (T_{max}) after a single oral dose of 40 mg/kg, was 1 hour (data not shown). Similar data were obtained in the 26-week rat study where absorption of enclomiphene citrate was rapid, and the plasma levels of enclomiphene (C_{max} and AUC_{0-24}) increased with increasing doses on all sampling days (days 1, 14, 90, and 180). The maximum exposure to enclomiphene (C_{max}) on Day 1 was greater than proportional compared to the administered dose but became proportional in the mid and high-dose groups on Days 14, 90, and 180 (Table 1).

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[8] The 2018 EMA Assessment Report is available to the public at: https://www.ema.europa.eu/en/documents/assessment-report/encyzix-epar-public-assessment-report_en.pdf. Accessed Apr 18, 2022.

Table 1: Pharmacokinetics of Enclomiphene in Rats Administered Enclomiphene Citrate Orally for 6 Months (EMA 2018)

Dose (mg/kg/day)	Day	C_{max} (ng/mL)	T_{max} (hr)	AUC_{0-24} (ng•hr/mL)	r2	$t_{1/2}$ (hr)
0.5	1	5.82	1	28.2		
	14	22.1	4	85.7		
	90	10.8	1	46.1	0.9217	5.9
	180	9.31	2	48.1		
5	1	42.6	1	307	0.9978	3.2
	14	136	1	694	0.9548	2.9
	90	157	1	1,046	0.9239	3.7
	180	165	2	999	0.9779	4.5
10	1	133	1	726	0.9825	3.1
	14	270	1	1,931	0.9991	3.2
	90	259	4	2,496	0.9770	4.3
	180	319	1	2,105	0.9925	5.2

A comparison between the maximum exposure (C_{max}) to enclomiphene and its metabolites in the rat and humans following oral exposure is shown below (Table 2). The data indicate that enclomiphene C_{max} was higher in the rat than in humans. In addition, the levels of enclomiphene metabolites (4-OH, desethyl and 4-OH desethyl enclomiphene) were statistically significantly (marked with a * in the table below) decreased compared to enclomiphene citrate in the rat but not in humans.

Table 2: Oral C_{max} Levels of Enclomiphene and its Metabolites in Rats and Humans

Analyte	C_{max} in Rat 180 days 10 mg/kg/day	C_{max} in Human 25 mg daily 12 weeks	Overage
Enclomifene	319	5.79	55.1
4-OH enclomifene	47.9*	3.42	14
Desethyl enclomifene	23.9*	2.58	9. 3
4-OH desethyl enclomifene	9.90*	1.75	5.7

Enclomiphene was widely distributed in tissues and organs following a single oral dose administered to mice and was selectively associated with melanin-containing tissues. Elimination was essentially complete by 72 hours except for retention in melanin containing tissues.

c. Acute toxicity[9]

The oral lethal dose (LD_{50}) of the isomeric mixture of enclomiphene citrate and zuclomiphene is reported to be 1700 mg/kg in mice and 5750 mg/kg in rats.

d. Repeat dose toxicity[10]

Pivotal repeat dose toxicity studies have been conducted in rats and dogs.

Rat:
In a 26-week oral study in the rat, a no adverse effect level (NOAEL) could not be assigned due to drug-related toxicities that were recorded at all dose levels (0.5, 5, and 10 mg/kg/day). Test article-related findings included reduced body weight associated with reduced food consumption, organ weight decreases (prostate gland, pituitary gland, and epididymides), and histopathological findings (prostate, testes, seminal vesicles, and kidneys).

- The prostate glands, seminal vesicles, and coagulating glands - atrophy (minimal to severe, all dose levels).
- Leydig cell - atrophy and depletion in the testes (minimal, all dose levels).
- Preputial glands - subacute inflammation (0.5 and 10 mg/kg/day); abscess formation (minimal to mild, 0.5 and 5 mg/kg/day).
- Kidneys - tubular dilatation and minimal tubular mineralization (minimal to mild, all dose levels).

Dog:
In a 9-month oral dog study (2, 10, and 40 which was reduced to 20 mg/kg/day due to morbidities associated with the 40 mg/kg/day dose treatment), a NOAEL of 2 mg/kg/day was reported. Toxicities included organ weight changes (adrenal/liver/prostate gland), and delayed onset in puberty which was also evident in high-dose animals and correlated with changes noted in testes and epididymides. Deaths in high dose animals were related to hepatotoxicity. Ophthalmic abnormalities (cataracts) were widely reported among mid- and high dose treated males. A peer review of the eyes collected from the 9-month toxicity study concluded that these changes consisted of minimal lens fiber swelling. The peer reviewers also agreed that there was no progression in the lens fiber swelling between the interim 13-week necropsy and the terminal 9-month necropsy. There was no evidence of fiber fragmentation, lenticular disorganization, or lenticular epithelial proliferation. Another dog study was conducted which concluded that no lenticular lesions were seen in either the control group or at the highest dose of 10 mg/kg (representing 6 times the clinical safety margin) of enclomiphene citrate.

[9] Acute toxicity refers to adverse effects observed following administration of a single dose of a substance, or multiple doses given within a short period (approximately 24 hours). Endpoints captured in acute toxicity studies usually include mortality and gross clinical observations. Acute toxicity data are usually superseded by data obtained from longer term toxicity studies.
[10] Repeated-dose toxicity studies consist of in vivo animal studies that seek to evaluate the toxicity of the test substance by observing the changes that emerge in clinical observations, clinical chemistry, gross pathology, and histology endpoints when the test substance is repetitively administered daily for a predetermined period of time.

e. Genotoxicity[11]

A standard battery was conducted to assess the genotoxic potential of enclomiphene citrate. Under the conditions of the studies reported, no genotoxicity was reported (EMA 2018). Clomiphene citrate, which is a mixture of trans-clomiphene citrate (enclomiphene citrate) and cis-clomiphene citrate (zuclomiphene citrate), was associated with a significant increase in chromosomal aberrations and micronuclei in cultured human lymphocytes (Yilmaz et al. 2014).

Table 3: Genotoxicity of Enclomiphene (EMA 2018 report unless noted)

Type of test / study ID / GLP status	**Test system**	**Concentrations**	**Results**
Gene mutations in bacteria (Ames test) **ZN01-100** GLP	*S. typhimurium* (TA1535, TA1537, TA98, TA100) +/-S9	Enclomiphene citrate or Zuclomiphene citrate: 6.7, 10, 33, 67, 100, 333, 667, 1000, 3333 and 5000 µg/plate	Negative
L5178Y Tk+/- Mouse Lymphoma Forward Mutation Assay **769285 (21984)** GLP	L5178Y cell line +/- S9	500, 1500, 5000 µg/ml	Negative
In Vivo Rat Bone Marrow Micronucleus Assay **769290 (22008)** GLP	CD-1 mice	250, 500, and 1000 mg/kg and administered via oral gavage	Negative
Chromosomal aberrations and micronuclei (Yilmaz et al. 2014)	Cultured human lymphocytes	0.40, 0.80, 1.60, and 3.20µg/ml clomiphene citrate	Positive ($p < 0.01$ and $p < 0.001$)

[11] The genotoxicity assessment battery usually consists of a gene mutagenicity assay (for single dose trials) and a variety of clastogenicity/genotoxicity assays. To support multiple dose administration in humans, additional genotoxicity testing assessment is usually conducted to detect chromosomal damage in mammalian systems.

f. Developmental and reproductive toxicity[12]

A mouse oral fertility study did not have adverse effects on mating or fertility in either males or females at 40 or 100 mg/kg/day. The high dose of 200 mg/kg/day resulted in mortality in all males. Among lower dose groups, changes in sperm parameters, as well as increased resorptions and post-implantation loss were seen (NOAEL 100 mg/ml).

g. Carcinogenicity[13]

In a 26-week oral transgenic mouse carcinogenicity study (10, 50, 100, and 200 mg/kg/day enclomiphene), most high dose treated animals died prematurely (100 and 200 mg/kg). The cause of death was attributed to necrosis and inflammation of the intestines. Among surviving animals, a decrease in body weight gain was seen (statistically significant and dose-proportional in both sexes). Necropsy showed gross changes in the spleen, ovaries, seminal vesicles, skin, testes, thymus and uterus. An increased incidence of testicular interstitial cell adenomas was also seen. The study was considered negative because the findings were not dose-related and did not reach statistical significance compared to controls.

A 2-year oral rat carcinogenicity study (0, 0.0125, 0.025, and 0.05 mg/kg/day) did not show any gross pathological changes among enclomiphene-treated rats. A reduction in weight gain was seen in the enclomiphene-treated groups (both sexes) along with a decrease in food consumption, which was associated with prolonged survival over the control group. A dose-related increase in the incidence and severity of centrilobular hepatocellular vacuolation was seen in males at the highest dose tested. The incidence of tumors was not increased among rats treated with enclomiphene citrate compared to controls. The study was considered negative.

Conclusions: The nonclinical toxicity profile of enclomiphene citrate reflects its exaggerated pharmacological action as a selective estrogen receptor modulator. When tested via the oral route of administration in rats and dogs, enclomiphene citrate showed dose-related findings including reduced body weight, reduced food consumption, organ weight decreases (prostate gland, pituitary gland, and epididymides), and histopathological findings (prostate, testes, seminal vesicles, liver, and kidneys). Enclomiphene citrate was not genotoxic or carcinogenic under the conditions of the conducted studies.

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[12] Developmental and reproductive toxicity studies are usually designed to assess the potential adverse effects in humans of both sexes and include females from various age groups that will be exposed to the proposed substance. *Developmental toxicity* or *teratogenicity* refers to adverse effects (can include embryo-fetal mortality, structural abnormalities, functional impairment, or alterations to growth) and can occur in pups either as a result of the exposure of their parents to the substance, prior to the pups' birth, or by direct exposure of the pups to the substance after birth.

[13] Studies that assess cancer risk in animals are used as predictive tools to evaluate the potential for drugs to result in tumors when used by humans on a chronic basis. Carcinogenicity studies are conducted if the clinical use is expected to be continuous for a minimum of 6 months of life, or if intermittent clinical use is expected to total 6 months or more of life.

2. *Human Safety*

The following databases were consulted in the preparation of this section: PubMed, ClinicalTrials.gov, Embase, FDA Adverse Event Reporting System (FAERS), professional healthcare organization websites, and various online clinical references and websites.

a. Pharmacokinetic data

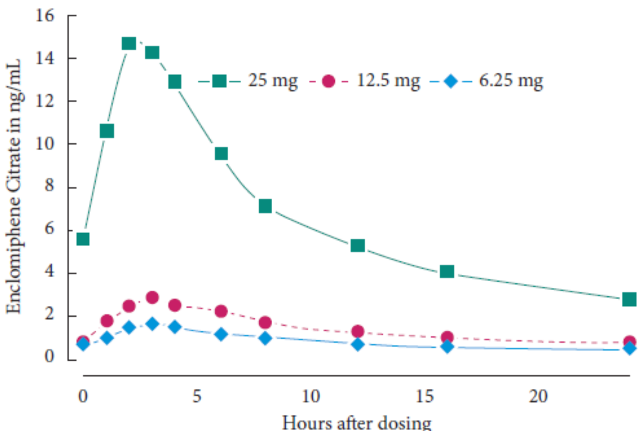
Oral enclomiphene citrate is rapidly absorbed and has a half-life of approximately 10 hours (Wiehle et al. 2014b). According to the EMA 2018 report, it is metabolized by CYP2D6, and its main metabolite appears to be 4-OH enclomiphene formed primarily by CYP2D6 and to a lesser extent by CYP2B6. It is also metabolized to desethyl enclomiphene by CYP3A4 and CYP3A5; this is then further metabolized to 4-OH desethyl enclomiphene, primarily by CYP2D6. EMA concluded that available PK data for enclomiphene citrate are considered incomplete and additional information would be required to exclude the possibility of a unique human metabolite of significance (EMA 2018). Maximum serum concentration occurs 2-3 hours after administration. Absorption is increased by the presence of food. Excretion is 61.5% in feces and 8.2% in urine (Earl and Kim 2019).

A randomized, single-blind, two-center phase II trial published by Wiehle et al. (2013) enrolled 48 men with secondary hypogonadism[14] to determine the PK and PD profiles of enclomiphene citrate. Forty-four of the 48 enrolled men completed the study, after being randomized to receive enclomiphene citrate 6.25 mg (n=12), enclomiphene citrate 12.5 mg (n=7), enclomiphene citrate 25 mg (n=12), or transdermal testosterone (AndroGel®) (n=13) daily for 6 weeks. Patients in the enclomiphene citrate arms underwent PK assessment at 6 weeks after taking a single oral dose of enclomiphene citrate (6.25 mg, 12.5 mg, and 25 mg). Individual and overall PK profiles showed a rapid rise of the drug and first order elimination (see Figure 3).

[14] For definition and further discussion of secondary hypogonadism, see Section II.C.1. and Appendix 1 of this evaluation.

Figure 3: Pharmacokinetics of Enclomiphene Citrate (Wiehle et al. 2013)

Fig. 4 Pharmacokinetics of serum enclomiphene citrate. After 6 weeks of continuous oral dosing at various dosages of enclomiphene citrate, serum samples were obtained at various time points for the assessment of serum drug levels. The levels of serum enclomiphene citrate are given for subjects taking 6.25 mg (filled diamonds), 12.5 mg (filled circles), and 25 mg (filled squares) of study drug.



Calculations of the mean area under the curve showed that the value for the 25 mg dose was about 4.5 times higher than for 12.5 mg, and 7.5 times higher than for the 6.25 mg dose. There was evidence of accumulation of enclomiphene citrate in the serum as serum levels did not return to baseline 24 hours after drug administration.

A randomized, placebo-controlled trial of 52 men with secondary hypogonadism published by Wiehle et al. (2014b) evaluated the safety, efficacy, and pharmacokinetics of enclomiphene citrate as an alternative to topical testosterone. Patients received oral enclomiphene citrate 12.5 mg (n=10), enclomiphene citrate 25 mg (n=11), or enclomiphene citrate 50 mg (n=11); topical testosterone gel (Androgel® 1% (5 g)) (n=10); or oral placebo (n=10) daily for 14 days (there was no topical placebo arm). All patients completed the initial study, and patients who requalified for the study based on testosterone levels were crossed over to receive open-label topical testosterone (Androgel® 1% (10 g)) daily for 14 days under an amended protocol (n=10). Only patients randomized to enclomiphene citrate or placebo arms were double-blinded. Mean peak concentration (C_{max}) increased on days 1 and 14 in a greater than dose proportional manner from 12.5 mg to 25 mg, and a less than dose proportional manner from 25 mg to 50 mg. Mean plasma concentration time profiles for enclomiphene citrate 25 mg and 50 mg were similar and were higher than the 12.5 mg group following single dose administration and after 14 days. The authors proposed that this might suggest tachyphylaxis as the uptake may become saturated between the 25 and 50 mg doses, and that higher doses may not be indicated. The authors observed that a non-dose-dependent steady-state level was maximal at the 25 mg dose. The authors concluded that the study suggests a “rapid but not immediate onset of action which is not lost for 7–10 days” following enclomiphene citrate treatment and supports further study of enclomiphene citrate in larger trials.

The EMA 2018 report reviewed PK studies which indicated higher exposure for hepatic and renally-impaired patients than for healthy subjects. EMA discussed that in patients with moderate hepatic and renal impairment, the consequences of higher plasma enclomiphene citrate concentrations on the cardiac/vascular system, ophthalmic system, breast and reproductive system, and bone in the context of chronic treatment is not known, and stated that further characterization of the PK profile was needed to inform the need for any dose adjustments in those with renal and hepatic impairment (EMA 2018). Of note, the label of the FDA-approved clomiphene citrate tablet, which is a mixture of two geometric isomers [cis (zuclomiphene) and trans (enclomiphene)], specifies it is only indicated in patients with normal liver function and contains a contraindication for use in patients with liver (hepatic) disease or a history of liver dysfunction. The label does not provide information for patients with renal disease.[15]

b. Reported adverse reactions (FAERS)

The Office of Surveillance and Epidemiology conducted a search of the FAERS database for reports of adverse events (AEs) for enclomiphene citrate through October 13, 2021. The search retrieved no reports.

c. Clinical trials assessing safety

Adverse Events Reported in Clinical Trials:
We found clinical trials in the medical literature that evaluated the safety of oral enclomiphene citrate in humans. AEs in trials that reported safety outcomes are summarized below.

In two parallel, randomized, double-blind, double-dummy, placebo-controlled, multi-center phase III trials (ZA-304 and ZA-305) published by Kim et al. (2016), 256 overweight men aged 18 to 60 years with secondary hypogonadism were treated for 16 weeks with oral enclomiphene citrate 12.5 mg (n=43), enclomiphene citrate 12.5 mg up-titrated to 25 mg (n=42), topical testosterone gel (n=85), or placebo (n=86), to determine the effects of treatment on serum total testosterone, LH, FSH, and sperm counts. There were 53 (21%) men who had AEs considered by the investigators to be possibly, probably, or definitely related to the study drug (no details provided in the publication); none of these were severe or serious, and there was no difference in AEs between treatment groups. There were 2 deaths and 11 patients discontinued (2- enclomiphene citrate 25 mg, 1- testosterone gel and 8- placebo or testosterone gel arm (not specified further in publication)). The following are AEs (1 each) reported per treatment arm, and no details were provided except where indicated:

- Enclomiphene citrate 25 mg:
  - high hematocrit or hemoglobin - patient discontinued due to AE
  - high prostate-specific antigen (PSA) level - patient discontinued due to AE
  - psoriatic arthropathy (severe AE)
  - depression (severe AE)

[15] See prescription label for clomiphene citrate tablets, ANDA 075528. NIH DailyMed. https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=be399623-6400-475d-93d3-1dedd4d43017. Accessed Jan 11, 2022.

- Enclomiphene citrate 12.5 mg:
  - death (fatal road traffic accident) - no details provided, unrelated per investigator
  - death (cerebrovascular accident) - ischemic stroke in a 59-year-old man with a history of type 2 diabetes, obesity, atrial fibrillation, sleep apnea, nephrolithiasis, and hyperlipidemia, treated with enclomiphene citrate 12.5 mg for 34 days. Per investigators, the cause of death was highly unlikely to be the study drug because of his many risk factors and limited exposure to the study drug.
  - hypertriglyceridemia (severe AE)
  - anxiety (severe AE)
- Testosterone gel:
  - high hematocrit or hemoglobin – patient discontinued due to AE
  - arthropod bite (severe AE)
  - coronary bypass (severe AE, no details provided)
- Placebo: muscle spasms (severe AE)

Per authors, changes in PSA and hematocrit seen with both enclomiphene citrate and testosterone gel are secondary to increases in total testosterone.

(Wiehle et al. 2014a) A randomized, placebo-controlled phase IIB trial (ZA-203) compared oral enclomiphene citrate and topical testosterone gel in 124 men with secondary hypogonadism and evaluated change in testosterone, FSH, and LH levels over a 3-month dosing period. Men with a morning serum testosterone level of <250 ng/dL were randomized to receive enclomiphene citrate 12.5 mg (n=29), enclomiphene citrate 25 mg (n=33), 1% topical testosterone gel (Testim®) (n=33), or oral placebo (n=29). Patients randomized to enclomiphene citrate or placebo arms were double-blinded. Investigators noted two mild to moderate AEs experienced by two patients in the enclomiphene citrate 25 mg group that led to discontinuation of the study and were considered possibly related to the study drug:

- inability to climax and loss of sensation during intercourse and
- mild nausea and mild dry heaving.

One patient in the enclomiphene citrate 25 mg group experienced mild hives, which was considered probably not related to the study drug.

Wiehle et al. (2014b) published a randomized, placebo-controlled trial of 52 men with secondary hypogonadism. Patients received oral enclomiphene citrate 12.5 mg (n=10), enclomiphene citrate 25 mg (n=11), or enclomiphene citrate 50 mg (n=11); topical testosterone gel (Androgel® 1% (5 g)) (n=10); or oral placebo (n=10) daily for 14 days (no topical placebo arm). The study aimed to evaluate safety, efficacy, and pharmacokinetics of enclomiphene citrate as an alternative to topical testosterone. All patients completed the initial study, and patients who requalified for the study based on testosterone levels were crossed over to receive open-label topical testosterone (Androgel® 1% (10 g)) daily for 14 days under an amended protocol (n=10). Only patients randomized to enclomiphene citrate or placebo arms were double-blinded. Treatment-emergent AEs were reported in 9 to 20% of patients and were similar among active treatment and placebo groups. No serious AEs were reported in patients receiving enclomiphene citrate. AEs reported in each group included (1 patient each):

- Enclomiphene citrate 12.5 mg: mildly increased estradiol;
- Enclomiphene citrate 25 mg: mild sinus headache;
- Enclomiphene citrate 50 mg: moderate headache;
- Topical testosterone (5 g): near syncopal episode which was related to sick sinus syndrome, and not study medication per investigators (serious AE); and
- Placebo: mild headache.

We found clinical trials on enclomiphene citrate in men with secondary hypogonadism on ClinicalTrials.gov that reported AEs in the posted study results.[16] These are not discussed in further detail because we did not find corresponding publications and causality assessment of AEs is not provided on ClinicalTrials.gov. However, one safety study from ClinicalTrials.gov was cited by the nominator (Empower Pharmacy) and is briefly described below.

An open-label, non-randomized phase III safety study (ZA-300), completed in 2013, evaluated the safety profile of enclomiphene citrate in 499 men ages 18 to 65 with secondary hypogonadism (ClinicalTrials.gov ID: NCT01534208). Patients were assigned to receive oral enclomiphene citrate 25 mg (n=283) or enclomiphene citrate 12.5 mg which could be increased to 25 mg if indicated[17] (n=216) daily for 26 weeks. AE data were collected from first administration of the study drug until 8 weeks after the end of treatment (8 months total). There were 15 patients with reports of serious AEs, 6 in the enclomiphene citrate 12.5 mg and 9 in the enclomiphene citrate 25 mg group.

The following are the serious AEs reported:

- Enclomiphene citrate 12.5 mg (1 report each):
    - Bradycardia
    - Chest pain
    - Biliary colic
    - Transient ischemic attack (TIA)
    - Seminoma
    - Dyspnea
    - Knee arthroplasty
    - Deep vein thrombosis (DVT)
    - Hypotension
- Enclomiphene citrate 25 mg (1 report each unless noted):
    - Atrial flutter
    - Cholelithiasis
    - Diverticulitis (2)
    - Food poisoning
    - Kidney infection
    - Pulmonary embolism (PE)
    - Cellulitis (2)
    - DVT

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[16] NIH U.S. National Library of Medicine. www.ClinicalTrials.gov, search term “enclomiphene citrate” and “enclomiphene.” Accessed Nov 3, 2021.

[17] Parameters to increase the dose were not specified on ClinicalTrials.gov.

Non-serious AEs reported were:

- Enclomiphene citrate 12.5 mg:
  - Hot flush (2)
  - Upper respiratory tract infection (URI) (26)
  - Influenza (7)
  - Sinusitis (7)
  - Muscle spasms (10)
  - Headache (12)
  - Dizziness (6)
  - Pollakiuria (daytime urinary frequency) (6)
- Enclomiphene citrate 25 mg:
  - Hot flush (9)
  - URI (27)
  - Influenza (5)
  - Sinusitis (6)
  - Muscle spasms (12)
  - Headache (23)
  - Dizziness (5)
  - Pollakiuria (5)

d. Other safety information

<u>Adverse Event and Safety Information from European Medicines Agency:</u>
In addition to the information above, we found safety information from the EMA. As mentioned above, in 2018, the EMA evaluated enclomiphene (also spelled enclomifene, proposed trade name EnCyzix)[18] for marketing authorization to treat secondary hypogonadism. The EMA's Assessment Report concluded that the safety of the product was not sufficiently demonstrated.[19] The EMA documented the following summary of AEs in the "Unfavourable effects" section of the report:

> "In terms of adverse events, the most frequently reported treatment emergent adverse events were headache, hot flushes, nausea and muscle spasms. The most common adverse events leading to discontinuation were blurred vision, muscle spasm, headache and aggression.
>
> A number of adverse events that are known to be associated with testosterone replacement therapy were reported in the enclomifene clinical studies. These include venous thromboembolic events, cardiac disorders, increased haematocrit and PSA [prostate-specific antigen], eye disorders and psychiatric disorders. These events were reported with a higher frequency for enclomifene treated patients compared to the placebo group.
>
> Four cases of thromboembolic events were reported, with one fatality occurring due to this. In contrast, no events were reported in patients treated with testosterone or placebo, even though the treated population included in these trials at an increased risk for such events.

[18] International Non-proprietary Name/Common Name Enclomiphene; Active substance Enclomiphene citrate. The finished product was presented as capsules containing 8.5 mg or 17 mg of enclomiphene (as citrate) as active substance. Phase III clinical trials evaluated enclomiphene citrate dosage strengths of 12.5 mg and 25 mg.
[19] The 2018 EMA Assessment Report of EnCyzix is available to the public at: https://www.ema.europa.eu/en/documents/assessment-report/encyzix-epar-public-assessment-report_en.pdf.

> The incidence of cardiac events in the enclomifene group was also slightly increased when compared to patients treated with testosterone (0.9% vs 0.7%)."

EMA noted that it was unclear if the thromboembolic events occurred due to increased testosterone and resulting increased erythropoiesis, increased estradiol levels, co-morbid conditions such as obesity, or risks associated with the drug class. Of note, the FDA-approved clomiphene citrate tablet, which is a mixture of geometric isomers [cis (zuclomiphene) and trans (enclomiphene)], lists pulmonary embolism and retinal thrombosis in the Postmarketing Adverse Events section of its label.[20]

Ophthalmic abnormalities (cataracts) were reported in nonclinical studies, and therefore eye examinations were conducted during phase II and phase III trials. EMA concluded that the available data did not provide conclusive evidence that use of enclomiphene was associated with development of new or progression of existing cataracts, but recommended that ocular safety monitoring be included in a Risk Management Plan to collect additional data.

The EMA also concluded that the PK data for enclomiphene were incomplete and further characterization of the PK profile was needed to inform the need for dose adjustments in elderly patients, patients with renal and hepatic impairment, and poor CYP2D6 metabolizers; and to exclude the possibility of a unique human metabolite of significance.

e. Availability of alternative approved therapies that may be as safe or safer

There are drug products approved by FDA (listed below) for the treatment of male patients who have hypogonadism (low testosterone levels) caused by certain medical conditions that are considered to be safe treatment. Products approved for secondary hypogonadism (also called hypogonadotropic hypogonadism) include testosterone products and chorionic gonadotropin.

<u>Testosterone</u>

Testosterone has been approved in the United States since the 1950s as replacement therapy in men for conditions associated with a deficiency or absence of endogenous testosterone.[21] Example available formulations[22] of testosterone for various routes of administration include:

[20] See prescription label for clomiphene citrate tablets, ANDA 075528. NIH DailyMed. https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=be399623-6400-475d-93d3-1dedd4d43017. Accessed Jan 11, 2022.

[21] See FDA Briefing Information For The December 6, 2016 Meeting of the Bone, Reproductive and Urologic Drugs Advisory Committee (BRUDAC). https://www.fda.gov/media/101582/download.

[22] Orange Book. https://www.accessdata.fda.gov/scripts/cder/ob/index.cfm, search term "testosterone." Accessed Nov 2, 2021.

- Topical – gels (AndroGel®)[23], patches (ANDRODERM®)[24], and axillary solutions (Testosterone topical solution)[25]
- Intranasal – gel (Natesto®)[26]
- Oral – capsules (JATENZO®)[27]
- Subcutaneous implantation - implant pellets (TESTOPEL®)[28]
- Injections – intramuscular (Testosterone Cypionate)[29] and subcutaneous (XYOSTED®)[30]

Testosterone products are indicated for replacement therapy in males for conditions associated with a deficiency or absence of endogenous testosterone. Labeling lists the following indications and usage:

- Primary hypogonadism (congenital or acquired):[31] testicular failure due to conditions such as cryptorchidism, bilateral torsion, orchitis, vanishing testis syndrome, orchiectomy, Klinefelter's syndrome, chemotherapy, or toxic damage from alcohol or heavy metals. These men usually have low serum testosterone concentrations and gonadotropins (FSH, LH) above the normal range.
- Hypogonadotropic hypogonadism (congenital or acquired): gonadotropin or luteinizing hormone-releasing hormone (LHRH) deficiency or pituitary-hypothalamic injury from tumors, trauma, or radiation. These men have low testosterone serum concentrations but have gonadotropins in the normal or low range.

[23] See prescription label for AndroGel® (testosterone gel) 1.62% for topical use, NDA 022309/S-20. Drugs@FDA, https://www.accessdata.fda.gov/scripts/cder/daf/. Accessed Nov 2, 2021.
[24] See prescription label for ANDRODERM® (testosterone transdermal system), for topical use, NDA 020489/S-36. Drugs@FDA. https://www.accessdata.fda.gov/scripts/cder/daf/. Accessed Nov 2, 2021.
[25] See prescription label for Testosterone topical solution, ANDA 205328. NIH DailyMed. https://dailymed.nlm.nih.gov/dailymed/lookup.cfm?setid=7227ab2f-c8ef-4bab-92ab-3092182abcc9. Accessed Nov 2, 2021.
[26] See prescription label for NATESTO® (testosterone) nasal gel, NDA 205488/S-2. Drugs@FDA. https://www.accessdata.fda.gov/scripts/cder/daf/. Accessed Nov 2, 2021.
[27] See prescription label for JATENZO® (testosterone undecanoate) capsules, for oral use, NDA 206089. Drugs@FDA. https://www.accessdata.fda.gov/scripts/cder/daf/. Accessed Nov 2, 2021.
[28] See prescription label for TESTOPEL®- testosterone pellet, ANDA 205328. NIH DailyMed. https://dailymed.nlm.nih.gov/dailymed/lookup.cfm?setid=03b9c0b1-5884-11e4-8ed6-0800200c9a66. Accessed Nov 2, 2021.
[29] See prescription label for Testosterone Cypionate injection, ANDA 210362. NIH DailyMed. https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=78d55bad-4a5b-4e21-aeb8-a4c6346208be. Accessed Nov 2, 2021.
[30] See prescription label for XYOSTED® (testosterone enanthate) injection, for subcutaneous use, NDA 209863/S-2. Drugs@FDA. https://www.accessdata.fda.gov/scripts/cder/daf/. Accessed Nov 2, 2021.
[31] Congenital, referring to conditions that are present at birth, regardless of their causation. Acquired, denoting a disease, predisposition, or abnormality that is not inherited. See: Medical Dictionary. https://medical-dictionary.thefreedictionary.com/. Accessed Nov 10, 2021.

Human Chorionic Gonadotropin

Human chorionic gonadotropin (HCG) is a non-testosterone product approved for use in select cases of hypogonadotropic hypogonadism (hypogonadism secondary to a pituitary deficiency) in males.[32] HCG can simultaneously raise serum testosterone and restore spermatogenesis.[33]

Conclusions: Based on available clinical information, we conclude that safety concerns for enclomiphene citrate include cardiac and thromboembolic events, as reported adverse events in clinical trials included bradycardia, atrial flutter, hypotension, deep vein thrombosis, pulmonary embolism, and ischemic stroke; elevated estradiol; increased prostate-specific antigen; and increased hematocrit. Pharmacokinetic data are also limited, including information on dose adjustments for patients with renal or hepatic impairment. There are currently available FDA-approved therapies indicated for male patients with hypogonadism; these products meet established criteria for safety and efficacy and are labeled accordingly to inform safe use of the product.

C. Are there concerns about whether a substance is effective for a particular use?

The following databases were consulted in the preparation of this section: PubMed, ClinicalTrials.gov, Embase, professional healthcare organization websites, and various online clinical references and websites. In addition to a comprehensive review of pertinent information from these databases, this section provides a brief overview of male hypogonadism and treatment (see Appendix 1 for additional information), relevant regulatory history, and a discussion of the proposed use of enclomiphene citrate.

1. Background

Male hypogonadism results from failure of the testis to produce physiological concentrations of testosterone and/or a normal number of spermatozoa due to pathology in the HPG axis (Bhasin et al. 2018). Hypogonadism is classified as primary or secondary (Hill et al. 2009; Basaria 2014):

- Primary hypogonadism: dysfunction arising from the level of the testes; characterized by low serum testosterone levels and spermatogenesis, resulting in elevated levels of gonadotropins (high LH and FSH) in a stimulatory effort (hypergonadotropic hypogonadism).
- Secondary hypogonadism: dysfunction arising from the level of hypothalamus or pituitary; testosterone levels and spermatogenesis are low, with low or inappropriately normal gonadotropin levels (hypogonadotropic hypogonadism).

Diagnosis of male hypogonadism is based on assessment of signs and symptoms, and low morning total testosterone levels on at least two occasions (Bhasin et al. 2018). Laboratory definitions of low testosterone level vary; for example, the American Urologic Association

[32] See information from package insert label for chorionic gonadotropin, BLA 017067/S-57. Drugs@FDA, https://www.accessdata.fda.gov/scripts/cder/daf/. Accessed Oct 10, 2021.
[33] See FDA Briefing Information For The December 6, 2016 Meeting of the Bone, Reproductive and Urologic Drugs Advisory Committee (BRUDAC). https://www.fda.gov/media/101582/download.

(AUA)[34] recommends total testosterone level below 300 ng/dL to diagnose low testosterone (Mulhall et al. 2018), while the Endocrine Society[35] suggests a lower limit of 264 ng/dL (Bhasin et al. 2018). Measurement of gonadotropin levels (FSH and LH) helps to differentiate between primary and secondary hypogonadism (Bhasin et al. 2018).[36]

Signs and symptoms of hypogonadism vary depending on age of onset, severity of testosterone deficiency, androgen sensitivity, and previous use of testosterone-replacement therapy. Clinical manifestations with post-pubertal onset of hypogonadism may include decreased libido, decreased spontaneous erections, decrease in testicular volume, gynecomastia, hot flashes, decreased bone mass, height loss, decreased pubic or axillary hair, decreased muscle mass, and decreased energy and motivation (Basaria 2014).

Treatment of hypogonadism depends in part on the underlying etiology of the condition and on the patient's goals for immediate fertility.[37] Direct androgen replacement with testosterone is the only treatment option in primary hypogonadism (Hill et al. 2009). The focus of our subsequent discussion is on secondary hypogonadism. Products approved for the treatment of secondary hypogonadism include testosterone products and human chorionic gonadotropin. Secondary hypogonadism is often managed with testosterone (Rodriguez et al. 2016). However, because testosterone can impair spermatogenesis, there has been interest in developing non-testosterone alternatives for men with secondary hypogonadism, such as gonadotropins (e.g., chorionic gonadotropin) and SERMs like enclomiphene citrate.

There are no FDA-approved drug products containing enclomiphene citrate as the active ingredient. Repros Therapeutics, Inc. (Repros) submitted new drug application (NDA) 207959, enclomiphene citrate 12.5 mg and 25 mg oral capsules, for the proposed treatment of secondary hypogonadism in fertile men (men with more than 15 million sperm/ mL), younger than 60 years of age with a BMI over 25 kg/m^2.[38] This NDA was to be discussed during an FDA advisory committee meeting scheduled for November 2015; however, it was cancelled due to questions that arose late in the review regarding the bioanalytical method validation that could affect

[34] The American Urological Association is a urologic association with a mission to promote the highest standards of urological clinical care through education, research, and the formulation of health care policy (https://www.auanet.org/about-us/about-the-aua). Accessed Sep 20, 2021.

[35] The Endocrine Society is a not-for-profit organization representing basic, applied, and clinical interests in endocrinology (https://rarediseases.org/organizations/endocrine-society/). The society is devoted to advancing hormone research, excellence in the clinical practice of endocrinology, broadening understanding of the critical role hormones play in health, and advocating on behalf of the global endocrinology community (https://www.endocrine.org/about-us). Accessed Aug 20, 2021.

[36] Reference range for FSH in an adult male is 1.5 - 12.4 mIU/mL. Reference range for LH in an adult male is 1.7 - 8.6 mIU/mL. See: Follicle-stimulating Hormone (FSH). Labcorp. https://www.labcorp.com/tests/004309/follicle-stimulating-hormone-fsh. Accessed Nov 10, 2021. Luteinizing Hormone (LH). Labcorp. https://www.labcorp.com/tests/004283/luteinizing-hormone-lh. Accessed Nov 10, 2021.

[37] See FDA Briefing Information For The December 6, 2016 Meeting of the Bone, Reproductive and Urologic Drugs Advisory Committee (BRUDAC). https://www.fda.gov/media/101582/download.

[38] Notice of announcement of advisory committee meeting to discuss NDA 207959 can be found at https://www.regulations.gov/document/FDA-2015-N-0001-0074.

interpretability of certain pivotal study data.[39] In December 2015, Repros announced that it received a Complete Response Letter from FDA informing the company that the design of Phase III studies was no longer adequate to demonstrate clinical benefit and recommending that the company conduct an additional Phase III study or studies to support approval in the target population. FDA also noted concerns regarding study entry criteria, titration, and bioanalytical method validation in the Phase III program.[40] Earl and Kim (2019) referred to the trials submitted under the NDA and stated that although the Phase III trials demonstrated significant improvements with enclomiphene on serum testosterone levels and preservation of sperm concentrations compared to AndroGel® 1.62%, this primary endpoint was determined to be an insufficient basis for FDA-approval. The authors added that at question was the issue of demonstration of clinical benefit. FDA placed a particular emphasis on the lack of measurable symptomatic improvement.

In December 2016, the Bone, Reproductive and Urologic Drugs Advisory Committee (BRUDAC) met to discuss appropriate clinical trial design features, including acceptable endpoints for demonstrating clinical benefit, for drugs intended to treat secondary hypogonadism while preserving or improving testicular function.[41] Three drug companies, including Repros, collaborated to present their views on clinical trial design features. The meeting discussion did not focus on the benefit/risk assessment or safety profiles of individual drugs, although enclomiphene citrate was discussed as an example of a product being investigated to treat secondary hypogonadism.[42]

During the BRUDAC December 2016 meeting, committee members were asked, "For products intended to treat men with hypogonadism attributed to obesity, is raising serum testosterone concentrations into the normal range for young, healthy eugonadal men and preservation of spermatogenesis, as assessed by maintenance of sperm concentrations, sufficient for establishing evidence of clinical benefit?" A majority of the committee voted that raising testosterone concentrations and maintaining spermatogenesis is not sufficient evidence of clinical benefit.[43] Additionally, a majority of the committee voted that raising the sperm concentration for men

[39] Repros Therapeutics Announces Cancellation of FDA Advisory Committee Meeting to Review Enclomiphene for the Treatment of Secondary Hypogonadism. U.S. Securities and Exchange Commission (SEC) Web site. Available at https://www.sec.gov/Archives/edgar/data/897075/000117184315005822/newsrelease.htm. Accessed Aug 3, 2021.

[40] Repros Therapeutics Receives Complete Response Letter From FDA for Enclomiphene. SEC Web site. Available at https://www.sec.gov/Archives/edgar/data/897075/000117184315006596/newsrelease.htm. Accessed Aug 3, 2021.

[41] See Summary Minutes of the Bone, Reproductive and Urologic Drugs Advisory Committee (BRUDAC) Meeting December 6, 2016, available at https://wayback.archive-it.org/7993/20170403223743/https://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/ReproductiveHealthDrugsAdvisoryCommittee/ucm507639.htm.

[42] See BRUDAC Transcript, December 6, 2016, available at https://wayback.archive-it.org/7993/20170403223743/https://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/ReproductiveHealthDrugsAdvisoryCommittee/ucm507639.htm.

[43] See Summary Minutes of the Bone, Reproductive and Urologic Drugs Advisory Committee (BRUDAC) Meeting December 6, 2016, available at https://wayback.archive-it.org/7993/20170403223743/https://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/ReproductiveHealthDrugsAdvisoryCommittee/ucm507639.htm.

with “non-classic”[44] secondary hypogonadism and azoospermia[45] or oligospermia[46] above a specific threshold would not provide evidence of clinical benefit, and that pregnancy outcomes would be needed.

In 2016, Renable Pharma Limited applied to the EMA for marketing authorization of enclomiphene. The proposed therapeutic indication was for, “treatment of hypogonadotropic hypogonadism (secondary hypogonadism) in adult men aged ≤60 years with a BMI ≥25 kg/m^2 which has been confirmed by clinical features and biochemical tests in patients which have not responded to diet and exercise.” On January 25, 2018, EMA published an Assessment Report issuing a negative opinion and recommending the refusal of the authorization. The EMA determined that the safety and efficacy of the product was not sufficiently demonstrated.[47]

Proposed Use

Enclomiphene citrate was nominated for the use, “to increase serum testosterone, LH and FS [follicle-stimulating hormone, FSH] to normal levels and maintain sperm concentrations within the normal range.” The proposed use describes a proposed action (i.e., increase serum testosterone, LH, and FSH), without specifying a medical condition in which the substance might be used. However, the primary condition in which this action would be applicable is for secondary hypogonadism (see above in Section II.C.1 and Appendix 1 to this evaluation memo for information on male hypogonadism). The publications submitted by the nominator all refer to enclomiphene citrate use in the condition of secondary hypogonadism. For example, the publication by Wiehle et al. (2013), which is cited by the nominator in the “proposed use” section of the nomination, states that, “[e]nclomiphene citrate is proposed for the treatment of some men who have secondary hypogonadism, especially that caused by dysfunctional, but reversible hypothalamus/pituitary activity. These men present with low total testosterone and low or inappropriately normal gonadotropin levels (LH and FSH).” Therefore, our evaluation focuses on the use of enclomiphene citrate in secondary hypogonadism, also known as hypogonadotropic hypogonadism.

The proposed use included the wording “maintain sperm concentrations within the normal range”; however, the clinical benefit of a “normal range” is unclear. A lower reference limit (fifth percentile) for sperm concentration of 15 million per mL is suggested by the World Health Organization (WHO) (Cooper et al. 2009).[48] It is important to note that sperm concentrations and other parameters assessed on semen analysis evaluate aspects of testicular function and are not tests of fertility. A man can have a normal semen analysis but still be infertile because of other sperm abnormalities that may either require further clinical and laboratory assessments or

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[44] “Non-classic” hypogonadism, which is also called functional hypogonadism, is not associated with an intrinsic defect of the HPG axis. See Appendix 1 to this evaluation memo for additional information.
[45] Azoospermia, complete absence of sperm in the semen. See: Medical Dictionary, https://medical-dictionary.thefreedictionary.com/azoospermia, accessed Dec 27, 2021.
[46] Oligospermia, a subnormal concentration of spermatozoa in the penile ejaculate. See: Medical Dictionary, https://medical-dictionary.thefreedictionary.com/oligospermia, accessed Dec 27, 2021.
[47] The 2018 EMA Assessment Report of EnCyzix is available to the public at: https://www.ema.europa.eu/en/documents/assessment-report/encyzix-epar-public-assessment-report_en.pdf.
[48] Lower reference limit values were generated from men whose partners had time-to-pregnancy ≤12 months.

to date be undetected.[49] One study found that 12% of infertile men and only 41% of fertile men had normal sperm parameters according to the WHO reference criteria (Boeri et al. 2020).

FDA issued a guidance for industry in May 2018 to provide recommendations for establishing clinical effectiveness for drugs intended to treat male hypogonadotropic hypogonadism associated with obesity and other conditions that do not cause structural disorders of the hypothalamus or pituitary.[50] This guidance incorporates advice FDA received at the BRUDAC December 2016 meeting on hypogonadotropic hypogonadism. In the guidance, FDA states that changes in semen parameters alone are not sufficient for establishing efficacy of these drugs, since the intent of the drug is to improve fertility, and improvement in semen parameters does not ensure fertility. Therefore, we evaluated the following use, "To increase serum testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) to normal levels in the treatment of secondary hypogonadism," and we did not evaluate "maintain sperm concentrations within the normal range" as part of the proposed use.

Finally, enclomiphene citrate was nominated for oral use. Searches performed for this evaluation did not find any information on the use of enclomiphene citrate by other routes of administration.

2. *Reports of trials, clinical evidence, and anecdotal reports of effectiveness, or lack of effectiveness, of the bulk drug substance*

Published clinical trials and publications of enclomiphene citrate:
The nomination for enclomiphene citrate made reference to three publications evaluating efficacy of enclomiphene citrate (Wiehle et al. 2013; Wiehle et al. 2014a; and Kim et al. 2016). Our search of published medical literature retrieved two additional studies on enclomiphene citrate (Kaminetsky et al. 2013; Wiehle et al. 2014b). We discuss the studies in chronological order.

Kaminetsky et al. (2013) conducted a proof-of-principle, randomized, open-label, active-control, two-center phase IIB trial of enclomiphene citrate in 12 men with secondary hypogonadism who had been using a topical testosterone therapy for at least 6 months. After discontinuing topical testosterone, serum testosterone levels were checked following a 7-14 day washout period; men with levels <300 ng/dL[51] were then randomized to receive daily oral enclomiphene citrate 25 mg (n=7) or topical testosterone gel (n= 5) for 6 months. The trial aimed to evaluate the difference in changes in hormone levels and seminal parameters between enclomiphene citrate and topical testosterone gel treatment. After 6 months of treatment, serum total testosterone levels were 545 ± 269 ng/dL for the testosterone gel and 525 ± 256 ng/dL for enclomiphene citrate groups. LH and FSH increased in men in the enclomiphene citrate group at 3 and 6 months (values not listed in text). Two of the men who received enclomiphene citrate did not have total testosterone

[49] See FDA Briefing Information For The December 6, 2016 Meeting of the Bone, Reproductive and Urologic Drugs Advisory Committee (BRUDAC). https://www.fda.gov/media/101582/download.
[50] See final guidance for industry entitled, Establishing Effectiveness for Drugs Intended to Treat Male Hypogonadotropic Hypogonadism Attributed to Nonstructural Disorders at https://www.regulations.gov/document/FDA-2017-D-6759-0004.
[51] The AUA recommends using a total testosterone level below 300 ng/dL to diagnose low testosterone (Mulhall et al. 2018).

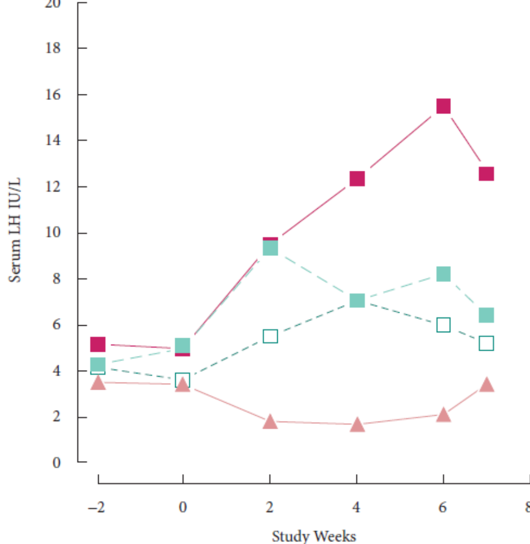
levels exceed 300 ng/dL after 3 or 6 months of treatment, suggesting a lack of efficacy in these patients. The authors theorized that these two men may have had undisclosed primary hypogonadism or were insensitive to enclomiphene citrate. While the authors concluded that enclomiphene citrate increased testosterone stating that the concomitant changes in LH and FSH suggested normalization of endogenous testosterone production, enclomiphene citrate failed to increase testosterone levels in a subset of patients. The authors state that the low number of men in this trial clearly limits the conclusions that can be drawn, and another trial in a larger number of men would be conclusive.

In a randomized, single-blind, two-center, phase II study, Wiehle et al. (2013) evaluated the effects of three different oral dosage strengths of enclomiphene citrate and transdermal testosterone on 24-hour LH and total testosterone, in men ages 18 to 65 with low morning testosterone levels (defined in the study as ≤350 ng/dL) and low or normal LH (<12 IU/L). Forty-four of the 48 enrolled men completed the 6-week study, after being randomized to receive enclomiphene citrate 6.25 mg (n=12), enclomiphene citrate 12.5 mg (n=7), enclomiphene citrate 25 mg (n=12), or transdermal testosterone (AndroGel®) (n=13). Patients underwent a 24-hour assessment of total testosterone, LH, and FSH at week 0 and week 6. Mean testosterone levels increased in all treatment groups at 6 weeks. The percentage of patients with a mean total testosterone concentration >350 ng/dL at 6 weeks in each group was as follows:

- Enclomiphene citrate 6.25 mg: 50%
- Enclomiphene citrate 12.5 mg: 57%
- Enclomiphene citrate 25 mg: 100%
- Transdermal testosterone: 85%

In the transdermal testosterone group, investigators observed greater variability in serum testosterone levels, with a higher percentage of samples with serum total testosterone <300 ng/dL or >1000 ng/dL, and a higher range of total testosterone concentration. LH levels increased in all enclomiphene citrate groups (see Figure 4 below).

Figure 4: Effect of Enclomiphene Citrate and Transdermal Testosterone Treatment on LH Levels (Wiehle et al. 2013)



FSH levels increased in the enclomiphene citrate groups. The authors concluded that enclomiphene citrate increased serum total testosterone into normal range, and increased LH and FSH above the normal range, with the effects on LH and total testosterone persisting for at least 7 days after cessation of treatment.

Wiehle et al. (2014a) published a randomized, placebo-controlled, parallel, multi-center phase IIB trial (ZA-203) to determine the effect of oral enclomiphene citrate in men with secondary hypogonadism. Men with a morning serum testosterone level of <250 ng/dL were randomized to receive enclomiphene citrate 12.5 mg (n=29), enclomiphene citrate 25 mg (n=33), 1% topical testosterone gel (Testim®) (n=33), or oral placebo (n=29). Patients randomized to enclomiphene citrate or placebo arms were double-blinded. The study population had either discontinued previous testosterone treatment for at least 6 months or had never been treated. Seventy-three of the 124 patients enrolled completed the study. The primary endpoint was the change in morning total testosterone level from baseline to the end of the 3-month dosing period. Changes in LH and FSH levels from baseline to month 3 were secondary endpoints.

Total testosterone level increased in all active treatment groups. LH and FSH levels increased in both enclomiphene citrate groups, and decreased in the topical testosterone group. According to the authors, estradiol and dihydrotestosterone increased with statistical significance in all three active treatment arms. Estradiol remained elevated compared to baseline with statistical significance after 1 month of enclomiphene citrate discontinuation. The authors concluded that enclomiphene citrate reverses low serum total testosterone levels and inappropriately low normal serum LH levels, while a topical testosterone product increases testosterone equally well but suppresses LH and FSH. According to the authors, the limitations of this study were several and

included that all hormone values were determined by immunologic-based assays, and the authors state they realize that liquid chromatography mass spectrometry assay are replacing older assays. Future phase III studies would benefit from more men enrolled.

In a randomized, placebo-controlled trial, 52 men ages 18-75 with low (<250 ng/dL) or borderline-low (250-350 ng/dL) serum testosterone were randomized to receive oral enclomiphene citrate 12.5 mg (n=10), enclomiphene citrate 25 mg (n=11), enclomiphene citrate 50 mg (n=11), topical testosterone gel (Androgel® 1% (5 g)) (n=10), or oral placebo (n=10) daily for 14 days (there was no topical placebo arm) (Wiehle et al. 2014b). The study aimed to evaluate safety, efficacy, and pharmacokinetics of enclomiphene citrate (referred to as Enclomid in Table 4) as an alternative to topical testosterone. All patients completed the initial study, and patients who requalified for the study based on testosterone levels were crossed over to receive open-label topical testosterone (Androgel® 1% (10 g)) daily for 14 days under an amended protocol (n=10). Patients randomized to enclomiphene citrate or placebo arms were double-blinded. Testosterone levels and other hormones including LH, FSH, and estradiol were measured at baseline and after 2 weeks. Serum total testosterone levels after 14 days increased with statistical significance in active treatment groups, as seen in the Table 4 below:

Table 4: Serum Total Testosterone at Baseline Day 1 and Day 15 (Wiehle et al. 2014b)

	Total serum testosterone (ng/dL) results					
Parameter	*Enclomid 12.5 mg* (n = *10*)	*Enclomid 25 mg* (n = *11*)	*Enclomid 50 mg* (n = *11*)	*Topical testosterone 1% 5 g* (n = *10*)	*Topical testosterone 1% 10 g* (n = *10*)	*Placebo* (n = *10*)
Screening mean (SD)	220.8 (54.8)	244.2 (55.4)	255.6 (59.9)	234.2 (65.7)	264.4 (103.1)	267.9 (57.7)
Day 1 mean (SD)	242.9 (102.4)	273.2 (63.7)	295.2 (109.6)	260.8 (90.9)	245.6 (103.4)	301.3 (72.7)
Day 15 mean (SD)	411.5 (219.4)	520.1 (160.3)	589.5 (172.5)	473.0 (289.3)	608.1 (322.8)	300.1 (108.6)
Day 15 change (SD)	168.6 (145.8)	246.9 (141.2)	294.3 (192.4)	212.2 (264.6)	362.5 (398.6)	−1.20 (73.7)
p-Value vs. baseline	$p = 0.0053$	$p = 0.0002$	$p = 0.0005$	$p = 0.0428$	$p = 0.0183$	$p = 0.9601$
p-Value vs. placebo	$p = 0.0041$	$p < 0.0001$	$p = 0.0004$	$p = 0.0437$	$p = 0.0183$	–
Follow-up mean (SD)	351.8 (140.9)	458.0 (104.0)	579.3 (136.9)	256.0 (124.1)	354.1 (273.5)	276.2 (86.5)

The increase in total testosterone was sustained through the follow-up visit 7-10 days after discontinuation of enclomiphene citrate. LH and FSH levels increased with statistical significance in all enclomiphene citrate groups compared to baseline and placebo. The authors concluded that treatment with enclomiphene citrate promoted normalization of endogenous testosterone pathways and increased testosterone, LH, and FSH.

In two parallel, randomized, double-blind, double-dummy, placebo-controlled, multi-center phase III trials (ZA-304 and ZA-305) published by Kim et al. (2016), 256 overweight men aged 18 to 60 years with secondary hypogonadism were treated for 16 weeks with oral enclomiphene citrate 12.5 mg (n=43), enclomiphene citrate 12.5 mg that was up-titrated to 25 mg at week 4 if total testosterone was <450 ng/dL (n=42), topical testosterone gel (Androgel® 1.62%) (n=85), or placebo (n=86). Study objectives were to compare the effects of treatment on total testosterone, LH, FSH, and sperm counts. Inclusion criteria included men with early morning serum total testosterone ≤300 ng/dL and low or inappropriately normal LH (<9.4 IU/L). Total testosterone levels increased in all active treatment groups. LH and FSH increased in the enclomiphene citrate groups and decreased in the topical testosterone group. After cessation of treatment, total testosterone levels in the pooled enclomiphene citrate groups remained higher than baseline for

at least 7 days, while in the topical testosterone group levels decreased. The authors concluded that enclomiphene citrate consistently increased total testosterone to normal levels, increased LH and FSH, and maintained sperm concentration in the normal range. Authors acknowledged that study limitations included the lack of patient-reported outcomes (PROs) evaluated, the lack of actual pregnancy or live birth data, and the short-term duration of the study compared to the length of therapy seen in clinical practice.

A review by Rodriguez et al. (2016) states that enclomiphene citrate, while incompletely studied, is effective in ameliorating testosterone deficiency. The authors note that weaknesses among studies include sample size and lack of comparisons with other SERMs, and that further work to determine the symptomatic benefits of enclomiphene is needed.

Earl and Kim (2019) examined the available literature on the trans-isomer enclomiphene. Their review of the literature included the five clinical trials described above (Kaminetsky et al. 2013, Wiehle et al. 2013, Wiehle et al. 2014a, Wiehle et al. 2014b, and Kim et al. 2016). The publication discusses the following points:

- Clomiphene citrate exists as a mixture of both the cis-isomer (zuclomiphene) and the trans-isomer (enclomiphene). According to the authors, the literature has suggested that most of the beneficial effects of clomiphene are due to the trans-isomer enclomiphene; zuclomiphene contributes little to the intended outcomes. The authors note that enclomiphene maintains the androgenic benefit of clomiphene citrate without the undesirable estrogen agonist effects attributable to zuclomiphene, while also stating in the review that the effects of zuclomiphene, "…are not fully understood."
- Per authors, enclomiphene has been shown to preserve sperm concentration when compared with exogenous testosterone replacement. Note that as discussed in Section II.C.1. of this evaluation, FDA does not consider that changes in semen parameters alone are sufficient for establishing efficacy of drugs intended to treat functional secondary hypogonadism, since the intent of the drug is to improve fertility, and improvement in semen parameters does not ensure fertility.
- The authors state, "[a]lthough the evidence is weak at best, early studies suggest that the side effect profile is not significantly worse than testosterone replacement therapy or clomiphene citrate. Ideally, future research will more clearly delineate and confirm this hypothesis." Note that FDA-approved therapies for secondary hypogonadism, such as testosterone products which are discussed in Section II.B.2., meet established criteria for safety and are labeled accordingly to inform safe use of the product.
- The authors conclude that phase II and phase III trials have shown that enclomiphene achieves comparable testosterone levels to transdermal testosterone replacement therapy while increasing physiologic production of LH and FSH; these effects persist for some time even after the cessation of therapy. According to the authors, this illustrates that enclomiphene acts through the restoration of the physiologic hypothalamic-pituitary-gonadal axis. The authors also state that further studies are necessary to fully characterize the impact on the subjective symptoms of hypogonadism, as well as to fully characterize the potential adverse effect profile.

A search of ClinicalTrials.gov retrieved several completed studies that posted results.[52] These are not discussed in further detail because we did not find corresponding publications and data analysis is not provided on ClinicalTrials.gov.

Considerations on Establishing Effectiveness of Therapy

It is important to note that while studies may demonstrate an increase in testosterone levels with enclomiphene citrate treatment in the study populations above, the clinical meaningfulness of this increase remains unclear. In an FDA guidance for industry (May 2018) which provides recommendations for establishing clinical effectiveness for drugs intended to treat male hypogonadotropic hypogonadism attributed to nonstructural disorders of the hypothalamus or pituitary, FDA notes that it is unclear whether low testosterone concentrations in this population are inappropriately low and whether increasing testosterone in these men confers clinical benefit.[53, 54] Since serum testosterone is not a validated surrogate endpoint for establishing efficacy in these patients, trials should show that a drug provides clinically meaningful improvement in at least one symptom or sign of hypogonadism, in addition to increasing serum testosterone. The guidance also notes that PRO instruments may play a central role in establishing efficacy because they provide direct evidence of how patients feel or function.

It should be noted that enclomiphene citrate trials described above did not evaluate improvement in hypogonadal symptoms or quality of life using validated metrics. A review by Rodriguez et al. (2016) notes, "…a paucity of data examining the symptomatic benefits of SERMs in hypogonadal men exists." As discussed above, Kim et al. (2016) acknowledge that a lack of evaluated PROs was a limitation of the phase III trials ZA-304 and ZA-305. The EMA 2018 Report concluded that, "…although the results of the clinical studies suggest that testosterone levels are increased after administration of enclomifene, there is no evidence that enclomifene provides clinically meaningful benefits on the typical symptoms and signs of secondary hypogonadism especially as validated PRO measures were not studied and patients were not specifically recruited into the studies based on signs and symptoms of secondary hypogonadism."[55] EMA also concluded that normalizing testosterone levels in the patient population included in the clinical trials was not considered sufficient to conclude translation into clinically meaningful benefits for patients with secondary hypogonadism.

Therefore, while studies may demonstrate that administration of enclomiphene citrate increases testosterone levels, this increase does not equate to evidence of clinical effectiveness, as increasing testosterone in these individuals is not a clear clinically relevant endpoint. These trials did not assess signs or symptoms of hypogonadism, patient reported outcomes, or fertility outcomes.

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[52] NIH U.S. National Library of Medicine. www.ClinicalTrials.gov, search term "enclomiphene citrate" and "enclomiphene". Accessed Nov 3, 2021.

[53] See final guidance for industry entitled, Establishing Effectiveness for Drugs Intended to Treat Male Hypogonadotropic Hypogonadism Attributed to Nonstructural Disorders at https://www.regulations.gov/document/FDA-2017-D-6759-0004.

[54] The guidance incorporates advice FDA received at a December 2014 advisory committee meeting on the appropriate indicated population for testosterone therapy and a December 2016 advisory committee meeting on hypogonadotropic hypogonadism.

[55] The 2018 EMA Assessment Report of EnCyzix is available to the public at: https://www.ema.europa.eu/en/documents/assessment-report/encyzix-epar-public-assessment-report_en.pdf.

3. *Whether the product compounded with this bulk drug substance is intended to be used in a serious or life-threatening disease*

Enclomiphene citrate has been evaluated to increase serum testosterone, LH and FSH to normal levels in the treatment of secondary hypogonadism. Low testosterone is a potentially serious medical condition that may result in reduced male fertility, sexual dysfunction, decreased muscle formation and bone mineralization, disturbances of fat metabolism and cognitive dysfunction.[56]

4. *Whether there are any alternative approved therapies that may be as effective or more effective*

There are FDA-approved products indicated for testosterone deficiency, which include testosterone products (in various dosage forms) and chorionic gonadotropin as described in Section II.B.2.e., Section II.C.1., and Appendix 1.

There are several FDA-approved drugs that have been used off-label to treat secondary hypogonadism, such as SERMs like clomiphene citrate. However, there are no data to prove that off-label therapies may be as effective or more effective.

Conclusions: We conclude that there is insufficient information to support the effectiveness of enclomiphene citrate for the proposed use. While studies may suggest that treatment with enclomiphene citrate may increase testosterone levels, with a concurrent increase in LH and FSH levels, it is unclear whether increasing testosterone concentrations alone in men with secondary hypogonadism equates to clinical effectiveness or confers clinical benefit. Clinical trials did not demonstrate that enclomiphene citrate provides clinically meaningful improvement in symptoms or signs of hypogonadism. There are currently FDA-approved therapies with established efficacy for the proposed use.

D. Has the substance been used historically in compounding?

The following databases were consulted in preparation of this section: PubMed, Natural Medicines, European Pharmacopoeia, Japanese Pharmacopoeia, UpToDate, Micromedex, and Google.

1. *Length of time the substance has been used in pharmacy compounding*

The nominator did not provide historical use data. Literature shows that enclomiphene citrate, previously referred to as trans-clomiphene, was studied in humans as early as 1969 for a variety of conditions including polycystic ovarian syndrome, primary ovarian failure, post-steroid amenorrhea, anovulatory cycles, and uterine synechiae (Charles et al. 1969).

Although enclomiphene citrate has been studied for several decades, there is insufficient information available to determine how long enclomiphene citrate has been used specifically in pharmacy compounding.

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[56] European Association of Urology (EAU) Guidelines on Male Hypogonadism. EAU Website. https://uroweb.org/guideline/male-hypogonadism/. Accessed Nov 12, 2021.

2. The medical condition(s) it has been used to treat

According to literature, enclomiphene citrate has been studied for its effects on follicular development, ovulation induction, and secondary hypogonadism (Glasier et al. 1989; Gupta and Kriplani 2013; and Wiehle et al. 2014b); however, the source of the drug product used in these studies is not specified, making it is unclear whether the enclomiphene citrate was compounded.

In Glasier et al. (1989), the authors examined follicular development in women awaiting donor insemination. The women received oral clomiphene citrate and, following a washout period, received either the "En or the Zu isomer" [enclomiphene or zuclomiphene, respectively].

In Gupta and Kriplani (2013), the authors examined the effects of enclomiphene citrate and clomiphene citrate on ovulation induction in "women with unexplained infertility." The women were randomized to receive either oral enclomiphene citrate or clomiphene citrate "from cycle day 3 to day 7."

In the 2014 trial by Wiehle et al., the authors examined enclomiphene citrate in patients with secondary hypogonadism. Patients received either oral enclomiphene citrate, topical testosterone gel, or an oral placebo.

In a 2018 review article by Gupta and Khanna, the authors discussed oral enclomiphene citrate use in studies related to the above conditions as well as briefly reviewed enclomiphene use in "hypogonadism," "male infertility," and "syndrome X."[57]

Enclomiphene citrate use has been discussed on the websites of medical clinics and compounding pharmacies in the United States. For example, one medical clinic website states "Enclomiphene citrate is often suggested as treatment dysfunctional hypothalamus and pituitary function, which occurs in some males who are suffering from secondary hypogonadism."[58] In addition, a compounding pharmacy discussed the use of enclomiphene citrate oral capsules "in the process of treating male hypogonadism" in one of their catalogs.[59]

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[57] Syndrome X is the co-occurrence of metabolic risk factors for both type 2 diabetes and cardiovascular disease, also called metabolic syndrome or insulin resistance syndrome. See: Metabolic syndrome (insulin resistance syndrome or syndrome X). UpToDate. www.uptodate.com. Subscription required; Accessed Nov 30, 2021.

[58] Enclomiphene Citrate Alternative to HCG. Florida Alternative Medicine and Weight Loss. https://flalternativemeds.com/enclomiphene-citrate-alternative-to-hcg/. Accessed Nov 30, 2021.

[59] See Tailor Made Compounding Catalog 2021. https://imcwc.bpl.fyi/wp-content/uploads/2021/11/TMC_Fall-Catalog.pdf. Accessed Nov 30, 2021.

3. *How widespread its use has been*

According to FDA outsourcing facility product reporting data from January 2017 to June 2021, there were no reported compounded drug products containing enclomiphene citrate.[60]

Insufficient data are available from which to draw conclusions about the extent of use of enclomiphene citrate in compounded drug products within and outside of the United States.

4. *Recognition of the substance in other countries or foreign pharmacopeias*

A search of the European Pharmacopeia (10th Edition, 10.7) and the Japanese Pharmacopeia (17th Edition) did not show any monograph listings for enclomiphene citrate.

In January 2018, enclomiphene citrate (brand name "EnCyzix") was denied marketing authorization by the EMA's Committee of Medicinal Products for Human Use. The Committee stated that the studies performed did not examine if the drug product "would improve symptoms such as bone strength, weight, gain, impotence, and libido" and that "there is a risk of venous thromboembolism."[61]

Conclusions: Enclomiphene citrate has been studied for several decades primarily for its use in ovulation induction and secondary hypogonadism. It has been advertised by clinics and compounding pharmacies in the U.S.; however, there is insufficient information about the length and extent of its use in the U.S. There is also insufficient information about its use in compounding internationally. Enclomiphene citrate is not recognized in the European or Japanese pharmacopeias.

III. RECOMMENDATION

We have balanced the criteria described in Section II above to evaluate enclomiphene citrate for the 503A Bulks List. After considering the information currently available, a balancing of the criteria ***weighs against*** enclomiphene citrate being placed on that list based on the following:

1. Enclomiphene citrate is a well-characterized small molecule and is expected to be stable under ordinary storage conditions when protected from light.

2. The nonclinical toxicity profile of enclomiphene citrate reflects its exaggerated pharmacological action as a selective estrogen receptor modulator. When tested in rats and dogs, enclomiphene citrate showed dose-related findings including reduced body weight, reduced food consumption, organ weight decreases (prostate gland, pituitary

[60] The Drug Quality and Security Act, signed into law on November 27, 2013, created a new section 503B in the Federal Food, Drug, and Cosmetic Act. Under section 503B, a compounder can become an outsourcing facility. Outsourcing facilities are required to provide FDA with a list of drugs they compounded during the previous six-month period upon initial registration and in June and December each year. This retrospective information does not identify drugs that outsourcing facilities intend to produce in the future.

[61] EnCyzix. European Medicines Agency. https://www.ema.europa.eu/en/medicines/human/EPAR/encyzix. Accessed Nov 30, 2021.

gland, and epididymides), and histopathological findings (prostate, testes, seminal vesicles, and kidneys). Enclomiphene citrate was not genotoxic or carcinogenic under the conditions of the conducted studies.

Based on available clinical information, we conclude that safety concerns for enclomiphene citrate include cardiac and thromboembolic events, as reported adverse events in clinical trials included bradycardia, atrial flutter, hypotension, deep vein thrombosis, pulmonary embolism, and ischemic stroke; elevated estradiol, increased prostate-specific antigen, and hematocrit. Pharmacokinetic data are also limited, including information on dose adjustments for patients with renal or hepatic impairment. There are currently available FDA-approved therapies indicated for male patients with hypogonadism; these products meet established criteria for safety and efficacy and are labeled accordingly to inform safe use of the product.

3. There is insufficient information to support the effectiveness of enclomiphene citrate for the proposed use. While studies may suggest that treatment with enclomiphene citrate may increase testosterone levels, with a concurrent increase in LH and FSH levels, it is unclear whether increasing testosterone concentrations alone in men with secondary hypogonadism equates to clinical effectiveness or confers clinical benefit. Clinical trials did not demonstrate that enclomiphene citrate provides clinically meaningful improvement in symptoms or signs of hypogonadism. There are currently FDA-approved therapies with established efficacy for the proposed use.

4. Enclomiphene citrate has been studied for several decades primarily for its use in ovulation induction and secondary hypogonadism. It has been advertised by clinics and compounding pharmacies in the U.S.; however, there is insufficient information about the length and extent of its use in the U.S. There is also insufficient information about its use in compounding internationally.

Based on this information the Agency has considered, a balancing of the four evaluation criteria ***weighs against*** enclomiphene citrate being added to the 503A Bulks List.

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APPENDIX 1: MALE HYPOGONADISM

Background, classification, clinical features, and etiology

The hypothalamic-pituitary-testicular axis, also referred to as the hypothalamic-pituitary-gonadal (HPG) axis, regulates testosterone synthesis (Majzoub and Sabanegh 2016). The hypothalamus releases gonadotropin-releasing hormone, which stimulates the release of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH stimulates testicular Leydig cells to produce testosterone. FSH, in conjunction with intratesticular testosterone, stimulates spermatogenesis by acting on testicular Sertoli cells and seminiferous tubules. Testosterone is metabolized to dihydrotestosterone and estradiol. Estradiol (an estrogen hormone) and testosterone provide negative feedback at the level of the hypothalamus and pituitary to inhibit gonadotropin secretion (Basaria 2014) (see Figure 5 below).

Figure 5: The Hypothalamic-Pituitary-Gonadal Axis (Majzoub and Sabanegh 2016)

Figure 1 The hypothalamopituitary gonadal axis. GnRH, gonadotropin releasing hormone; GnH, gonadotropin hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; DHT, dihydrotestosterone.

Male hypogonadism is a clinical syndrome that results from failure of the testis to produce physiological concentrations of testosterone and/or a normal number of spermatozoa due to pathology in the HPG axis (Bhasin et al. 2018). Hypogonadism is classified as primary or secondary (Hill et al. 2009; Basaria 2014):

- Primary hypogonadism: dysfunction arising from the level of the testes; characterized by low serum testosterone levels and spermatogenesis, resulting in elevated levels of gonadotropins (high LH and FSH) in a stimulatory effort (hypergonadotropic hypogonadism).
- Secondary hypogonadism: dysfunction arising from the level of hypothalamus or pituitary; testosterone levels and spermatogenesis are low, with low or inappropriately normal gonadotropin levels (hypogonadotropic hypogonadism).

Signs and symptoms of hypogonadism vary depending on age of onset, severity of testosterone deficiency, androgen sensitivity, and previous use of testosterone-replacement therapy. Clinical manifestations with post-pubertal onset of hypogonadism may include decreased libido, decreased spontaneous erections, decrease in testicular volume, gynecomastia, hot flashes, decreased bone mass, height loss, decreased pubic or axillary hair, decreased muscle mass, and decreased energy and motivation (Basaria 2014). The most sensitive symptoms supporting a diagnosis of hypogonadism are erectile dysfunction and decreased libido (Rodriguez et al. 2016).

Both primary and secondary hypogonadism can be classified as organic (caused by intrinsic pathology such as congenital, structural, or destructive disorders) or functional (not associated with an intrinsic defect of the HPG axis) (Bhasin et al. 2018); potential causes are listed in the table below. Causes of primary and secondary hypogonadism can also be differentiated by congenital or acquired etiologies.

Table 5: Causes of Primary and Secondary Hypogonadism (Bhasin et al. 2018)

Table 1. Classification of Hypogonadism and Causes of Primary and Secondary Hypogonadism

Primary Hypogonadism	Secondary Hypogonadism
ORGANIC	
KS	Hypothalamic/pituitary tumor
Cryptorchidism, myotonic dystrophy, anorchia	Iron overload syndromes
Some types of cancer chemotherapy, testicular irradiation/damage, orchidectomy	Infiltrative/destructive disease of hypothalamus/pituitary
Orchitis	Idiopathic hypogonadotropic hypogonadism
Testicular trauma, torsion	
Advanced age	
FUNCTIONAL	
Medications (androgen synthesis inhibitors)	Hyperprolactinemia
End-stage renal disease[a]	Opioids, anabolic steroid use, glucocorticoids
	Alcohol and marijuana abuse[a]
	Systemic illness[a]
	Nutritional deficiency/excessive exercise
	Severe obesity, some sleep disorders
	Organ failure (liver, heart, and lung)[a]
	Comorbid illness associated with aging[a]

[a]Combined primary and secondary hypogonadism, but classified to usual predominant hormonal pattern. Adapted with permission from Bhasin *et al.* (7).

Note: KS, Klinefelter Syndrome

Functional causes of secondary hypogonadism, such as obesity and aging, have garnered increasing attention. The European Male Aging Study, a large, population-based study, found men with a high Body Mass Index over 25 kg/m^2 are more likely to have secondary hypogonadism, and concluded that obesity is the most powerful predictor of low testosterone due to HPG dysregulation (Tajar et al. 2010). Additionally, in recent years, testosterone use increased markedly among middle-aged and elderly men for a controversial condition sometimes referred to as 'andropause,' 'late-onset hypogonadism,' or 'age-related hypogonadism.' This condition refers to men who have low serum testosterone concentrations for no apparent

reason other than advancing age, and who experience non-specific symptoms that could be consistent with the low testosterone concentrations but could also result from aging or comorbidities, such as decreases in energy level, sexual function, bone mineral density, muscle mass and strength, and increases in fat mass. Serum concentrations of testosterone decrease as men age, and can fall below the lower limit of the normal range for younger, healthy men. Whether these signs and symptoms are a clinical consequence of this age-related decline in endogenous testosterone is unclear, and the clinical benefit of replacing or supplementing testosterone in these older men has not been clearly established.[62]

Diagnosis

Diagnosis of male hypogonadism is based on assessment of signs and symptoms, and low morning total testosterone levels on at least two occasions (Bhasin et al. 2018). Laboratory definitions of low testosterone level vary; for example, the American Urologic Association (AUA)[63] recommends total testosterone level below 300 ng/dL to diagnose low testosterone (Mulhall et al. 2018), while the Endocrine Society[64] suggests a lower limit of 264 ng/dL (Bhasin et al. 2018). Serum total testosterone is the best screening test; however, because 40% of testosterone is bound to sex-hormone-binding globulin (SHBG), free or bioavailable testosterone should be measured if abnormalities in SHBG are suspected.[65] Measurement of gonadotropin levels (FSH and LH) helps to differentiate between primary and secondary hypogonadism (Bhasin et al. 2018). Reference ranges for FSH and LH levels in adult males are:

- FSH: 1.5 - 12.4 mIU/mL[66]
- LH: 1.7 - 8.6 mIU/mL[67]

Treatment of hypogonadism

Treatment of hypogonadism depends in part on the underlying etiology of the condition and on the patient's goals for immediate fertility.[68] Direct androgen replacement with testosterone is the only treatment option in primary hypogonadism (Hill et al. 2009). The focus of our subsequent discussion is on secondary hypogonadism. Products approved for the treatment of secondary hypogonadism include testosterone products and human chorionic gonadotropin. Secondary hypogonadism is often managed with testosterone (Rodriguez et al. 2016). However, because

[62] See FDA Briefing Information For The December 6, 2016 Meeting of the Bone, Reproductive and Urologic Drugs Advisory Committee (BRUDAC). https://www.fda.gov/media/101582/download.

[63] The American Urological Association is a urologic association with a mission to promote the highest standards of urological clinical care through education, research, and the formulation of health care policy (https://www.auanet.org/about-us/about-the-aua). Accessed Sep 20, 2021.

[64] The Endocrine Society is a not-for-profit organization representing basic, applied, and clinical interests in endocrinology (https://rarediseases.org/organizations/endocrine-society/).The society is devoted to advancing hormone research, excellence in the clinical practice of endocrinology, broadening understanding of the critical role hormones play in health, and advocating on behalf of the global endocrinology community (https://www.endocrine.org/about-us). Accessed Aug 20, 2021.

[65] Conditions that can alter concentrations of SHBG in serum include aging, obesity, thyroid disease, diabetes, HIV, exogenous administration of glucocorticoids, androgens, and progestins (Basaria 2014).

[66] Follicle-stimulating Hormone (FSH). Labcorp. https://www.labcorp.com/tests/004309/follicle-stimulating-hormone-fsh. Accessed Nov 10, 2021.

[67] Luteinizing Hormone (LH). Labcorp. https://www.labcorp.com/tests/004283/luteinizing-hormone-lh. Accessed Nov 10, 2021.

[68] See FDA Briefing Information For The December 6, 2016 Meeting of the Bone, Reproductive and Urologic Drugs Advisory Committee (BRUDAC). https://www.fda.gov/media/101582/download.

testosterone can impair spermatogenesis, there has been interest in developing non-testosterone alternatives for men with secondary hypogonadism who desire to maintain fertility, such as gonadotropins (e.g., chorionic gonadotropin) and estrogen receptor modulators. These treatments are discussed in additional detail below.

Testosterone Replacement Therapy
Testosterone has been approved in the United States since the 1950s as replacement therapy (often referred to as testosterone replacement therapy (TRT)) in men for conditions associated with a deficiency or absence of endogenous testosterone.[69] A clinical practice guideline from the Endocrine Society recommends testosterone therapy in hypogonadal men to induce and maintain secondary sex characteristics and correct symptoms of testosterone deficiency (Bhasin et al. 2018). Available formulations of testosterone include transdermal gels and patches, axillary solutions, implant pellets, nasal gels, oral capsules, and intramuscular and subcutaneous injections.[70] However, exogenous testosterone treatment inhibits the HPG axis, decreasing LH and FSH and decreasing spermatogenesis. Most men who discontinue testosterone replacement will have a return of normal sperm production within one year (Crosnoe et al. 2013). The Endocrine Society recommends against starting testosterone therapy in patients who desire fertility in the near term (Bhasin et al. 2018).

Gonadotropins
In men with secondary hypogonadism who desire fertility, gonadotropin therapy is the treatment of choice (Corona et al. 2015). Human chorionic gonadotropin (HCG) is the only FDA-approved non-testosterone treatment for testosterone deficiency in men (Krzastek and Smith 2020).[71] HCG functions as an LH analog that stimulates endogenous testosterone production and initiates spermatogenesis, but concurrent use of FSH may be required to induce spermatogenesis (Majzoub and Sabanegh 2016). If no sperm are detected by semen analysis after six months of treatment with HCG, an FSH product can be added to the regimen. Several recombinant FSH products (e.g., Follistim®[72], Gonal-F®[73]) are approved by the FDA for induction of spermatogenesis.[74] Additionally, the combination of lower doses of HCG while on testosterone replacement has been evaluated as an alternative approach to preserve fertility (Majzoub and Sabanegh 2016).

Estrogen Receptor Agonists/Antagonists
Selective estrogen receptor modulators act as estrogen receptor agonists in some tissues and antagonists in others. Some of these products are believed to block the negative feedback effect of estradiol at the level of the hypothalamus/pituitary gland, increasing FSH and LH secretion

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[69] See FDA Briefing Information For The December 6, 2016 Meeting of the Bone, Reproductive and Urologic Drugs Advisory Committee (BRUDAC). https://www.fda.gov/media/101582/download.
[70] Orange Book. https://www.accessdata.fda.gov/scripts/cder/ob/search_product.cfm, search term "testosterone." Accessed Nov 2, 2021. See Section II.B.2.e. for links to sample labels.
[71] See information from package insert label for chorionic gonadotropin, BLA 017067/S-57. Drugs@FDA, https://www.accessdata.fda.gov/scripts/cder/daf/. Accessed Oct 6, 2021.
[72] See information from label for FOLLISTIM AQ, BLA 021211/S-11. Drugs@FDA, https://www.accessdata.fda.gov/scripts/cder/daf/. Accessed Nov 2, 2021.
[73] See information from label for GONAL-F (follitropin alfa for injection), for subcutaneous use, BLA 020378/S-75. Drugs@FDA, https://www.accessdata.fda.gov/scripts/cder/daf/. Accessed Nov 2, 2021.
[74] See FDA Briefing Information For The December 6, 2016 Meeting of the Bone, Reproductive and Urologic Drugs Advisory Committee (BRUDAC). https://www.fda.gov/media/101582/download.

and stimulating endogenous testosterone production. These products have been of interest as an alternative to TRT because they do not suppress the HPG axis and should, therefore, not inhibit spermatogenesis (Rambhatla et al. 2016). However, the HPG axis needs to be relatively intact so that sufficient FSH and LH can be produced and so that the testes can sufficiently respond. Therefore, such products are unlikely to be useful in men with primary hypogonadism or in those with organic hypogonadism. Instead, there is interest in using such therapies for the treatment of men with functional causes of secondary hypogonadism, such as obesity.[75] These therapies include compounds such as clomiphene citrate and enclomiphene citrate.

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[75] See FDA Briefing Information For The December 6, 2016 Meeting of the Bone, Reproductive and Urologic Drugs Advisory Committee (BRUDAC). https://www.fda.gov/media/101582/download.

Tab 2

Glutathione

Tab 2a

Glutathione Nominations

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September 30, 2014

VIA ELECTRONIC SUBMISSION

Division of Dockets Management [HFA-305]
Food and Drug Administration
5630 Fishers Lane, Room 1061
Rockville, MD 20852

Re: Bulk Drug Substances That May Be Used To Compound Drug Products in Accordance With Section 503A of the Federal Food, Drug, and Cosmetic Act; Revised Request for Nominations

Docket No. FDA-2013-N-1525

Dear Sir/Madam:

The Alliance for Natural Health USA ("ANH-USA") submits this comment on the Notice: "Bulk Drug Substances That May Be Used To Compound Drug Products in Accordance With Section 503A of the Federal Food, Drug, and Cosmetic Act; Revised Request for Nominations" published in the Federal Register of July 2, 2014 by the Food and Drug Administration ("FDA" or the "Agency").

ANH-USA appreciates this opportunity to comment on the list of bulk drug substances that may be used to compound drug products pursuant to Section 503A of the FD&C Act ("FDCA"), 21 U.S.C. §353a (hereinafter the "503A List"). This list of ingredients is crucial to patients who require compounded substances, in particular those substances that are available only across state lines. ANH-USA therefore write to request that the Agency:

A) Extend the deadline for nominations by at least 90 days;
B) Maintain the 1999 List; and
C) Accept the ingredients set forth herein and in the attached submissions as nominations for inclusion in the 503A List.

As discussed in detail below, in the interest compiling a comprehensive 503B List, more time is needed to provide the required information. This will benefit both FDA, by reducing the subsequent number of petitions for amendments, and consumers, by allowing continued access to important substances.

Organizational Background of Commenter Alliance for Natural Health USA

ANH-USA is a membership-based organization with its membership consisting of healthcare practitioners, food and dietary supplement companies, and over 335,000 consumer advocates. ANH-USA focuses on the protection and promotion of access to healthy foods, dietary nutrition, and natural compounded medication that consumers need to maintain optimal health. Among ANH-USA's members are medical doctors who prescribe, and patients who use, compounded medications as an integral component of individualized treatment plans.

ANH-USA's Request and Submissions Regarding Docket No. FDA-2013-N-1525

A) Extend the deadline for nominations by at least 90 days

This revised request for nominations follows the initial notice published in the Federal Register of December 4, 2013. Like the initial notice, this revised request provides only a 90 day response period. However, FDA is requiring more information than it sought originally and yet providing the same amount of time for the submission of nominations. The September 30, 2014 deadline for such a complex and expansive request is unreasonably burdensome and woefully insufficient.

The task set forth by FDA to nominate bulk drug substances for the 503A List places an undue burden on those who are responding. The Agency requires highly technical information for each nominated ingredient, including data about the strength, quality and purity of the ingredient, its recognition in foreign pharmacopeias and registrations in other countries, history with the USP for consideration of monograph development, and a bibliography of available safety and efficacy data, including any peer-reviewed medical literature. In addition, FDA is requiring information on the rationale for the use of the bulk drug substance and why a compounded product is necessary.

For the initial request for nomination, it was estimated that compiling the necessary information for just one nominated ingredient would require five to ten hours. With the revised request requiring more information, the time to put together all of the data for a single nomination likely will be higher. Given that it is necessary to review all possible ingredients and provide the detailed support, or risk losing important therapeutic ingredients, this task requires more time than has been designated by the Agency. While ANH-USA recognizes there will be additional opportunities to comment and petition for amendments after the 503A List is published, the realities of substances not making the list initially makes this request for more time imperative. For example, if a nomination for a substance cannot be completed in full by the current September 30, 2014 deadline, doctors and patients will lose access to such clinically important substances and face the

administrative challenges in obtaining an ingredient listing once the work of the advisory committee is completed. There is no regulatory harm in providing additional time to compile a well-researched and comprehensive initial 503A List.

B) Rescind the withdrawal of the ingredient list published on January 7, 1999

In the revised request for nomination, the Agency references in a footnote its withdrawal of the proposed ingredient list that was published on January 7, 1999. ANH-USA argued against this in its March 4, 2014 comment and would like to reiterate its opposition to the withdrawal. There is no scientific or legal justification to require discarding the work that lead to the nominations and imposing the burden on interested parties to begin the process all over again.

C) Accept the ingredients set forth herein and in the attached submissions as nominations for inclusion in the 503A List

ANH-USA submits the following ingredients for nomination for the 503B list:

1. The attached Excel spreadsheets for 21 nominated ingredients prepared by IACP in support of its petition for the nomination of these ingredients; and
2. The submissions for Copper Hydrosol and Silver Hydrosol from Natural Immunogenics Corp.,[1] with their Canadian Product Licenses as proof of safety and efficacy.

In conclusion, Alliance for Natural Health USA requests that FDA provide a more realistic time frame, adding at least 90 days to the current deadline; rescind the withdrawal of the ingredient list published on January 7, 1999; and accept the ingredient nominations for approval for use.

Sincerely,

Gretchen DuBeau, Esq.
Executive and Legal Director
Alliance for Natural Health USA

[1] As of October 1, 2014, the address for Natural Immunogenics Corp. will be 7504 Pennsylvania Ave., Sarasota, FL 34243.

Column A—What information is requested?	Column B—Put data specific to the nominated substance
What is the name of the nominated ingredient?	Reduced L-glutathione
Is the ingredient an active ingredient that meets the definition of ‘‘bulk drug substance’’ in § 207.3(a)(4)?	Yes. Glutathione disulfide is a component of FDA-approved product.
Is the ingredient listed in any of the three sections of the Orange Book?	Glutathione disulfide is a component of a FDA-approved irrigation product.
Were any monographs for the ingredient found in the USP or NF monographs?	There is a Dietary Supplement monograph in the USP for this substance
What is the chemical name of the substance?	2-amino-5-[(R)-1-(carboxymethylamino)-3-mercapto-1-oxopropan-2-ylamino]-5-oxo, (S); N-(N-L- -Glutamyl-L-cysteinyl)glycine; Glycine, N-(N-L-gamma-glutamyl-L-cysteinyl)
What is the common name of the substance?	Reduced L-glutathione
Does the substance have a UNII Code?	GAN16C9B8O
What is the chemical grade of the substance?	Dietary Supplement grade
What is the strength, quality, stability, and purity of the ingredient?	A valid Certificate of Analysis accompanies each lot of raw material received. This bulk drug substance is a generally recognized as safe (GRAS) dietary supplement.
How is the ingredient supplied?	Glutathione is supplied as a white crystalline powder
Is the substance recognized in foreign pharmacopeias or registered in other countries?	EINECS: This product is on the European Inventory of Existing Commercial Chemical Substances (EINECS No. 200-725-4). Canada: Listed on Canadian Domestic Substance List (DSL). China: Listed on National Inventory. Japan: Listed on National Inventory (ENCS). Korea: Listed on National Inventory (KECI). Philippines: Listed on National Inventory (PICCS). Australia: Listed on AICS.
Has information been submitted about the substance to the USP for consideration of monograph development?	A Dietary Supplement monograph for this bulk drug substance is available in the USP.
What dosage form(s) will be compounded using the bulk drug substance?	Injection, inhalation
What strength(s) will be compounded from the nominated substance?	Strengths can range from 60 mg/mL (up to 1.8g/30 mL) to 200 mg/mL (up to 6 g/30 mL) in various sizes of multiple dose or preservative free vials.
What are the anticipated route(s) of administration of the compounded drug product(s)?	Intravenous, intramuscular or inhalation

Are there safety and efficacy data on compounded drugs using the nominated substance?	Mol Aspects Med. 2009;30(1-2):1-12.[PubMed 18796312] 2. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr. 2004;134(3):489-492.[PubMed 14988435] 3. Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K, Hammond CL. Glutathione dysregulation and the etiology and progression of human diseases. Biol Chem. 2009;390(3):191-214.[PubMed 19166318] 4. Micke P, Beeh KM, Buhl R. Effects of long-term supplementation with whey proteins on plasma glutathione levels of HIV-infected patients. Eur J Nutr. 2002;41(1):12-18.[PubMed 11990003] 5. Simoni RD, Hill RL, Vaughan M. The discovery of glutathione by F. Gowland Hopkins and the beginning of biochemistry at Cambridge University. J Biol Chem. 2002;277(24):e13. 6. Pompella A, Visvikis A, Paolicchi A, De Tata V, Casini AF. The changing faces of glutathione, a cellular protagonist. Biochem Pharmacol. 2003;66(8):1499-1503.[PubMed 14555227] 7. Martin HL, Teismann P. Glutathione—a review on its role and significance in Parkinson's disease. FASEB J. 2009;23(10):3263-3272.[PubMed 19542204] 8. Pocernich CB, Butterfield DA. Elevation of glutathione as a therapeutic strategy in Alzheimer disease.
Has the bulk drug substance been used previously to compound drug product(s)?	Yes
What is the proposed use for the drug product(s) to be compounded with the nominated substance?	Glutathione (typically at 60 to 200 mg/ml for IV administration, or 60 to 300 mg per dose for inhalation) is a tripeptide synthesized from Glycine, Glutamic acid and Cysteine. It is a primary intracellular antioxidant that is essential to life. It is useful to prevent radiation injury before radiation treatment is started. It helps reduce the side effects of chemotherapy treatments. It can prevent or reverse alcohol induced fatty liver cirrhosis, hepatitis, and liver lesions. It inhibits chemical induced carcinogenesis. It improves prognosis of stroke victims. It has been used in patients with COPD or chronic lung disease to improve lung functions and minimize oxygen-dependency. It is one of the main anti-oxidation protocols in children with Autistic Syndrome Disorder. Generally, It is useful in any condition where there is a risk for oxidative stress/damage.
What is the reason for use of a compounded drug product rather than an FDA-approved product?	Various liver disease protocols can include anti-viral agents, chemotherapy, lactulose, beta blockers, diuretics, and antibiotics. Cancer treatments protocols include surgery, chemotherapy, and radiation. Stroke treatments can include aspirin, TPA, and more invasive procedures depending upon the acute nature of the symptoms. Glutathione would be used in conjunction with the above protocols to reduce the intensity of symptoms of the disease processes, and to reduce the severity of the side effects of the pharmaceutical protocols. There are no FDA-approved drug products including Reduced L-glutathione for injection, inhalation.

| Is there any other relevant information? | appears much more attractive than a foreign chemical with possible toxic side effects, acute and chronic. Most or all patients with Autistic Syndrome Disorder receives glutathione therapy. 20 - 30 % of patients with COPD or chronic lung disease may benefit from inhaled glutathione as an adjuvant therapy. Libyan J Med. 2014 Jun 12;9:23873. doi: 10.3402/ljm.v9.23873. eCollection 2014.
Oxidative stress and lung function profiles of male smokers free from COPD compared to those with COPD: a case-control study.
Ben Moussa S1, Sfaxi I2, Tabka Z3, Ben Saad H4, Rouatbi S3.
Author information 1Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; bm.syrine@gmail.com.2Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia.3Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; Laboratory of Physiology, Faculty of Medicine of Sousse, University of Sousse, Sousse, Tunisia.4Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; Laboratory of Physiology, Faculty of Medicine of Sousse, University of Sousse, Sousse, Tunisia; Research Unit: Secondary Prevention after Myocardial Infarction, Faculty of Medicine of Sousse, Sousse, Tunisia.
Abstract
BackGROUND: The mechanisms of smoking tobacco leading to chronic obstructive pulmonary disease (COPD) are beginning to be understood. However, conclusions about the role of blood or lung oxidative stress markers were disparate.

AIMS: To investigate the oxidative stress in blood or lung associated with tobacco smoke and to evaluate its effect on pulmonary function data and its relation with physical activity.

METHODS: It is a case-control study. Fifty-four male-smokers of more than five pack-years (PY) and aged 40-60 years were included (29 Non-COPD, 16 COPD). Physical activity score was determined. Blood sample levels of malondialdehyde (MDA), protein-cys SH (PSH), and Glutathione (GSH) were measured. Fractional exhaled nitric oxide (FeNO) and plethysmographic measurements were performed. Correlation coefficients (r) evaluated the association between oxidative stress markers and independent variables |
| | |

September 30, 2014

Division of Dockets Management (HFA-305)
Food and Drug Administration
Department of Health and Human Services
5630 Fishers Lane, Room 1061
Rockville, MD 20852

Re: Docket FDA-2013-N-1525

> "Bulk Drug Substances That May Be Used to Compound Drug Products in Accordance With Section 503A of the Federal Food, Drug, and Cosmetic Act; Revised Request for Nominations"

To Whom It May Concern:

The American Association of Naturopathic Physicians (AANP) appreciates the opportunity to address the FDA's request for nominations of bulk drug substances that may be used to compound drug products that are neither the subject of a United States Pharmacopeia (USP) or National Formulary (NF) monograph nor components of FDA-approved drugs.

This is a significant issue for our members and their patients. AANP strongly supports efforts to ensure that the drug products dispensed to patients are safe and effective.

Background: AANP Submissions to Date

On January 30, 2014, we submitted comments to Docket FDA-2013-D-1444, "Draft Guidance: Pharmacy Compounding of Human Drug Products Under Section 503A of the Federal Food, Drug, and Cosmetic Act; Withdrawal of Guidances" relating to congressional intent in crafting HR 3204. These comments highlighted the fact that, for compounding pharmacies subject to Section 503A, Congress intended that States continue to have the authority to regulate the availability of safely compounded medications obtained by physicians for their patients. As we further noted, compounded medications that are formulated to meet unique patient needs, and that can be administered immediately in the office, help patients receive the products their physicians recommend and reduce the medical and financial burden on both the patient and

doctor that restrictions on office use would impose. Such medications, we emphasized, provide a unique benefit to patients and have an excellent track record of safety when properly produced and stored.

AANP also (on March 4, 2014) nominated 71 bulk drug substances. We identified 21 more where we did not have the capacity to research and present all the necessary documentation within the timeframe the Agency was requiring. We estimated, at that time, that at least 6 hours per ingredient would be needed to do so – time that our physician members simply do not have in their day-to-day business of providing patient care. Thus, AANP sought a 90-day extension to more completely respond to the Agency's request.

In this renomination, we have narrowed our focus to 42 bulk drug substances that are most important for the patients treated by naturopathic doctors. Twenty-one of these bulk drug substances are formally nominated in the attachments as well as noted by name in this letter. Given the limitations imposed by the fact that our physician members spend the majority of their day providing patient care, however, AANP again found that the span of time the Agency provided for renominations was insufficient to prepare the documentation needed for the remaining 21 bulk drug substances.

We now request that FDA extend the deadline for which comments are due by 120 days, so that we may provide this further documentation. We have determined that as much as 40 hours per ingredient will be needed to do so – time that our physician members simply do not have in their day-to-day business of providing patient care. Thus, AANP respectfully seeks an additional 120-day period for the purpose of gathering this essential information.

Naturopathic Medicine and Naturopathic Physicians

A word of background on our profession is in order. AANP is a national professional association representing 4,500 licensed naturopathic physicians in the United States. Our members are physicians trained as experts in natural medicine. They are trained to find the underlying cause of a patient's condition rather than focusing solely on symptomatic treatment. Naturopathic doctors (NDs) perform physical examinations, take comprehensive health histories, treat illnesses, and order lab tests, imaging procedures, and other diagnostic tests. NDs work collaboratively with all branches of medicine, referring patients to other practitioners for diagnosis or treatment when appropriate.

NDs attend 4-year, graduate level programs at institutions recognized through the US Department of Education. There are currently 7 such schools in North America. Naturopathic medical schools provide equivalent foundational coursework as MD and DO schools. Such coursework includes cardiology, neurology, radiology, obstetrics, gynecology, immunology, dermatology, and pediatrics. In addition, ND programs provide extensive education unique to the naturopathic approach, emphasizing disease prevention and whole person wellness. This includes the prescription of clinical doses of vitamins and herbs and safe administration via oral, topical, intramuscular (IM) and intravenous (IV) routes.

Degrees are awarded after extensive classroom study and clinical training. In order to be licensed to practice, an ND must also pass an extensive postdoctoral exam and fulfill annual continuing education requirements. Currently, 20 states and territories license NDs to practice.

Naturopathic physicians provide treatments that are effective and safe. Since they are extensively trained in pharmacology, NDs are able to integrate naturopathic treatments with prescription medications, often working with conventional medical doctors and osteopathic doctors, as well as compounding pharmacists, to ensure safe and comprehensive care.

Characteristics of Patients Seen by Naturopathic Physicians

Individuals who seek out NDs typically do so because they suffer from one or more chronic conditions that they have not been able to alleviate in repeated visits to conventional medical doctors or physician specialists. Such chronic conditions include severe allergies, asthma, chronic fatigue, chronic pain, digestive disorders (such as irritable bowel syndrome), insomnia, migraine, rashes, and other autoimmune disorders. Approximately three-quarters of the patients treated by NDs have more than one of these chronic conditions. Due to the fact that their immune systems are often depleted, these individuals are highly sensitive to standard medications. They are also more susceptible to the numerous side effects brought about by mass-produced drugs.

Such patients have, in effect, fallen through the cracks of the medical system. This is why they seek out naturopathic medicine. Safely compounded medications – including nutritional, herbal, and homeopathic remedies – prove efficacious to meet their needs every day in doctors' offices across the country. Such medications are generally recognized as safe (GRAS), having been used safely for decades in many cases. As patients' immune function improves, and as they work with their ND to improve their nutrition, get better sleep, increase their exercise and decrease their stress, their health and their resilience improves. This is the 'multi-systems' approach of naturopathic medicine – of which compounded drugs are an essential component.

Bulk Drug Substances Nominated at this Time

Notwithstanding the concerns expressed and issues highlighted in the foregoing, AANP nominates the following 21 bulk drug substances for FDA's consideration as bulk drug substances that may be used in pharmacy compounding under Section 503A. Thorough information on these substances is presented in the spreadsheets attached with our comments. The documentation is as complete and responsive to the Agency's criteria as we can offer at this time.

The bulk drug substances nominated are:

Acetyl L Carnitine

Alanyl L Glutamine
Alpha Lipoic Acid
Artemisia/Artemisinin
Boswellia
Calcium L5 Methyltetrahydrofolate
Cesium Chloride
Choline Chloride
Curcumin
DHEA
Dicholoroacetic Acid
DMPS
DMSA
Germanium Sesquioxide
Glutiathone
Glycyrrhizin
Methylcobalamin
MSM
Quercitin
Rubidium Chloride
Vanadium

As explained above, we did not have sufficient opportunity to provide all the required information for many of the bulk drug substances identified as essential for treating the patients of naturopathic doctors. AANP wishes to specify these 21 ingredients so that we may, with sufficient opportunity to carry out the extensive research required, provide the necessary documentation to support their nomination. The additional bulk drug substances include:

7 Keto Dehydroepiandrosterone
Asparagine
Calendula
Cantharidin
Choline Bitartrate
Chromium Glycinate
Chromium Picolinate
Chrysin
Co-enzyme Q10
Echinacea
Ferric Subsulfate
Iron Carbonyl
Iscador
Pantothenic Acid
Phenindamine Tartrate
Piracetam
Pterostilbene

Pyridoxal 5-Phosphate
Resveratrol
Salicinium
Thymol Iodide

AANP Objects to Unreasonable Burden

AANP believes it necessary and proper to lodge an objection to FDA's approach, i.e., the voluminous data being required in order for bulk drug substances to be considered by the Agency for approval. FDA is placing the entire burden of documentation of every element in support of the clinical rationale and scientific evidence on already overtaxed health professionals. Given that many of the persons most knowledgeable about and experienced in the application of compounded medications are either small business owners or busy clinicians, and given the extent and detail of information on potentially hundreds of ingredients as sought by FDA, this burden is unreasonable. The approach has no basis in the purpose and language of the Drug Quality and Security Act ("Act") – particularly for drugs that have been safely used for years, not only with the Agency's implicit acceptance, but without any indication of an unacceptable number of adverse patient reactions.

The volume of data being required in this rulemaking is contrary to the manner in which FDA has approached such reviews in the past. For example, to accomplish the Drug Efficacy Study Implementation (DESI) program, the Agency contracted with the National Academy of Science/National Research Council (NAS/NRC) to make an initial evaluation of the effectiveness of over 3,400 products that were approved only for safety between 1938 and 1962. Unlike the compounding industry, most pharmaceuticals under review were manufactured by pharmaceutical companies with the resources to seek regulatory approvals. The FDA's analysis of the costs of regulatory compliance did not appear to include an examination of the impacts on the industry. The initial or continuing notice for nominations did not analyze this under the Executive Regulatory Flexibility Act (5 U.S.C. 601-612) nor the Unfunded Mandates Reform Act of 1995 (Pub. L. 104-4).

The burden on respondents to this current rulemaking is further aggravated by the FDA's complete absence of consideration of the harm that will be caused if needed drugs are removed from the market. The "Type 2" errors caused by removing important agents from clinical use could far exceed the "Type 1" errors of adverse reactions, particularly given the strong track record of safely compounded medications. The infectious contamination that gave rise to the Act has little to do with the process set out by FDA for determining which ingredients may be compounded. Yet the Agency has offered little consideration of the respective risks and benefits of its approach. Based on the fact that compounding pharmacies and physicians are carrying the full burden of proof, as well as how much time it is likely to take for the process of documentation and evaluation to conclude, the Agency itself may well find that it has caused more harm to patients' clinical outcomes than provided a bona fide contribution to patient safety.

Conclusion

AANP appreciates the Agency's consideration of the arguments and objection presented herein, the request for an extension of time to gather the documentation that FDA is seeking, and the nominations made and referenced at this time.

We look forward to continued dialogue on these matters. As AANP can answer any questions, please contact me (jud.richland@naturopathic.org; 202-237-8150).

Sincerely,

Jud Richland, MPH
Chief Executive Officer

Column A—What information is requested?	Column B—Put data specific to the nominated substance
What is the name of the nominated ingredient?	Reduced L-glutathione
Is the ingredient an active ingredient that meets the definition of ''bulk drug substance'' in § 207.3(a)(4)?	Yes. Glutathione disulfide is a component of FDA-approved product.
Is the ingredient listed in any of the three sections of the Orange Book?	Glutathione disulfide is a component of a FDA-approved irrigation product.
Were any monographs for the ingredient found in the USP or NF monographs?	There is a Dietary Supplement monograph in the USP for this substance
What is the chemical name of the substance?	2-amino-5-[(R)-1-(carboxymethylamino)-3-mercapto-1-oxopropan-2-ylamino]-5-oxo, (S); N-(N-L- -Glutamyl-L-cysteinyl)glycine; Glycine, N-(N-L-gamma-glutamyl-L-cysteinyl)
What is the common name of the substance?	Reduced L-glutathione
Does the substance have a UNII Code?	GAN16C9B8O
What is the chemical grade of the substance?	Dietary Supplement grade
What is the strength, quality, stability, and purity of the ingredient?	A valid Certificate of Analysis accompanies each lot of raw material received. This bulk drug substance is a generally recognized as safe (GRAS) dietary supplement.
How is the ingredient supplied?	Glutathione is supplied as a white crystalline powder
Is the substance recognized in foreign pharmacopeias or registered in other countries?	EINECS: This product is on the European Inventory of Existing Commercial Chemical Substances (EINECS No. 200-725-4). Canada: Listed on Canadian Domestic Substance List (DSL). China: Listed on National Inventory. Japan: Listed on National Inventory (ENCS). Korea: Listed on National Inventory (KECI). Philippines: Listed on National Inventory (PICCS). Australia: Listed on AICS.
Has information been submitted about the substance to the USP for consideration of monograph development?	A Dietary Supplement monograph for this bulk drug substance is available in the USP.
What dosage form(s) will be compounded using the bulk drug substance?	Injection, inhalation
What strength(s) will be compounded from the nominated substance?	Strengths can range from 60 mg/mL (up to 1.8g/30 mL) to 200 mg/mL (up to 6 g/30 mL) in various sizes of multiple dose or preservative free vials.
What are the anticipated route(s) of administration of the compounded drug product(s)?	Intravenous, intramuscular or inhalation

Are there safety and efficacy data on compounded drugs using the nominated substance?	Mol Aspects Med. 2009;30(1-2):1-12.[PubMed 18796312] 2. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr. 2004;134(3):489-492.[PubMed 14988435] 3. Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K, Hammond CL. Glutathione dysregulation and the etiology and progression of human diseases. Biol Chem. 2009;390(3):191-214.[PubMed 19166318] 4. Micke P, Beeh KM, Buhl R. Effects of long-term supplementation with whey proteins on plasma glutathione levels of HIV-infected patients. Eur J Nutr. 2002;41(1):12-18.[PubMed 11990003] 5. Simoni RD, Hill RL, Vaughan M. The discovery of glutathione by F. Gowland Hopkins and the beginning of biochemistry at Cambridge University. J Biol Chem. 2002;277(24):e13. 6. Pompella A, Visvikis A, Paolicchi A, De Tata V, Casini AF. The changing faces of glutathione, a cellular protagonist. Biochem Pharmacol. 2003;66(8):1499-1503.[PubMed 14555227] 7. Martin HL, Teismann P. Glutathione—a review on its role and significance in Parkinson's disease. FASEB J. 2009;23(10):3263-3272.[PubMed 19542204] 8. Pocernich CB, Butterfield DA. Elevation of glutathione as a therapeutic strategy in Alzheimer disease.
Has the bulk drug substance been used previously to compound drug product(s)?	Yes
What is the proposed use for the drug product(s) to be compounded with the nominated substance?	Glutathione (typically at 60 to 200 mg/ml for IV administration, or 60 to 300 mg per dose for inhalation) is a tripeptide synthesized from Glycine, Glutamic acid and Cysteine. It is a primary intracellular antioxidant that is essential to life. It is useful to prevent radiation injury before radiation treatment is started. It helps reduce the side effects of chemotherapy treatments. It can prevent or reverse alcohol induced fatty liver cirrhosis, hepatitis, and liver lesions. It inhibits chemical induced carcinogenesis. It improves prognosis of stroke victims. It has been used in patients with COPD or chronic lung disease to improve lung functions and minimize oxygen-dependency. It is one of the main anti-oxidation protocols in children with Autistic Syndrome Disorder. Generally, It is useful in any condition where there is a risk for oxidative stress/damage.
What is the reason for use of a compounded drug product rather than an FDA-approved product?	Various liver disease protocols can include anti-viral agents, chemotherapy, lactulose, beta blockers, diuretics, and antibiotics. Cancer treatments protocols include surgery, chemotherapy, and radiation. Stroke treatments can include aspirin, TPA, and more invasive procedures depending upon the acute nature of the symptoms. Glutathione would be used in conjunction with the above protocols to reduce the intensity of symptoms of the disease processes, and to reduce the severity of the side effects of the pharmaceutical protocols. There are no FDA-approved drug products including Reduced L-glutathione for injection, inhalation.

Is there any other relevant information?	appears much more attractive than a foreign chemical with possible toxic side effects, acute and chronic. Most or all patients with Autistic Syndrome Disorder receives glutathione therapy. 20 - 30 % of patients with COPD or chronic lung disease may benefit from inhaled glutathione as an adjuvant therapy. Libyan J Med. 2014 Jun 12;9:23873. doi: 10.3402/ljm.v9.23873. eCollection 2014. Oxidative stress and lung function profiles of male smokers free from COPD compared to those with COPD: a case-control study. Ben Moussa S1, Sfaxi I2, Tabka Z3, Ben Saad H4, Rouatbi S3. Author information 1Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; bm.syrine@gmail.com.2Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia.3Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; Laboratory of Physiology, Faculty of Medicine of Sousse, University of Sousse, Sousse, Tunisia.4Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; Laboratory of Physiology, Faculty of Medicine of Sousse, University of Sousse, Sousse, Tunisia; Research Unit: Secondary Prevention after Myocardial Infarction, Faculty of Medicine of Sousse, Sousse, Tunisia. Abstract BackGROUND: The mechanisms of smoking tobacco leading to chronic obstructive pulmonary disease (COPD) are beginning to be understood. However, conclusions about the role of blood or lung oxidative stress markers were disparate. AIMS: To investigate the oxidative stress in blood or lung associated with tobacco smoke and to evaluate its effect on pulmonary function data and its relation with physical activity. METHODS: It is a case-control study. Fifty-four male-smokers of more than five pack-years (PY) and aged 40-60 years were included (29 Non-COPD, 16 COPD). Physical activity score was determined. Blood sample levels of malondialdehyde (MDA), protein-cys SH (PSH), and Glutathione (GSH) were measured. Fractional exhaled nitric oxide (FeNO) and plethysmographic measurements were performed. Correlation coefficients (r) evaluated the association between oxidative stress markers and independent variables

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September 30, 2014

Division of Dockets Management (HFA-305)
Food And Drug Administration
Department of Health and Human Services
5630 Fishers Lane, Room 1061
Rockville, MD 20852
Re: Docket FDA-2013-N-1525

“Bulk Drug Substances That May Be Used to compound Drug Products in Accordance With Section 503B of Federal Food, Drug, Drug, and Cosmetic Act; Revised Request for Nominations”

To Whom It May Concern:

The American College for Advancement in Medicine (ACAM) is a prominent and active medical education organization involved in instructing physicians in the proper use of oral and intravenous nutritional therapies for over forty years. We have also been involved in clinical research sponsored by the National Heart Lung and Blood Institute. We have a strong interest in maintaining the availability of compounded drug products.

We appreciate the opportunity to address the FDA’s request for nominations of bulk drug substances that may be used by compounding facilities to compound drug products. To meet what appear to be substantial requirements involved in this submittal, the FDA has given compounding pharmacists (in general a small business operation) and physicians very limited time to comply with onerous documentation. The Agency has requested information for which no single pharmacy or physician organization can easily provide in such a contracted time frame. As such this time consuming process requires significant coordination from many practicing professionals for which adequate time has not been allotted.

This issue is of great importance and has the potential to drastically limit the number of available compounded drugs and drug products thus limiting the number of individualized treatments that compounded medicines offer to patients.
ACAM and its physician members have not had the time to collect, review and assess all documentation necessary to submit for the intended list of compounded drugs required to assure all patient therapies are represented in our submission. We respectfully seek an additional 120 day period to educate and coordinate our physicians on the issue at hand and to gather the essential information necessary to provide the Agency with the most comprehensive information. In an attempt to comply with the current timeframe established, a collaborative effort resulted in the attached nominations prepared for bulk drug substances that may be used in pharmacy compounding under Section 503B.

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It is not clear whether the current submission will be the final opportunity to comment or communicate with the Agency. Will a deficiency letter be provided if the initial nomination information was inadequate or will a final decision to reject a nominated substance be made without the opportunity to further comment? ACAM respectfully requests that the FDA issue a deficiency letter should the submitted documentation for a nomination be considered inadequate.

Sincerely,

Neal Speight, MD
(Immediate Past President) for
Allen Green, MD
President and CEO
The American College for Advancement in Medicine

Column A—What information is requested?	Column B—Put data specific to the nominated substance
What is the name of the nominated ingredient?	Reduced L-glutathione
Is the ingredient an active ingredient that meets the definition of ''bulk drug substance'' in § 207.3(a)(4)?	Yes. Glutathione disulfide is a component of FDA-approved product.
What is the chemical name of the substance?	2-amino-5-[(R)-1-(carboxymethylamino)-3-mercapto-1-oxopropan-2-ylamino]-5-oxo, (S); N-(N-L- -Glutamyl-L-cysteinyl)glycine; Glycine, N-(N-L-gamma-glutamyl-L-cysteinyl)
What is the common name of the substance?	Glutathione
Does the substance have a UNII Code?	GAN16C9B8O
What is the chemical grade of the substance?	Dietary Supplement grade
What is the strength, quality, stability, and purity of the ingredient?	A valid Certificate of Analysis accompanies each lot of raw material received. This bulk drug substance is a generally recognized as safe (GRAS) dietary supplement.
How is the ingredient supplied?	Glutathione is supplied as a white crystalline powder
Is the substance recognized in foreign pharmacopeias or registered in other countries?	EINECS: This product is on the European Inventory of Existing Commercial Chemical Substances (EINECS No. 200-725-4). Canada: Listed on Canadian Domestic Substance List (DSL). China: Listed on National Inventory. Japan: Listed on National Inventory (ENCS). Korea: Listed on National Inventory (KECI). Philippines: Listed on National Inventory (PICCS). Australia: Listed on AICS.
Has information been submitted about the substance to the USP for consideration of monograph development?	A Dietary Supplement monograph for this bulk substance is available in the USP.

What medical condition(s) is the drug product compounded with the bulk drug substances intended to treat?	Glutathione (typically at 60 to 200 mg/ml for IV administration, or 60 to 300 mg per dose for inhalation) is a tripeptide synthesized from Glycine, Glutamic acid and Cysteine. It is a primary intracellular antioxidant that is essential to life. It is useful to prevent radiation injury before radiation treatment is started. It helps reduce the side effects of chemotherapy treatments. It can prevent or reverse alcohol induced fatty liver cirrhosis, hepatitis, and liver lesions. It inhibits chemical induced carcinogenesis. It improves prognosis of stroke victims. It has been used in patients with COPD or chronic lung disease to improve lung functions and minimize oxygen-dependency. It is one of the main anti-oxidation protocols in children with Autistic Syndrome Disorder. Generally, It is useful in any condition where there is a risk for oxidative stress/damage.
Are there other drug products approved by FDA to treat the same medical condition?	There are various FDA-approved liver disease protocols can include anti-viral agents, chemotherapy, lactulose, beta-blockers, diuretics, and antibiotics. Cancer treatments protocols include surgery, chemotherapy, and radiation. Stroke treatments can include aspirin, TPA, and more invasive procedures depending upon the acute nature of the symptoms.
If there are FDA-approved drug products that address the same medical condition, why is there a clinical need for a compounded drug product? Provide a justification for clinical need, including an estimate of the size of the population that would need the compounded drug.	Glutathione would be used in conjunction with the above protocols to reduce the intensity of symptoms of the disease processes, and to reduce the severity of the side effects of the pharmaceutical protocols. If a chemotherapeutic agent, and/or a radiation protocol, causes symptomology from oxidative stress, glutathione may be useful to prevent the severity of these side effects. Since glutathione naturally occurs in the liver, a boost in glutathione can help preserve liver function in patients undergoing intense hepatic disease protocols. Most or all patients with Autistic Syndrome Disorder receives glutathione therapy. 20 - 30 % of patients with COPD or chronic lung disease may benefit from inhaled glutathione as an adjuvant therapy.

Are there safety and efficacy data on compounded drugs using the nominated substance?	1. Forman HJ, Zhang H, Rinna A. Glutathione: overview of its protective roles, measurement, and biosynthesis. Mol Aspects Med. 2009;30(1-2):1-12.[PubMed 18796312] 2. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr. 2004;134(3):489-492.[PubMed 14988435] 3. Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K, Hammond CL. Glutathione dysregulation and the etiology and progression of human diseases. Biol Chem. 2009;390(3):191-214.[PubMed 19166318] 4. Micke P, Beeh KM, Buhl R. Effects of long-term supplementation with whey proteins on plasma glutathione levels of HIV-infected patients. Eur J Nutr. 2002;41(1):12-18.[PubMed 11990003] 5. Simoni RD, Hill RL, Vaughan M. The discovery of glutathione by F. Gowland Hopkins and the beginning of biochemistry at Cambridge University. J Biol Chem. 2002;277(24):e13. 6. Pompella A, Visvikis A, Paolicchi A, De Tata V, Casini AF. The changing faces of glutathione, a cellular protagonist. Biochem Pharmacol. 2003;66(8):1499-1503.[PubMed 14555227] 7. Martin HL, Teismann P. Glutathione—a review on its role and significance in Parkinson's disease. FASEB J. 2009;23(10):3263-3272.[PubMed 19542204] 8. Pocernich CB, Butterfield DA. Elevation of glutathione as a therapeutic strategy in Alzheimer disease. Biochim Biophys Acta. 2012;1822(5):625-630.[PubMed 22015471] 9. Hauser RA, Lyons KE, McClain T, Carter S, Perlmutter D. Randomized, double-blind, pilot evaluation of intravenous glutathione in Parkinson's disease. Mov Disord. 2009;24(7):979-983.[PubMed 19230029] 10. Okun MS, Lang A, Jankovic J. Reply: Based on the available randomized trial patients should say no to glutathione for Parkinson's disease. Mov Disord. 2010;25(7):961-962; author reply 962-963.[PubMed 20461816] 11. Naito Y, Matsuo K, Kokubo Y, Narita Y, Tomimoto H. Higher-dose glutathione therapy for Parkinson's disease in Japan: is it really safe? Mov Disord. 2010;25(7):962; author reply 962-963.[PubMed 20131395] 12. Berk M, Ng F, Dean O, Dodd S, Bush AI. Glutathione: a novel treatment target in psychiatry. Trends Pharmacol Sci. 2008;29(7):346-351.[PubMed 18538422] 13. Lavoie S, Murray MM, Deppen P, et al. Glutathione precursor, N-acetyl-cysteine, improves mismatch negativity in schizophrenia patients. Neuropsychopharmacology. 2008;33(9):2187-2199.[PubMed 18004285] 14. Balendiran GK, Dabur R, Fraser D. The role of glutathione in cancer. Cell Biochem Funct. 2004;22(6):343-352.[PubMed 15386533] 15. Singh S, Khan AR, Gupta AK. Role of glutathione in cancer pathophysiology and therapeutic interventions. J Exp Ther Oncol. 2012;9(4):303-316.[PubMed 22545423] 16. Zhang ZJ, Hao K, Shi R, et al. Glutathione S-transferase M1 (GSTM1) and glutathione S-transferase T1 (GSTT1) null polymorphisms, smoking, and their interaction in oral cancer: a HuGE review and meta-analysis. Am J Epidemiol. 2011;173(8):847-857.[PubMed 21436184] 17. Miko Enomoto T, Johnson T, Peterson N, Homer L, Walts D, Johnson N. Combination glutathione and anthocyanins as an alternative for skin care during external-beam radiation. Am J Surg. 2005;189(5):627-630; discussion 630-631.[PubMed 15862509] 18. Raza A, Galili N, Smith S, et al. Phase 1 multicenter dose-escalation study of ezatiostat hydrochloride (TLK199 tablets), a novel glutathione analog prodrug, in patients with myelodysplastic syndrome. Blood.
If there is an FDA-approved drug product that includes the bulk drug substance nominated, is it necessary to compound a drug product from the bulk drug substance rather than from the FDA-approved drug product?	There are no FDA-approved drug products including Reduced L-glutathione for injection, inhalation.
What dosage form(s) will be compounded using the bulk drug substance?	Injection, inhalation

What strength(s) will be compounded from the nominated substance?	Strengths can range from 60 mg/mL (up to 1.8g/30 mL) to 200 mg/mL (up to 6 g/30 mL) in various sizes of multiple dose or preservative free vials.
What are the anticipated route(s) of administration of the compounded drug product(s)?	Injection, inhalation
Has the bulk drug substance been used previously to compound drug product(s)?	Yes
Is there any other relevant information?	appears much more attractive than a foreign chemical with possible toxic side effects, acute and chronic. Reduced L-glutathione is produced by the liver for detoxification purposes. This therapy appears much more attractive than a foreign chemical with possible toxic side effects, acute and chronic. Most or all patients with Autistic Syndrome Disorder receives glutathione therapy. 20 - 30 % of patients with COPD or chronic lung disease may benefit from inhaled glutathione as an adjuvant therapy. Libyan J Med. 2014 Jun 12;9:23873. doi: 10.3402/ljm.v9.23873. eCollection 2014. Oxidative stress and lung function profiles of male smokers free from COPD compared to those with COPD: a case-control study. Ben Moussa S1, Sfaxi I2, Tabka Z3, Ben Saad H4, Rouatbi S3. Author information 1Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; bm.syrine@gmail.com.2Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia.3Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; Laboratory of Physiology, Faculty of Medicine of Sousse, University of Sousse, Sousse, Tunisia.4Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; Laboratory of Physiology, Faculty of Medicine of Sousse, University of Sousse, Sousse, Tunisia; Research Unit: Secondary Prevention after Myocardial Infarction, Faculty of Medicine of Sousse, Sousse, Tunisia. Abstract BackGROUND: The mechanisms of smoking tobacco leading to chronic obstructive pulmonary disease (COPD) are beginning to be understood. However, conclusions about the role of blood or lung oxidative stress markers were disparate. AIMS: To investigate the oxidative stress in blood or lung associated with tobacco smoke and to evaluate its effect on pulmonary function data and its relation with physical activity. METHODS: It is a case-control study. Fifty-four male-smokers of more than five pack-years (PY) and aged 40-60 years were included (29 Non-COPD, 16 COPD). Physical activity score was determined. Blood sample levels of malondialdehyde (MDA), protein-cys SH (PSH), and Glutathione (GSH) were measured. Fractional exhaled nitric oxide (FeNO)

VIA WWW.REGULATIONS.COM

September 30, 2014

Division of Dockets Management (HFA-305)
Food and Drug Administration
Department of Health and Human Services
5630 Fishers Lane, Room 1061
Rockville, MD 20852

Re: Docket FDA-2013-N-1525
Bulk Drug Substances That May Be Used To Compound Drug Products in Accordance With Section 503A of the Federal Food, Drug and Cosmetic Act, Concerning Outsourcing Facilities; Request for Nominations.

To Whom It May Concern:

The Integrative Medicine Consortium (IMC) appreciates the opportunity to address the Food and Drug Administration's request for the submission of ingredients to be listed as allowed for compounding by compounding pharmacies pursuant to Section 503A of the Food Drug and Cosmetic Act. IMC represents the interests of over 6,000 medical and naturopathic physicians and their patients. As we noted in our submission of March 4, 2014, we know from extensive experience that the appropriate availability of compounded drugs offers significant clinical benefits for patients and raise certain objections to the manner in which the FDA is proceeding on these determinations.

First, we note that we are in support of and incorporate by reference the comments and proposed ingredients submitted by our member organization, the American Association of Naturopathic Physicians (AANP), as well as the International Association of Compounding Pharmacists (IACP), and the Alliance for Natural Health-USA (ANH-USA). We also write on behalf of the Academy of Integrative Health and Medicine (AIHM), a merger of the American Holistic Medical Association and the American Board of Integrative and Holistic Medicine.

We also write to raise objections to:

A) The ingredient submission process the FDA is following on this docket, which places the burden entirely on small industry and practicing physicians to review and support ingredient nominations rather than devoting Agency resources to the task.

B) The withdrawal of approval for bulk ingredients that had been previously allowed until the

process is completed, leaving a void whose harm far outweighs the risks presented by these ingredients.

C) The lack of findings of the economic impact of this regulation with regard to the Executive Regulatory Flexibility Act (5 U.S.C. 601-612) or the Unfunded Mandates Reform Act of 1995 (Pub. L. 104-4).

Further, we write to ask that FDA:

D) Keep the record open for an additional 120 days for the submission of additional materials.

E) Address the outstanding issues we raised in our submission of March 4, 2014.

F) Accept the attached nominations.

G) Accept allergenic extracts as a class without requiring individual nominations and approval.

Commenter Organizational Background: The Integrative Medicine Consortium

The Integrative Medicine Consortium (IMC) began in 2006 when a group of Integrative Medicine leaders joined together to give a common voice, physician education and support on legal and policy issues. Our comment is based on the collective experience of over 6,000 doctors from the following seven organizations:

American Academy of Environmental Medicine (AAEM) www.aaemonline.org
American Association of Naturopathic Physicians (AANP) www.naturopathic.org
American College for Advancement in Medicine (ACAM) www.acam.org
International College of Integrative Medicine (ICIM) www.icimed.com
International Hyperbaric Medical Association (IHMA) www.hyperbaricmedicalassociation.org
International Organization of Integrative Cancer Physicians (IOIP) www.ioipcenter.org

The IMC has been involved in the assessment of risk as applied to the integrative field generally, including participation in the design of malpractice policies suited to the practice of integrative care along with quality assurance efforts for the field such as initiating the move toward developing a professional board certification process. IMC and its member organizations have collectively held over a hundred conferences, attended by tens of thousands of physicians, in which clinical methods that involve the proper use of compounded drugs are a not infrequent topic and subject to Category

I CME credit. Our collective experience on these matters is thus profound, well-credentialed and well-documented.

IMC Objections and Requests Regarding Docket FDA-2013-N-1525

A) The ingredient submission process the FDA is following on this docket, inappropriately places the burden entirely on small industry and practicing physicians to review and support ingredient nominations rather than devoting Agency resources to the task.

We wish to lodge our objection to FDA's approach to its data collection about drugs that will be placed on the list of permitted ingredients. The FDA is placing the entire burden of documentation of every element in support of the clinical rationale and scientific evidence on already overtaxed health professionals. Given that many of those knowledgeable and experienced in compounded pharmaceuticals are either small businesses or busy physicians, and given the significant quality and quantity of information on potentially hundreds of ingredients requested by FDA, this burden is unreasonable. This approach has no basis in the purpose and language of the Drug Quality and Security Act ("Act"), particularly for drugs that have been in use for years, not only with FDA's at least implicit acceptance, but without any indication of an unacceptable level of adverse reactions.

This is contrary to the manner in which FDA has approached such reviews in the past. For example, to accomplish the Drug Efficacy Study Implementation (DESI) program, FDA contracted with the National Academy of Science/National Research Council (NAS/NRC) to make an initial evaluation of the effectiveness of over 3,400 products that were approved only for safety between 1938 and 1962. Unlike the compounding industry, most pharmaceuticals under review were manufactured by pharmaceutical companies with the resources to seek regulatory approvals.

B) The withdrawal of approval for bulk ingredients that had been previously allowed until the process is completed, leaving a void whose harm far outweighs the risks presented by these ingredients.

Given that the Act arose from Good Manufacturing Practice violations and not concern for any specific drug ingredient, the requirement that ingredients not the subject of a USP monograph or a component of approved drugs be withdrawn pending these proceedings has no legislative basis or rationale. The hiatus in availability and inappropriate shift of burden to the compounding industry is further aggravated by the complete absence of consideration by the FDA of the harm caused by the removal of needed drugs from practice. The "Type 2" errors caused by removing important agents from clinical use could far exceed the "Type 1" errors of adverse reactions, particularly given the

track record in this industry. This is particularly true given that the infectious contamination that gave rise to the Act has little to do with the approval process for which ingredients may be compounded. Yet FDA has offered little consideration of the respective risks and benefits of its approach, and with pharmacies and physicians carrying the full burden of proof and the time expected for the advisory process to conclude, the FDA will likely itself cause more patient harm than provide a contribution to safety.

C) The lack of findings of the economic impact of this regulation with regard to the Executive Regulatory Flexibility Act (5 U.S.C. 601-612) or the Unfunded Mandates Reform Act of 1995 (Pub. L. 104-4).

The FDA's analysis of the costs of regulatory compliance did not appear to include an examination of the impacts on the industry. The initial or continuing notice for nominations did not analyze this under the Executive Regulatory Flexibility Act (5 U.S.C. 601-612) nor the Unfunded Mandates Reform Act of 1995 (Pub. L. 104-4). While the FDA made this assessment for "Additions and Modifications to the List of Drug Products That Have Been Withdrawn or Removed From the Market for Reasons of Safety or Effectiveness," 79 FR 37687, in which 25 drugs were added to the list of barred drugs, it has not done so for the much broader issue of upending the compounding pharmaceutical industry, which bears costs both in preparation of detailed submissions on potentially hundreds of ingredients, loss of sales of ingredients no longer approved, the economic consequence to physicians of not being to prescribe these drugs, and the economic impacts of health difficulties and added expense that will result from the withdrawal of drugs from clinical use. The Agency needs to address these concerns.

D) Extend the deadline for which comments are due by 120 days.

IMC's March 4, 2014 submission, along with AANP and ANH-USA nominated 71 bulk drug substances. IMC identified 21 more where we did not have the capacity to research and present all the necessary documentation within the timeframe the Agency was requiring.[1] We had determined that at least 6 hours per ingredient would be needed to do so, time that our physician members simply do not have in their day-to-day business of providing patient care. Thus, IMC sought a 90

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[1] For example, other nominations would include 7 Keto Dehydroepiandrosterone; Asparagine; Calendula; Cantharidin; Choline Bitartrate; Chromium Glycinate; Chromium Picolinate; Chrysin; Co-enzyme Q10; Echinacea; Ferric Subsulfate; Iron Carbonyl; Iscador; Pantothenic Acid; Phenindamine Tartrate; Piracetam; Pterostilbene; Pyridoxal 5-Phosphate; Resveratrol; Thymol Iodide.

day extension to more completely respond to the Agency's request.

In the renomination, we have narrowed our focus to the attached 21 bulk drug substances given restraints on available resources. These bulk drug substances are documented in the attachment. Given the limitations imposed by the fact that our physician members spent the majority of their day providing patient care, however, we have found that the span of time the Agency provided for renominations was insufficient.

We now request that FDA extend the deadline for which comments are due by at least 120 days, so that we may provide additional documentation. The FDA can certainly begin work on those nominations it has received, but nominations should remain open. We have determined that as much as 40 hours per ingredient will be needed to do, particularly given the lack of resources being offered by the Agency, time that our physician members simply do not have in their day-to-day business of providing patient care. Thus, IMC respectfully seeks an additional 120 day period - if not greater - for the purpose of gathering this essential information. If such an extension is not granted, we will explore the prospect of submitting a Citizen's Petition along with AANP and other interested parties.

E) Address the outstanding issues we raised in our submission of March 4, 2014.

In our submission of March 4, 2014, we raised a number of additional considerations, in particular citing a number of monographs, compendia and other authoritative sources that should be considered proper sources for authorized compounding in addition to the U.S. Pharmacopeia. We urge FDA to reach this issue as a means of allowing substances in long use on the market without undue delay or ambiguity.

F) Accept the attached nominations.

Notwithstanding the concerns expressed and issues highlighted in the foregoing, IMC nominates the bulk drug substances in the attachment for FDA's consideration as bulk drug substances that may be used in pharmacy compounding under Section 503A.

G) Accept allergenic extracts as a class without requiring individual nominations and acceptance.

In addition, we ask the FDA clarify its view of, and accept as appropriate for use, the category of materials that have been long used in the compounding of allergenic extracts for immunotherapy.

This should particularly be the case where such substances are compounded in manner consistent, where appropriate under its terms, with USP Monograph 797. Given both long-standing safe use, the nature of the materials and methods of clinical use,[2] and the safety assurances contained in this monograph, we believe that individual nominations and approval should not be imposed upon this form of treatment.

As explained above, we did not have sufficient opportunity to provide all the required information for many of the bulk drug substances identified as essential for treating patients. IMC wishes to identify these additional ingredients so that we may, with sufficient opportunity to carry out the extensive research required, provide the necessary documentation to support their nomination.

Sincerely,

Michael J. Cronin, N.D.
Chair, Integrative Medical Consortium

Enclosures:
Nominations

[2] Such as environmental and body molds, dust mites, grasses, grass terpenes, weeds, trees, foods, as well as hormone, neurotransmitter, and chemical antigens that are used in various forms of immunotherapy and desensitization.

Column A—What information is requested?	Column B—Put data specific to the nominated substance
What is the name of the nominated ingredient?	Reduced L-glutathione
Is the ingredient an active ingredient that meets the definition of ‘‘bulk drug substance’’ in § 207.3(a)(4)?	Yes. Glutathione disulfide is a component of FDA-approved product.
Is the ingredient listed in any of the three sections of the Orange Book?	Glutathione disulfide is a component of a FDA-approved irrigation product.
Were any monographs for the ingredient found in the USP or NF monographs?	There is a Dietary Supplement monograph in the USP for this substance
What is the chemical name of the substance?	2-amino-5-[(R)-1-(carboxymethylamino)-3-mercapto-1-oxopropan-2-ylamino]-5-oxo, (S); N-(N-L- -Glutamyl-L-cysteinyl)glycine; Glycine, N-(N-L-gamma-glutamyl-L-cysteinyl)
What is the common name of the substance?	Reduced L-glutathione
Does the substance have a UNII Code?	GAN16C9B8O
What is the chemical grade of the substance?	Dietary Supplement grade
What is the strength, quality, stability, and purity of the ingredient?	A valid Certificate of Analysis accompanies each lot of raw material received. This bulk drug substance is a generally recognized as safe (GRAS) dietary supplement.
How is the ingredient supplied?	Glutathione is supplied as a white crystalline powder
Is the substance recognized in foreign pharmacopeias or registered in other countries?	EINECS: This product is on the European Inventory of Existing Commercial Chemical Substances (EINECS No. 200-725-4). Canada: Listed on Canadian Domestic Substance List (DSL). China: Listed on National Inventory. Japan: Listed on National Inventory (ENCS). Korea: Listed on National Inventory (KECI). Philippines: Listed on National Inventory (PICCS). Australia: Listed on AICS.
Has information been submitted about the substance to the USP for consideration of monograph development?	A Dietary Supplement monograph for this bulk drug substance is available in the USP.
What dosage form(s) will be compounded using the bulk drug substance?	Injection, inhalation
What strength(s) will be compounded from the nominated substance?	Strengths can range from 60 mg/mL (up to 1.8g/30 mL) to 200 mg/mL (up to 6 g/30 mL) in various sizes of multiple dose or preservative free vials.
What are the anticipated route(s) of administration of the compounded drug product(s)?	Intravenous, intramuscular or inhalation

Are there safety and efficacy data on compounded drugs using the nominated substance?	Mol Aspects Med. 2009;30(1-2):1-12.[PubMed 18796312] 2. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr. 2004;134(3):489-492.[PubMed 14988435] 3. Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K, Hammond CL. Glutathione dysregulation and the etiology and progression of human diseases. Biol Chem. 2009;390(3):191-214.[PubMed 19166318] 4. Micke P, Beeh KM, Buhl R. Effects of long-term supplementation with whey proteins on plasma glutathione levels of HIV-infected patients. Eur J Nutr. 2002;41(1):12-18.[PubMed 11990003] 5. Simoni RD, Hill RL, Vaughan M. The discovery of glutathione by F. Gowland Hopkins and the beginning of biochemistry at Cambridge University. J Biol Chem. 2002;277(24):e13. 6. Pompella A, Visvikis A, Paolicchi A, De Tata V, Casini AF. The changing faces of glutathione, a cellular protagonist. Biochem Pharmacol. 2003;66(8):1499-1503.[PubMed 14555227] 7. Martin HL, Teismann P. Glutathione—a review on its role and significance in Parkinson's disease. FASEB J. 2009;23(10):3263-3272.[PubMed 19542204] 8. Pocernich CB, Butterfield DA. Elevation of glutathione as a therapeutic strategy in Alzheimer disease.
Has the bulk drug substance been used previously to compound drug product(s)?	Yes
What is the proposed use for the drug product(s) to be compounded with the nominated substance?	Glutathione (typically at 60 to 200 mg/ml for IV administration, or 60 to 300 mg per dose for inhalation) is a tripeptide synthesized from Glycine, Glutamic acid and Cysteine. It is a primary intracellular antioxidant that is essential to life. It is useful to prevent radiation injury before radiation treatment is started. It helps reduce the side effects of chemotherapy treatments. It can prevent or reverse alcohol induced fatty liver cirrhosis, hepatitis, and liver lesions. It inhibits chemical induced carcinogenesis. It improves prognosis of stroke victims. It has been used in patients with COPD or chronic lung disease to improve lung functions and minimize oxygen-dependency. It is one of the main anti-oxidation protocols in children with Autistic Syndrome Disorder. Generally, It is useful in any condition where there is a risk for oxidative stress/damage.
What is the reason for use of a compounded drug product rather than an FDA-approved product?	Various liver disease protocols can include anti-viral agents, chemotherapy, lactulose, beta blockers, diuretics, and antibiotics. Cancer treatments protocols include surgery, chemotherapy, and radiation. Stroke treatments can include aspirin, TPA, and more invasive procedures depending upon the acute nature of the symptoms. Glutathione would be used in conjunction with the above protocols to reduce the intensity of symptoms of the disease processes, and to reduce the severity of the side effects of the pharmaceutical protocols. There are no FDA-approved drug products including Reduced L-glutathione for injection, inhalation.

Is there any other relevant information?	appears much more attractive than a foreign chemical with possible toxic side effects, acute and chronic. Most or all patients with Autistic Syndrome Disorder receives glutathione therapy. 20 - 30 % of patients with COPD or chronic lung disease may benefit from inhaled glutathione as an adjuvant therapy. Libyan J Med. 2014 Jun 12;9:23873. doi: 10.3402/ljm.v9.23873. eCollection 2014. Oxidative stress and lung function profiles of male smokers free from COPD compared to those with COPD: a case-control study. Ben Moussa S1, Sfaxi I2, Tabka Z3, Ben Saad H4, Rouatbi S3. Author information 1Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; bm.syrine@gmail.com.2Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia.3Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; Laboratory of Physiology, Faculty of Medicine of Sousse, University of Sousse, Sousse, Tunisia.4Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; Laboratory of Physiology, Faculty of Medicine of Sousse, University of Sousse, Sousse, Tunisia; Research Unit: Secondary Prevention after Myocardial Infarction, Faculty of Medicine of Sousse, Sousse, Tunisia. Abstract BackGROUND: The mechanisms of smoking tobacco leading to chronic obstructive pulmonary disease (COPD) are beginning to be understood. However, conclusions about the role of blood or lung oxidative stress markers were disparate. AIMS: To investigate the oxidative stress in blood or lung associated with tobacco smoke and to evaluate its effect on pulmonary function data and its relation with physical activity. METHODS: It is a case-control study. Fifty-four male-smokers of more than five pack-years (PY) and aged 40-60 years were included (29 Non-COPD, 16 COPD). Physical activity score was determined. Blood sample levels of malondialdehyde (MDA), protein-cys SH (PSH), and Glutathione (GSH) were measured. Fractional exhaled nitric oxide (FeNO) and plethysmographic measurements were performed. Correlation coefficients (r) evaluated the association between oxidative stress markers and independent variables

September 30, 2014

Division of Dockets Management (HFA-305)
Food and Drug Administration
Department of Health and Human Services
5630 Fishers Lane, Room 1061
Rockville, MD 20852

McGUFF
COMPOUNDING
PHARMACY
SERVICES, INC.

Re: Docket FDA-2013-N-1525

"Bulk Drug Substances That May Be Used to Compound Drug Products in Accordance With Section 503A of the Federal Food, Drug, and Cosmetic Act; Revised Request for Nominations"

To Whom It May Concern:

McGuff Compounding Pharmacy Services, Inc. (McGuff CPS) appreciates the opportunity to address the FDA's request for nominations of bulk drug substances that may be used by compounding facilities to compound drug products.

Request for Extension

The Agency has indicated the majority of compounding pharmacies are small businesses. McGuff CPS is a small business and has found that the requirements to assemble the requested documentation have been particularly onerous. The Agency has requested information for which no one particular pharmacy, physician or physician organization can easily assemble and must be sought through coordination with the various stakeholders. To collect the information required is a time consuming process for which many practicing professionals have indicated that the time allotted for comment to the Docket has been too limited.

This is an issue of great importance which will limit the number of available compounded drugs products available to physicians and, therefore, will limit the number of individualized treatments to patients. McGuff CPS and physician stakeholders have not had the time to collect, review, and collate all documentation necessary to submit the intended list of compounded drugs required to assure all patient therapies are represented in our submission. McGuff CPS respectfully seeks an additional 120 day period for the purpose of coordinating the various stakeholders and gathering the essential information necessary to provide the Agency with the most comprehensive information.

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The Agency has not announced the process of follow on communication or failure e.g. what happens if a nominated substance needs more detailed information of a particular nature? Will the whole effort be rejected or will a "deficiency letter" be issued to the person or organization that submitted the nomination? The Agency issues "deficiency letters" for NDA and ANDA submissions and this appears to be appropriate for compounded drug nominations. McGuff CPS respectfully requests the FDA issue "deficiency letters" to the person or organization that submitted the nomination so that further documentation may be provided.

Nominations

To comply with the current time limits established by the Docket, attached are the nominations prepared to date for bulk drug substances that may be used in pharmacy compounding under Section 503A.

Sincerely,

Ronald M. McGuff

Ronald M. McGuff
President/CEO
McGuff Compounding Pharmacy Services, Inc.

Column A—What information is requested?	Column B—Put data specific to the nominated substance
What is the name of the nominated ingredient?	Reduced L-glutathione
Is the ingredient an active ingredient that meets the definition of ''bulk drug substance'' in § 207.3(a)(4)?	Yes. Glutathione disulfide is a component of FDA-approved product.
Is the ingredient listed in any of the three sections of the Orange Book?	Glutathione disulfide is a component of a FDA-approved irrigation product.
Were any monographs for the ingredient found in the USP or NF monographs?	There is a Dietary Supplement monograph in the USP for this substance
What is the chemical name of the substance?	2-amino-5-[(R)-1-(carboxymethylamino)-3-mercapto-1-oxopropan-2-ylamino]-5-oxo, (S); N-(N-L- -Glutamyl-L-cysteinyl)glycine; Glycine, N-(N-L-gamma-glutamyl-L-cysteinyl)
What is the common name of the substance?	Reduced L-glutathione
Does the substance have a UNII Code?	GAN16C9B8O
What is the chemical grade of the substance?	Dietary Supplement grade
What is the strength, quality, stability, and purity of the ingredient?	A valid Certificate of Analysis accompanies each lot of raw material received. This bulk drug substance is a generally recognized as safe (GRAS) dietary supplement.
How is the ingredient supplied?	Glutathione is supplied as a white crystalline powder
Is the substance recognized in foreign pharmacopeias or registered in other countries?	EINECS: This product is on the European Inventory of Existing Commercial Chemical Substances (EINECS No. 200-725-4). Canada: Listed on Canadian Domestic Substance List (DSL). China: Listed on National Inventory. Japan: Listed on National Inventory (ENCS). Korea: Listed on National Inventory (KECI). Philippines: Listed on National Inventory (PICCS). Australia: Listed on AICS.
Has information been submitted about the substance to the USP for consideration of monograph development?	A Dietary Supplement monograph for this bulk drug substance is available in the USP.
What dosage form(s) will be compounded using the bulk drug substance?	Injection, inhalation
What strength(s) will be compounded from the nominated substance?	Strengths can range from 60 mg/mL (up to 1.8g/30 mL) to 200 mg/mL (up to 6 g/30 mL) in various sizes of multiple dose or preservative free vials.
What are the anticipated route(s) of administration of the compounded drug product(s)?	Intravenous, intramuscular or inhalation

Are there safety and efficacy data on compounded drugs using the nominated substance?	Mol Aspects Med. 2009;30(1-2):1-12.[PubMed 18796312] 2. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr. 2004;134(3):489-492.[PubMed 14988435] 3. Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K, Hammond CL. Glutathione dysregulation and the etiology and progression of human diseases. Biol Chem. 2009;390(3):191-214.[PubMed 19166318] 4. Micke P, Beeh KM, Buhl R. Effects of long-term supplementation with whey proteins on plasma glutathione levels of HIV-infected patients. Eur J Nutr. 2002;41(1):12-18.[PubMed 11990003] 5. Simoni RD, Hill RL, Vaughan M. The discovery of glutathione by F. Gowland Hopkins and the beginning of biochemistry at Cambridge University. J Biol Chem. 2002;277(24):e13. 6. Pompella A, Visvikis A, Paolicchi A, De Tata V, Casini AF. The changing faces of glutathione, a cellular protagonist. Biochem Pharmacol. 2003;66(8):1499-1503.[PubMed 14555227] 7. Martin HL, Teismann P. Glutathione—a review on its role and significance in Parkinson's disease. FASEB J. 2009;23(10):3263-3272.[PubMed 19542204] 8. Pocernich CB, Butterfield DA. Elevation of glutathione as a therapeutic strategy in Alzheimer disease.
Has the bulk drug substance been used previously to compound drug product(s)?	Yes
What is the proposed use for the drug product(s) to be compounded with the nominated substance?	Glutathione (typically at 60 to 200 mg/ml for IV administration, or 60 to 300 mg per dose for inhalation) is a tripeptide synthesized from Glycine, Glutamic acid and Cysteine. It is a primary intracellular antioxidant that is essential to life. It is useful to prevent radiation injury before radiation treatment is started. It helps reduce the side effects of chemotherapy treatments. It can prevent or reverse alcohol induced fatty liver cirrhosis, hepatitis, and liver lesions. It inhibits chemical induced carcinogenesis. It improves prognosis of stroke victims. It has been used in patients with COPD or chronic lung disease to improve lung functions and minimize oxygen-dependency. It is one of the main anti-oxidation protocols in children with Autistic Syndrome Disorder. Generally, It is useful in any condition where there is a risk for oxidative stress/damage.
What is the reason for use of a compounded drug product rather than an FDA-approved product?	Various liver disease protocols can include anti-viral agents, chemotherapy, lactulose, beta blockers, diuretics, and antibiotics. Cancer treatments protocols include surgery, chemotherapy, and radiation. Stroke treatments can include aspirin, TPA, and more invasive procedures depending upon the acute nature of the symptoms. Glutathione would be used in conjunction with the above protocols to reduce the intensity of symptoms of the disease processes, and to reduce the severity of the side effects of the pharmaceutical protocols. There are no FDA-approved drug products including Reduced L-glutathione for injection, inhalation.

Is there any other relevant information?	appears much more attractive than a foreign chemical with possible toxic side effects, acute and chronic. Most or all patients with Autistic Syndrome Disorder receives glutathione therapy. 20 - 30 % of patients with COPD or chronic lung disease may benefit from inhaled glutathione as an adjuvant therapy. Libyan J Med. 2014 Jun 12;9:23873. doi: 10.3402/ljm.v9.23873. eCollection 2014. Oxidative stress and lung function profiles of male smokers free from COPD compared to those with COPD: a case-control study. Ben Moussa S1, Sfaxi I2, Tabka Z3, Ben Saad H4, Rouatbi S3. Author information 1Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; bm.syrine@gmail.com.2Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia.3Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; Laboratory of Physiology, Faculty of Medicine of Sousse, University of Sousse, Sousse, Tunisia.4Service of Physiology and Functional Explorations, Farhat HACHED Hospital, Sousse, Tunisia; Laboratory of Physiology, Faculty of Medicine of Sousse, University of Sousse, Sousse, Tunisia; Research Unit: Secondary Prevention after Myocardial Infarction, Faculty of Medicine of Sousse, Sousse, Tunisia. Abstract BackGROUND: The mechanisms of smoking tobacco leading to chronic obstructive pulmonary disease (COPD) are beginning to be understood. However, conclusions about the role of blood or lung oxidative stress markers were disparate. AIMS: To investigate the oxidative stress in blood or lung associated with tobacco smoke and to evaluate its effect on pulmonary function data and its relation with physical activity. METHODS: It is a case-control study. Fifty-four male-smokers of more than five pack-years (PY) and aged 40-60 years were included (29 Non-COPD, 16 COPD). Physical activity score was determined. Blood sample levels of malondialdehyde (MDA), protein-cys SH (PSH), and Glutathione (GSH) were measured. Fractional exhaled nitric oxide (FeNO) and plethysmographic measurements were performed. Correlation coefficients (r) evaluated the association between oxidative stress markers and independent variables

Submitted electronically via www.regulations.gov

September 30, 2014

Division of Dockets Management (HFA-305)
Food and Drug Administration
5630 Fishers Lane, rm. 1061
Rockville, MD 20852

Re: Docket No.: FDA-2013-N-1525: ***Bulk Drug Substances That May Be Used To Compound Drug Products in Accordance With Section 503A of the Federal Food, Drug and Cosmetic Act; Revised Request for Nominations***

Dear Sir or Madam:

The National Community Pharmacists Association (NCPA) is writing today to nominate specific bulk drug substances that may be used to compound drug products, although they are neither the subject of a United States Pharmacopeia (USP) or National Formulary (NF) monograph nor components of FDA-approved drugs. As the FDA considers which drugs nominated will be considered for inclusion on the next published bulk drugs list, NCPA is committed to working with the FDA and other interested stakeholders on these critical issues.

NCPA represents the interests of pharmacist owners, managers and employees of more than 23,000 independent community pharmacies across the United States. Independent community pharmacies dispense approximately 40% of the nation's retail prescription drugs, and, according to a NCPA member survey, almost 89% of independent community pharmacies engage in some degree of compounding.

Regarding specific nominations, NCPA would like to reference the attached spreadsheet as our formal submission of bulk drug substances (active ingredients) that are currently used by compounding pharmacies and are not, to the best of our knowledge, the subject of a USP or NF monograph nor are components of approved products.

All nominated substances on the attached spreadsheet are active ingredients that meet the definition of "bulk drug substance" to the best of our knowledge, and we have searched for the active ingredient in all three sections of the Orange Book, and the substances did not appear in any of those searches, confirming that the substance is not a component of any FDA-approved product. In addition, we have searched USP and NF monographs, and the substances are not the subject of such monographs to our best knowledge.

100 Daingerfield Road
Alexandria, VA 22314-2888
(703) 683-8200 PHONE
(703) 683-3619 FAX

Regarding the request for chemical grade information pertaining to the submitted ingredients, NCPA would like to stress that chemical grades of bulk active products vary according to manufacturing processes, and products are often unassigned. When compounding products for patient use, pharmacists use the highest grade ingredients available, typically USP/NF, USP/GenAR, ACS, or FCC, among others, depending on the chemical. The same standard applies for all of the bulk active ingredients submitted on the attached list.

Related to rationale for use, including why a compounded drug product is necessary, NCPA would like to stress that many of the attached listed products are unavailable commercially in traditional dosage forms and must therefore be compounded using bulk ingredients. For other listed products, the use of bulk ingredients allows compounders to create an alternate dosage form and/or strength for patients who are unable to take a dosage form that is commercially available.

NCPA would like to strongly recommend that FDA institute a formal process by which the list is updated and communicated to the compounding community. We would recommend an annual process that can be anticipated and acted upon in order to ensure maximum understanding and adherence to the list. The FDA should issue such request via *The Federal Register* and review and consider all updates to the list with the Pharmacy Compounding Advisory Committee (PCAC). No changes to the list should occur without the input and review of the PCAC.

NCPA is very disappointed that despite a call for nominations to the PCAC which we submitted in March 2014, no appointments have been made nor has the Committee been formed to do the work that Congress requires of the Agency. Without formation of this Committee, FDA is unable to consult the Committee regarding the submitted lists. NCPA strongly recommends that FDA consult with the PCAC related to every single submission the Agency receives in relation to FDA-2013-N-1525. It is only through complete consultation with the PCAC that each substance can be appropriately evaluated.

NCPA is committed to working with the FDA and other stakeholders regarding these important matters. We appreciate your consideration of our comments.

Sincerely,

Steve Pfister
Senior Vice President, Government Affairs

Attachment

Ingredient Name	Chemical Name	Common Name	UNII Code	Description of strength, quality, stability and	Ingredient Format(s)	Recognition in Pharmacopeias	Final Compounded Formulation Dosage Form(s)	Final Compounded Formulation Strength	Final Compounded Formulation Route(s) of	Bibliographies on Safety and Efficacy Data	Final Compounded Formulation Clinical Rationale and History of Past Use
Glutathione	N-(N-L-gamma-glutamyl-L-cysteinyl)glycine	Glutathione	GAN16C9B8O	From PCCA Certificate of Analysis: 99.1% Assay; From PCCA MSDS: 100% by weight and stable.	Powder	EP, not yet submitted to USP (Disulfide - salt form - is in 2 FDA-approved products)	Capsules/troches, cream/gel, solution, suppositories	Capsules/Troches: 25-500mg, Cream/gel: 1-20%, Solution: 1-20%, Suppositories: 25-500mg	Oral, sublingual, topical, injection, ophthalmic, nasal spray, rectal	Main PA, et al. The potential role of the antioxidant and detoxification properties of glutathione in autism spectrum disorders: a systematic review and meta-analysis. Nutr Metab (Lond). 012 Apr 24;9:35. [http://www.ncbi.nlm.nih.gov/pubmed/22524510] ; Kern JK, et al. A clinical trial of glutathione supplementation in autism spectrum disorders. Med Sci Monit. 2011 Dec;17(12):CR677-82. [http://www.ncbi.nlm.nih.gov/pubmed/22129897]; Prousky J. The treatment of pulmonary diseases and respiratory-related conditions with inhaled (nebulized or aerosolized) glutathione. Evid Based Complement Alternat Med. 2008 Mar;5(1):27-35. [http://www.ncbi.nlm.nih.gov/pubmed/18317545]	An antioxidant supplement used in Autism Spectrum Disorders, Antineoplastic Toxicity, COPD & Lung Disorders, Heavy Metal Poisoning

September 30, 2014

Submitted electronically via www.regulations.gov

Division of Dockets Management (HFA-305)
Food and Drug Administration
Department of Health and Human Services
5630 Fishers Lane, Rm. 1061
Rockville, MD 20852

[Docket No. FDA-2013-N-1525]

Re: FDA-2013-N-1525; List of Bulk Drug Substances That May Be Used in Pharmacy Compounding in Accordance with Section 503A

Dear Sir or Madam:

PCCA respectfully submits the following list of nineteen chemicals to be considered for the List of Bulk Drug Substances that may be used in Pharmacy Compounding in accordance with Section 503A.

PCCA provides its more than 3,600 independent community compounding pharmacy members across the United States with drug compounding ingredients, equipment, extensive education, and consulting expertise and assistance.

Regarding the specific nominations, we would like to reference the attached spreadsheet and point out a couple of facts regarding our research. To the best of our knowledge, all items submitted:

- Do not appear in any of the three sections of the Orange Book.
- Do not currently have a USP or NF monograph.
- Meet the criteria of a "bulk drug substance" as defined in § 207.3(a)(4).

In regards to the request for chemical grade information, we would like to point out that many of the items submitted do not currently have a chemical grade. PCCA believes that pharmacists should use the highest grade chemical available on the market for all aspects of pharmaceutical compounding and we continue to actively source graded chemicals from FDA-registered manufacturers. However, in the current marketplace, some graded chemicals cannot be obtained for various reasons. PCCA actively tests all products received to ensure they meet our required standards to ensure our members receive the highest quality chemicals possible.

We would like to echo the concerns, voiced by NCPA and others in our industry, the strong recommendation to formalize the process by which the list is updated and communicated to the pharmacy industry. We also recommend an annual process to ensure understanding and adherence to the list. All submissions and updates to the list should be reviewed by the Pharmacy Compounding Advisory Committee (PCAC) and no changes to the list should occur with input and review by the PCAC.

We are also dismayed in the fact that no appointments have been made to the PCAC despite the call for nominations closing in March 2014. Without these appointments, FDA is unable to consult the Committee regarding this list, as outlined in the Act. PCCA, along with industry partners, strongly recommends that the FDA consult with the PCAC related to every single submission the Agency received in relation to FDA-2013-N-1525.

We appreciate this opportunity to submit this list for consideration and we look forward to continuing to work with the FDA in the future on this and other important issues as they relate to the practice of pharmacy compounding.

Sincerely,

Aaron Lopez
Senior Director of Public Affairs
PCCA

John Voliva, R.Ph.
Director of Legislative Relations
PCCA

PCCA USA: 9901 S. Wilcrest Drive Houston, Texas 77099 | 800.331.2498 (f) 800.874.5760 | www.pccarx.com
PCCA Canada: 744 Third Street London, ON Canada N5V 5J2 | 800.668.9453 (f) 800.799.4537 | www.pccarx.ca
PCCA Australia: Unit 1, 73 Beauchamp Road Matraville NSW 2036 Australia | 02.9316.1500 (f) 02.9316.7422 | www.pccarx.com.au

PCCA Submission for Docket No. FDA-2013-N-1525: Bulk Drug Substances That May Be Used To Compound Drug Products in Accordance With Section 503A of the Federal Food, Drug and Cosmetic Act; Revised Request for Nominations

Ingredient Name	Glutathione
Is it a "bulk drug substance"	Yes
Is it listed in the Orange Book	Yes - salt form (Glutathione Disulfide) is contained in two FDA approved products - Endosol Extra (Application Number N020079) & BSS Plus (Application Number N018469)
Does it have a USP or NF Monograph	No
Chemical Name	*N* -(*N* -l-γ-Glutamyl-l-cysteinyl)glycine
Common Name(s)	Glutathione
UNII Code	GAN16C9B8O
Chemical Grade	N/A
Strength, Quality, Stability, and Purity	Assay, Description, Melting Point, Solubility; Example of PCCA Certificate of Analysis for this chemical is attached.
How supplied	Powder
Recognition in foreign pharmcopeias or registered in other countries	EP monograph; USP Dietary Supplement monograph; Available in sixteen countries
Submitted to USP for monograph consideration	No
Compounded Dosage Forms	Capsule/Troches, Cream/Gel, Solution, Suppository
Compounded Strengths	Capsule/Troche: 25 – 500 mg; Cream / Gel: 1-20%; Solution: 1-20%; Suppository: 25 – 500 mg
Anticipated Routes of Administration	Oral, Sublingual, Topical, Injection, Ophthalmic, Nasal Spray, Rectal

Saftey & Efficacy Data

Kern JK, et al. A clinical trial of glutathione supplementation in autism spectrum disorders. Med Sci Monit. 2011 Dec;17(12):CR677-82. [http://www.ncbi.nlm.nih.gov/pubmed/22129897]

Main PA, et al. The potential role of the antioxidant and detoxification properties of glutathione in autism spectrum disorders: a systematic review and meta-analysis. Nutr Metab (Lond). 2012 Apr 24;9:35. [http://www.ncbi.nlm.nih.gov/pubmed/22524510]

Prousky J. The treatment of pulmonary diseases and respiratory-related conditions with inhaled (nebulized or aerosolized) glutathione. Evid Based Complement Alternat Med. 2008 Mar;5(1):27-35. [http://www.ncbi.nlm.nih.gov/pubmed/18317545]

Bagnato GF. Effect of inhaled glutathione on airway response to 'Fog' challenge in asthmatic patients. Respiration. 1999 Nov-Dec;66(6):518-21. [http://www.ncbi.nlm.nih.gov/pubmed/10575337]

Cascinu S, et al. Neuroprotective effect of reduced glutathione on oxaliplatin-based chemotherapy in advanced colorectal cancer: a randomized, double-blind, placebo-controlled trial. J Clin Oncol. 2002 Aug 15;20(16):3478-83. [http://www.ncbi.nlm.nih.gov/pubmed/12177109]

Used Previously to compound drug products	Autism Spectrum Disorders, Antineoplastic Toxicity, COPD & Lung Disorders, Heavy Metal Poisoning
Proposed use	Autism Spectrum Disorders, Antineoplastic Toxicity, COPD & Lung Disorders, Heavy Metal Poisoning
Reason for use over and FDA-approved product	Treatment failures and/or patient unable to take FDA approved product

Other relevant information - Stability information	Unless other studies performed / found: Capsule/Troche: USP <795> recommendation of BUD for nonaqueous formulations – “no later than the time remaining until the earliest expiration date of any API or 6 months, whichever is earlier. Topical: USP <795> recommendation of BUD for water containing topical formulations – “no later than 30 days." Injection/Ophthalmic: USP <797> recommendations for high risk level compounded sterile products

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Houston, TX 77099
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Unit 1, 73 Beauchamp Road
Matraville, NSW 2036
Tel: 02.9316.1500
Fax: 02.9316.7422

CERTIFICATE OF ANALYSIS

PRODUCT: GLUTATHIONE (L) REDUCED
ITEM NUMBER: 30-2284
LOT NUMBER: C160619
MFG. DATE: 02/14/2013
EXPIRATION: 02/13/2015

CAS: 70-18-8
MW: 307.3300000000
FORMULA: C10H17N3O6S

TEST	SPECIFICATIONS	RESULTS
Arsenic	<=1 ppm *<= 1 ppm*	1 ppm
Assay	>= 98.0 %	99.1 %
Description	pass *WHITE POWDER OR CRYSTALLINE POWDER;ODORLESS*	pass *White powder*
Heavy metals	<=5 ppm *<= 5 ppm*	5 ppm
Identification	pass *IR*	pass
Iron	<=5 ppm *<= 5 ppm*	5 ppm
Lead Acetate	pass *Positive*	pass
Loss on Drying	<= 0.5 %	0.1 %
Melting point	pass celsius	pass celsius *187.7C*
Ninhydrin Test	pass *Positive*	pass
Optical Rotation	-17.5--15.5 degrees	-16.3 degrees
Residue on Ignition	<= 0.1 %	0.01 %
Solubility	pass *FREELY SOLUBLE IN WATER;SPARINGLY SOLUBLE IN ALCOHOL*	pass
Solution	pass *Clear Solution*	pass

QC APPROVED
PRINT DATE: 3/3/2014

The above test results have been obtained by our supplier or in our quality control laboratory.
This analysis is not to be construed as a warranty, expressed or implied.

Tab 2b

Glutathione
Nomination Clarification

Alliance for Natural Health USA
3525 Piedmont Road NE
Building 6, Suite 310
Atlanta, GA 30305

email: office@anh-usa.org
tel: 800.230.2762
202.803.5119
fax: 202.315.5837
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ANH-USA is a regional office of ANH-Intl
INTERNATIONAL
anhinternational.org

March 2, 2018

Toni Hallman, MS, BSN, RN
LT USPHS
Project Manager, PCAC
CDER/OC/OPRO
10903 New Hampshire Avenue
Building 51, Room 3249
Silver Spring, MD 20903

RE: Docket FDA-2015-N-3534

Dear Ms. Hallman:

The Alliance for Natural Health USA (ANH-USA) is responding to FDA's questions regarding the nomination of Reduced L-glutathione for inclusion on the 503A bulk drug substances list.

ANH-USA is an independent, nonprofit watchdog organization of more than 550,000 members nationally that protects consumer access to natural health services, practitioners, and resources. Safely compounded medications, as provided by integrative physicians, fulfill an important clinical need for many of our members. These are patients who have not found relief for their health conditions through conventional means. Such patients often have an adverse reaction to mass-manufactured drugs, and require a more individualized treatment regimen.

Before providing our responses, we wish to object to what has apparently evolved into a new request for a disease indication rather than simply a use for the ingredient. The implication is that FDA approval will be based upon a disease indication when functional and nutraceutical uses have substantial clinical value and are plainly lawful under the Food, Drug, and Cosmetic Act.

Responses:

Q1. Does Alliance for Natural Health USA still want to pursue review by the FDA and consideration by the PCAC of glutathione for inclusion on the 503A bulks list?

A. Yes

Q2. Please submit in writing the disease state(s) or health condition(s) that you are proposing for FDA's review, the dosage form and strength/concentration proposed for each use, and clinical, if available and other scientific articles in support of each use. If this information is not submitted for a proposed use, FDA does not intend to review the nominated substance for that use.

A. ANH-USA cites the responses of the American Association of Naturopathic Physicians, the Integrative Medicine Consortium, the Professional Compounding Centers of America, and McGuff Compounding Pharmacy, all of which possess the necessary expertise on these matters.

Q3. Glutathione is unlikely to be stable under ordinary storage conditions when compounded as a solution, cream, or gel, which are among the nominated dosage forms. Please provide any information available about how these issues are addressed for compounded products.

A. ANH-USA cites the responses of McGuff Compounding Pharmacy and the Professional Compounding Centers of America, which possess the necessary expertise on this matter.

ANH-USA appreciates the FDA's and its Pharmacy Compounding Advisory Committee's (PCAC) consideration of this further information in support of the nomination of glutathione for inclusion on the 503A bulk drug substances list. We would like to reiterate that the Agency's original request asked only for ingredients' proposed use, not the disease condition or indication.

If you have further questions, please contact me.

Sincerely,

Michael Jawer
Deputy Director

Email: mike@anh-usa.org
Phone: 240-396-2171

March 2, 2018

VIA EMAIL
toni.hallman@fda.hhs.gov

Toni Hallman, MS, BSN, RN
LT USPHS
Project Manager, PCAC
CDER/OC/OPRO
Food and Drug Administration
10903 New Hampshire Ave., Bldg 51, Rm 3249
Silver Spring, MD 20903

Re: Response to Requests for More Information on Nominations for Glutathione
Docket FDA-2015-N-3534

Dear LT. Hallman:

I write on behalf of the American Association of Naturopathic Physicians ("AANP") and its partner in these submissions, the Integrative Medicine Consortium ("IMC") in response to your requests for more information about the nominations of the above-named ingredient. It is correct that IMC and AANP maintain this nomination as an ingredient that should be placed on the 503A positive list.

Enclosed please find our submission regarding glutathione in which we address the questions raised for today's date. We are also in support of submissions made by co-nominators the Pharmacy Compounding Centers of America, Alliance for Natural Health and McGuff Compounding Pharmacy including assessments of stability.

Continuing Objection as to Insufficient Notice

IMC and AANP appreciate that FDA is seeking additional information as it weighs this nomination, but we maintain our continuing objection to the lack of sufficient notice to properly prepare and fully appraise FDA of the information it seeks to be able to make a proper decision that has tremendous implications for patient care.

Continuing Objection Over Apparent Exclusion of Functional Uses

We also continue our objection to the question about the use for these ingredients which, in the case of glutathione, asks for the "diseases or health conditions" for which its use is proposed. This request inappropriately narrows allowed use. As we noted in our letter of January 26, 2018 regarding alpha-lipoic acid, methylcobalamin and choline chloride, incorporated herein by reference, the law also provides for approval for functional benefits. In addition to our previous citations, we note that the relevant Guidance Document defines "[a] bulk drug substance is defined as meaning 'the same as active pharmaceutical ingredient as defined in 21 CFR § 207.1(b).' See 21 CFR § 207.3. Active pharmaceutical ingredient is defined as "any substance that is intended for incorporation into a finished drug product and is intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, *or to affect the structure or any function of the body...*" (Emphasis added.) Interim Policy on Compounding Using Bulk Drug Substances Under Section 503A of the Federal Food, Drug, and Cosmetic Act: Guidance for Industry, January 2017 at 2.

Glutathione is a particular case-in-point; as the attached bibliography and materials show, it has a number of functional uses that are widely considered by the community of naturopathic, integrative and functional medicine physicians as having highly critical and needed functional uses. Its ability to address oxidative stress is well-documented and is a valid clinical end-point in and of itself, as demonstrated by significant literature describing not only the role of oxidative stress in numerous disease conditions but also in supporting and maintaining metabolic and immune functions that may be precursors to as well as components of illness or in sustaining wellness. With the functional effects demonstrated clinically and in the literature, and the importance of these effects noted in numerous disease conditions, the need to then meet an additional burden of showing randomized clinical trials on each and every condition would not only be an improperly high threshold but one that is plainly legally unnecessary given that a functional use, by itself and without more, is sufficient to sustain the nomination.

We have provided some of the referenced articles in full text. Please contact me if further information would be helpful.

Sincerely,

Alan Dumoff

Submission of American Association of Naturopathic Physicians and Integrative Medicine Consortium; Response to Requests for More Information on Nomination for Glutathione FDA Docket 2015-N-3534
March 2, 2018

Cancer Treatment; Enhancement of Treatment and Alleviation of Adverse Chemotherapy Effects

Traverso N, Ricciarelli R, Nitti M at al. Role of glutathione in cancer progression and chemoresistance. Oxid Med Cell Longev. 2013;2013:972913. May 20.

Böhm S, Oriana S, Spatti G at al. Dose intensification of platinum compounds with glutathione protection as induction chemotherapy for advanced ovarian carcinoma. Oncology. 1999;57(2):115-20.

Tew KD. Glutathione-Associated Enzymes In Anticancer Drug Resistance. Cancer Res. 2016 Jan 1;76(1):7-9. doi: 10.1158/0008-5472.CAN-15-3143. Epub 2016 Jan 3.

Meijerman I, Beijnen JH, Schellens JH. Combined action and regulation of phase II enzymes and multidrug resistance proteins in multidrug resistance in cancer. Cancer Treat Rev. 2008 Oct;34(6):505-20. Epub 2008 Apr 14.

Cascinu S., et al. Neuroprotective effect of reduced glutathione on oxaliplatin-based chemotherapy in advance colorectal cancer: a randomized, double-blind, placebo-controlled trial. J Clin Oncol. Aug 15, 2002; @0(16):3478-34383.

J. F. Smyth, A. Bowman, T. Perren, P.Wilkinson, R. J. Prescott, K.J. Quinn & M. Tedeschi. Glutathione reduces the toxicity and improves quality of life of women diagnosed with ovarian cancer treated with cisplatin: Results of a doubleblind, randomised trial. Annals of Oncology 8: 569-573, 1997.

Frank P. T. Hamers, Jan H. Brakkee, Ennio Cavalietti, Michele Tedeschi, Laura Marmonti, Gabriella Pezzoni, Jan P. Neijt, and Willem H. Gispen. Reduced glutathione protects against cisplatin-induced neurotoxicity in rats. Cancer Research 53. 544-549. February 1, 1993.

Ping Chen, Jennifer Stone, Garrett Sullivan, Jeanne A. Drisko Qi Chen. Anti-cancer effect of pharmacologic ascorbate and its interaction with supplementary parenteral glutathione in preclinical cancer models. Free Radical Biology and Medicine
Volume 51, Issue 3, 1 August 2011, Pages 681-687.
https://doi.org/10.1016/j.freeradbiomed.2011.05.031

Balendiran GK, Dabur R, Fraser D. The role of glutathione in cancer. Cell Biochem Funct. 2004;22(6):343-352.[PubMed 15386533]

Singh S, Khan AR, Gupta AK. Role of glutathione in cancer pathophysiology and therapeutic

interventions. J Exp Ther Oncol. 2012;9(4):303-316.[PubMed 22545423]

Zhang ZJ, Hao K, Shi R, et al. Glutathione S-transferase M1 (GSTM1) and glutathione S-transferase T1 (GSTT1) null polymorphisms, smoking, and their interaction in oral cancer: a HuGE review and meta-analysis. Am J Epidemiol. 2011;173(8):847-857.[PubMed 21436184]

Miko Enomoto T, Johnson T, Peterson N, Homer L, Walts D, Johnson N. Combination glutathione and anthocyanins as an alternative for skin care during external-beam radiation. Am J Surg. 2005;189(5):627-630; discussion 630-631.[PubMed 15862509]

Raza A, Galili N, Smith S, et al. Phase 1 multicenter dose-escalation study of ezatiostat hydrochloride (TLK199 tablets), a novel glutathione analog prodrug, in patients with myelodysplastic syndrome. Blood. 2009;113(26):6533-6540.[PubMed 19398716]

Oriana S, Bohm S, Spatti G, Zunino F, Di Re F. A preliminary clinical experience with reduced glutathione as protector against cisplatin-toxicity. Tumori. 1987;73(4):337-40. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3 660470 2.

Di Re F, Bohm S, Oriana S, Spatti GB, Zunino F. Efficacy and safety of high-dose cisplatin and cyclophosphamide with glutathione protection in the treatment of bulky advanced epithelial ovarian cancer. Cancer Chemother Pharmacol. 1990;25(5):355-60. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=2 306797 3.

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Prevention after Myocardial Infarction, Faculty of Medicine of Sousse, Sousse, Tunisia.
BackGROUND: The mechanisms of smoking tobacco leading to chronic obstructive pulmonary disease (COPD) are beginning to be understood. However, conclusions about the role of blood or lung oxidative stress markers were disparate.

AIMS: To investigate the oxidative stress in blood or lung associated with tobacco smoke and to evaluate its effect on pulmonary function data and its relation with physical activity.

METHODS: It is a case-control study. Fifty-four male-smokers of more than five pack-years (PY) and aged 40-60 years were included (29 Non-COPD, 16 COPD). Physical activity score was determined. Blood sample levels of malondialdehyde (MDA), protein-cys-SH (PSH), and Glutathione (GSH) were measured. Fractional exhaled nitric oxide (FeNO) and plethysmographic measurements were performed. Correlation coefficients (r) evaluated the association between oxidative stress markers and independent variables (plethysmographic data and physical activity score).

RESULTS: Non-COPD (48 ± 6 years) and COPD (49 ± 5 years) groups had similar tobacco consumption patterns, that is, 27 ± 14 PY versus 30 ± 19 PY, respectively. Compared to the Non-COPD group, the COPD group had significantly lower levels of GSH and PSH, that is, mean ± SE were 40 ± 6 versus 25 ± 5 µg/mL and 54 ± 10 versus 26 ± 5 µg/g of hemoglobin, respectively. However, MDA level and FeNO values were similar. In the COPD group, none of the oxidative stress markers was significantly correlated with plethysmographic data or physical activity score. In the Non-COPD group, GSH was significantly correlated with physical activity score (r = 0.47) and PSH was significantly correlated with total lung capacity (TLC) (r = -0.50), residual volume (r = 0.41), and physical activity score (r = 0.62). FeNO was significantly correlated with TLC of the COPD group (r = -0.48).

CONCLUSION: Compared to the Non-COPD group, the COPD group had a marked decrease in blood antioxidant markers (GSH and PSH) but similar blood oxidant (MDA) or lung (FeNO) burden.

Gluathione Comments Sent into AANP
Comments from Physicians about Current and Historical Uses

"Glutathione (typically at 60 to 200 mg/ml for IV administration, or 60 to 300 mg per dose for inhalation) is a tripeptide synthesized from Glycine, Glutamic acid and Cysteine. It is a primary intracellular antioxidant that is essential to life. It is useful to prevent radiation injury before radiation treatment is started. It helps reduce the side effects of chemotherapy treatments. It can prevent or reverse alcohol induced fatty liver cirrhosis, hepatitis, and liver lesions. It inhibits chemical induced carcinogenesis. It improves prognosis of stroke victims. It has been used in patients with COPD or chronic lung disease to improve lung functions and minimize oxygen-dependency. It is one of the main anti-oxidation protocols in children with Autistic Syndrome Disorder. Generally, it is useful in any condition where there is a risk for oxidative stress/damage.

Various liver disease protocols can include anti-viral agents, chemotherapy, lactulose, beta-blockers, diuretics, and antibiotics. Cancer treatments protocols include surgery, chemotherapy, and radiation. Stroke treatments can include aspirin, TPA, and more invasive procedures depending upon the acute nature of the symptoms. Glutathione would be used in conjunction with the above protocols to reduce the intensity of symptoms of the disease processes, and to reduce the severity of the side effects of the pharmaceutical protocols. There are no FDA-approved drug products including Reduced L-glutathione for injection, inhalation.
"Reduced L-glutathione is produced by the liver for detoxification purposes. This therapy appears much more attractive than a foreign chemical with possible toxic side effects, acute and chronic. Most or all patients with Autistic Syndrome Disorder receives glutathione therapy.

"There is no alternative to reduced glutathione, either by injection or nebulization, for patients who require effective physiological antioxidant activity for treating COPD, asthma and many other conditions including those related to unintended consequences of radiation therapy and certain types of chemo-therapy.

W Bruce Milliman, ND
Naturopathic Academy of Naturopathic Physicians
(NAPCP-Founding President)"

"GSH has been very important in the successful IV treatment of neurodegenerative disorders like Parkinson's and age related cognitive decline as well as toxicity rehabilitation from adverse reactions to quinolones such as Cipro, as well as environmental toxicity/heavy metal accumulation (over consumption of fish containing mercury, etc) detoxification treatments.

Angela Agrios, ND
Palisades Natural Medicine"

“I have used L-glutathione (L-GSH) IV to support detoxification as this is our bodies most powerful antioxidant, specifically in people who are detoxifying from drug/etoh use and heavy metal/chemical/mold exposures. 1000-2000 mg qwk GSH IV in Parkinson's disease has improved symptoms regardless if they are using carbidopa-levodopa or other pharmaceuticals to improve symptoms. In addition to IV GSH I typically add in oral GSH.

Audrey Schenewerk, ND, MS”

“About five years ago I had a cancer patient who almost died from the effects of conventional cancer treatments. This patient was able to continue those treatment after beginning bi-weekly then weekly 2 grams doses of IV Glutathione.

About eight years ago, a woman brought her autistic daughter in. She was working with another MD who as best I recall had her on an infrequent regimen of three chelating agents, DMPS, NAC and one other which I believe was Calcium Disodium EDTA, plus a bi-weekly IM/subcutaneous shot of 5 mg methylcobalamin. This child developed into a very artistic and energetic young woman.”

“We use Glutathione quite frequently in our clinic. We typically use it in our Lyme, chronic fatigue and multiple chemical sensitivity patients.

Typical dose is 1-2 grams in an IV push 1-2 times/week for 6-8 weeks.

As far as results the most promising ones were from those patients who had acute chemical exposures. One patient is a man who services pools for a living. He was exposed to some chlorinated chemicals and immediately developed SOB, irritability and hives. He came in that evening of exposure and was given a nutritional IV followed by 2 grams of glutathione. He immediately felt less agitated and had less labored breathing. He continued to do 2 more glutathione IV's at 2g 1x/week and was also placed on oral glutathione. He has no residual side effects from his exposure.

I have 2 current pts. with Cipro toxicity who 1-4x/month receive an IV push of glutathione at 1-2g. They both notice less neurological symptoms and brain fog after their IV's.

Another usage of glutathione is before and after surgery to mitigate the side effects many experience from anesthesia. We typically give 1-2g 1-4x before surgery and 1-2g 1-2x/week for 2-4 weeks after surgery which allows most to recover from the brain fog and "spaciness" they report after anesthesia.

Jennifer Wicher, N.D.
Synergy Natural Medicine”

“Reduced Glutathione: GSH has been very important in the successful IV treatment of neurodegenerative disorders like Parkinson's and age related cognitive decline as well as toxicity rehabilitation from adverse reactions to quinolones such as Cipro, as well as environmental toxicity/heavy metal accumulation (over consumption of fish containing mercury, etc) detoxification treatments.

Dr. Angela Agrios, ND
California Licensed Naturopathic Doctor (ND)”

An Example of Many Patient Experiences

Hello,

I'm a patient of Dr. Andria Orlowski, a Phoenix naturopath. She encouraged me to share my experiences with you concerning ingredients under review by FDA.

I have struggled with chronic illness that has resisted conventional medical diagnosis and caused a wide range of symptoms for the last 25 years of my life.

. . . I suffer from a variety of food and chemical sensitivities. Exposure to fragrances makes me sick, so does eating food that contains chemical additives. I have found that taking supplemental reduced L-Glutathione helps me rapidly to overcome symptoms in such a case. Glutathione supplements are a lifesaver for me when I have to travel by air, and forced to breathe jet fuel pollution and the chemical fragrances of the other travelers for an extended period of time. Without taking Glutathione, such exposure results in headaches, nausea, rapid heartbeats, abdominal pain, joint pain and breathing difficulties.

Thanks to a supplemental program including these ingredients, I am a functioning and active member of society.

You have my permission to share this information.

Sincerely.

R. Boerner

Neuroprotective Effect of Reduced Glutathione on Oxaliplatin-Based Chemotherapy in Advanced Colorectal Cancer: A Randomized, Double-Blind, Placebo-Controlled Trial

By Stefano Cascinu, Vincenzo Catalano, Luigi Cordella, Roberto Labianca, Paolo Giordani, Anna Maria Baldelli, Giordano D. Beretta, Emilio Ubiali, and Giuseppina Catalano

Purpose: We performed a randomized, double-blind, placebo-controlled trial to assess the efficacy of glutathione (GSH) in the prevention of oxaliplatin-induced neurotoxicity.

Patients and Methods: Fifty-two patients treated with a bimonthly oxaliplatin-based regimen were randomized to receive GSH (1,500 mg/m² over a 15-minute infusion period before oxaliplatin) or normal saline solution. Clinical neurologic evaluation and electrophysiologic investigations were performed at baseline and after four (oxaliplatin dose, 400 mg/m²), eight (oxaliplatin dose, 800 mg/m²), and 12 (oxaliplatin dose, 1,200 mg/m²) cycles of treatment.

Results: At the fourth cycle, seven patients showed clinically evident neuropathy in the GSH arm, whereas 11 patients in the placebo arm did. After the eighth cycle, nine of 21 assessable patients in the GSH arm suffered from neurotoxicity compared with 15 of 19 in the placebo arm. With regard to grade 2 to 4 National Cancer Institute common toxicity criteria, 11 patients experienced neuropathy in the placebo arm compared with only two patients in the GSH arm (*P* = .003). After 12 cycles, grade 2 to 4 neurotoxicity was observed in three patients in the GSH arm and in eight patients in the placebo arm (*P* = .004). The neurophysiologic investigations (sural sensory nerve conduction) showed a statistically significant reduction of the values in the placebo arm but not in the GSH arm. The response rate was 26.9% in the GSH arm and 23.1% in the placebo arm, showing no reduction in activity of oxaliplatin.

Conclusion: This study provides evidence that GSH is a promising drug for the prevention of oxaliplatin-induced neuropathy, and that it does not reduce the clinical activity of oxaliplatin.

J Clin Oncol 20:3478-3483. © 2002 by American Society of Clinical Oncology.

OXALIPLATIN, A new cytotoxic agent from the diaminocyclohexane platinum family, has a spectrum of activity and toxicity different from that of cisplatin and carboplatin, and it has demonstrated a lack of cross-resistance with other platinum compounds.[1] The role of oxaliplatin in colorectal cancer has been well established. In combination with fluorouracil (5FU), it represents an effective first-line therapy, and its addition to 5FU regimens also represents an active salvage therapy.[2-5] Furthermore, a combination of oxaliplatin and 5FU has proven beneficial in enabling surgical removal of hepatic resections in patients with previously unresectable liver metastases.[6] The coming years will probably expand the therapeutic potential of oxaliplatin in several other cancers, such as breast, ovarian, non–small-cell lung, prostate, and stomach cancers.[7-9]

The most common toxicity resulting from oxaliplatin therapy is neurotoxicity. There are two distinct types of neurotoxicity. There are cold-sensitive paresthesias, which are unique among the platinum complexes studied to date. They occur at low total cumulative doses, are always reversible, and do not require discontinuation of therapy. However, there is also a peripheral sensory neuropathy with symptoms similar to those seen with cisplatin. This form of neurotoxicity is the most important for its clinical implications. The risk of developing severe disturbance of neurologic function is related to the cumulative dose, generally becoming a clinical problem when the cumulative dose approximates 800 mg/m². It is reversible, but it may last for several months and can even require discontinuation of treatment.[10] The mechanism of neurotoxicity induced by platinum drugs has been proposed to involve the accumulation of platinum within the peripheral nervous system, especially in the dorsal root ganglia.[11] However, unlike the case with cisplatin, for oxaliplatin it seems that the greater retention of platinum is due to a slower clearance rather than a greater accumulation of oxaliplatin.[12] These data suggest that a strategy optimal for reducing the neurotoxicity associated with oxaliplatin may be the use of agents such as glutathione

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Submitted July 16, 2001; accepted February 20, 2002.

This article was published ahead of print at www.jco.org.

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0732-183X/02/2016-3478/$20.00

(GSH), which is able to prevent the initial accumulation of platinum adducts in the dorsal root ganglia.[13]

Clinical trials conducted to assess the neuroprotective efficacy of GSH in patients treated with cisplatin reported a lower incidence of neurotoxicity compared with placebo, without any negative interference in oncolytic activity.[14-17] On the basis of these premises, to assess the efficacy of GSH in preventing oxaliplatin-induced neuropathy, a double-blind, placebo-controlled trial was performed in patients with advanced colorectal cancer. All were treated with the same oxaliplatin-based regimen and were given either GSH or placebo.

PATIENTS AND METHODS

Patients with a histologically verified advanced colorectal carcinoma were eligible for the study. Other eligibility criteria included Eastern Cooperative Oncology Group performance status of 0 to 2 and normal bone marrow function (leukocyte count $> 4,000/\mu L$, platelet count $> 100,000/\mu L$), liver function (serum bilirubin < 1.5 mg/dL), renal function (creatinine < 1.5 mg/dL), and cardiac function (stable heart rhythm, no active angina, and no clinical evidence of congestive heart failure). Previous chemotherapy with 5FU, adjuvant or not, was allowed. Patients were excluded if they had established clinical neuropathy, diabetes mellitus, alcoholic disease, other neurologic disease, or brain involvement. Patients who received vitamin B_1, B_6, or B_{12} supplements or who followed other vitamin diets were also excluded.

Informed consent was obtained from all participants after the nature of the study had been fully explained. The protocol was approved by the institutional review board.

The chemotherapeutic regimen consisted of oxaliplatin 100 mg/m^2 on day 1, given as a 2-hour infusion in 250 mL of dextrose 5%, concurrent with 6-*S*-stereoisomer of leucovorin 250 mg/m^2 as a 2-hour infusion followed by a 24-hour infusion of 5FU 1,500 mg/m^2/d for 2 consecutive days. Therapy was repeated every 2 weeks. GSH was given at a dose of 1,500 mg/m^2 in 100 mL of normal saline over a 15-minute period immediately before each oxaliplatin administration, while normal saline solution was administered to placebo-randomized patients. Routine antiemetic prophylaxis with dexamethasone 8 mg and 5-hydroxytryptamine-3 receptor antagonist was used for both treatment arms.

Response was evaluated after four cycles of therapy according to the standard World Health Organization criteria.[18] Patients who showed responsive or stable disease received four further cycles of chemotherapy. Toxicity was assessed after every 2-week cycle using the National Cancer Institute's (NCI) common toxicity criteria (CTC).[19] Chemotherapy was delayed until recovery if the neutrophil count decreased to less than $1,500/\mu L$ or the platelet count decreased to less than $100,000/\mu L$. 5FU and oxaliplatin doses were reduced when NCI CTC grade 3 diarrhea, dermatitis, or stomatitis occurred. In the case of NCI grade 2 sensory neuropathy, the oxaliplatin dose was reduced to 75% of the previous dose; in the case of NCI grade 3 sensory neuropathy, oxaliplatin was omitted from the regimen until recovery. Patients who experienced NCI CTC grade 4 toxicity, apart from alopecia, were withdrawn from the study.

A complete standardized neurologic examination, including an evaluation of strength and deep tendon reflexes, was performed by the neurologists (L.C. and E.U.) involved in the study. Special care was devoted to the presence of symptoms of peripheral nervous system involvement and to the assessment of position and vibratory sensations. The degree of neurotoxicity was expressed according to the NCI CTC.[19] The neurophysiologic evaluation was based on the bilateral determination with surface electrodes of the sensory nerve conduction in the sural nerves. All neurophysiologic examinations were performed under constant conditions of skin temperature (34°C). The same examiners, blinded with respect to the group to which each patient belonged, always performed the neurologic and electrophysiologic evaluations. All the patients were examined before entry onto the study and after four, eight, and 12 cycles of chemotherapy within 2 weeks of the end of treatment.

The study was defined as a double-blind, randomized, phase III trial in which at least 25 patients were assigned to each of the two treatment arms. The sample size was determined to detect a 40% difference in the occurrence of grade 2 to 4 (NCI CTC) neurotoxicity between the two treatment arms, with alpha and beta errors of 0.05 and 0.1, respectively. Grade 2 to 4 toxicities were chosen because, in our experience, these degrees seem to impair the quality of life of patients.

Using cards from a computer-generated list in sealed envelopes, randomization was performed by a person not involved in the care or evaluation of the patients. The personnel who evaluated the efficacy and tolerability of the treatment did not know the drug administered because administration was performed by other staff members.

Analysis of variance with repeated measures and a supplementary two-sided paired *t* test were used to compare the neurophysiologic results of the two groups after four cycles (oxaliplatin cumulative dose, 400 mg/m^2) and eight cycles (oxaliplatin cumulative dose, 800 mg/m^2) of chemotherapy. A χ^2 test with Yates' correction and the Wilcoxon test were used to assess the difference in terms of clinical neurotoxicity between the two groups, both as overall incidence and as a score.[20] This score was derived from the sum of the degree of the worst neurologic toxicity, according to the NCI scale, for each patient divided by the number of assessable patients for each dose step (400 mg/m^2, 800 mg/m^2, and 1,200 mg/m^2).

RESULTS

Fifty-two patients were entered onto the study: 26 were assigned to the placebo arm and 26 to the GSH arm. The patients' characteristics are listed in Table 1. Twelve patients in the placebo arm and 11 in the GSH arm received a 5FU/leucovorin regimen as adjuvant treatment. Seventeen patients in the placebo arm and 19 in the GSH arm were treated with 5FU and leucovorin as first-line treatment at the time of relapse. At baseline, the distribution of the other clinicopathologic variables was comparable between the two groups, except for a major incidence of women in the GSH arm ($P = .09$). No patient was excluded from the study, and an intention-to-treat analysis was performed.

In the placebo arm, seven patients did not complete the second step of treatment (eight cycles): five showed progressive disease, and two patients complained of persistent grade 3 or 4 neurotoxicity. In the GSH arm, five patients did not complete the treatment: four showed progressive disease, and one refused further therapy without clinical signs of neurotoxicity or disease progression.

In the placebo arm, a total of 172 cycles were administered (median, eight); the median dose-intensity of oxali-

Table 1. Patient Characteristics

	Placebo Arm	GSH Arm
No. of patients	26	26
Age, years		
Median	65	65
Range	50-76	40-77
Sex male/female	19/7	12/14
ECOG performance status		
0	20	17
1	6	9
Primary site		
Colon	15	12
Rectum	11	14
Site of metastases		
Liver	18	16
Abdomen	8	10
Peritoneum	4	3
Lung	10	6
Lymph nodes	3	5
Others	3	1
No. of sites		
1	9	14
> 2	17	12
Previous treatment		
No	9	6
Yes	17	19
Adjuvant therapy		
No	14	15
Yes	12	11

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

platin was 38.8 mg/m^2/wk, and the median cumulative dose of oxaliplatin was 783 mg/m^2. In the GSH arm, a total of 175 cycles was administered (median, eight); the median dose-intensity of oxaliplatin was 39.2 mg/m^2/wk, while the median cumulative dose of oxaliplatin was 782 mg/m^2. The reduced dose-intensity of oxaliplatin was mainly due to neurotoxicity in the placebo arm and to other toxicities in the GSH arm. No statistical difference in number of cycles, dose-intensity, or cumulative dose of oxaliplatin between the two groups was observed. At baseline, no patient suffered from clinical neuropathy in either arm.

At the time of the second neurologic examination (four cycles), seven patients had a clinical neuropathy (grade 1 or 2) in the GSH arm (27%; 95% confidence interval [CI], 9.8% to 44%) compared with 11 patients in the placebo arm (42%; 95% CI, 23% to 61%) (Table 2).

After eight cycles of chemotherapy, nine patients (43%; 95% CI, 22% to 64%) had clinical neuropathy in the GSH arm (score, 0.52) compared with 15 patients (79%; 95% CI, 60% to 80%) in the placebo arm (score, 1.68) (P = .04). Remarkably, the incidence of moderate to severe (grade 2 to 4 NCI CTC) clinical neurotoxicity was present in 11 of 19 assessable patients (58%; 95% CI, 35% to 80%) in the placebo arm, compared with only two of 21 assessable patients (9.5%; 95% CI, 0% to 22%) in the GSH arm (P = .003). Furthermore, grade 3 or 4 neurotoxicity was not present in the GSH arm, while it was reported in five patients (26%) in the placebo arm (P = .01).

Only 18 patients received 12 cycles of treatment, 10 in the GSH arm and eight in the placebo arm. Grade 2 to 4 neurotoxicity was observed in only three patients in the GSH arm and in eight patients in the placebo arm (P = .004).

The most frequent neurologic symptoms were distal paresthesia, numbness in the legs, and ataxia, while the physical examinations generally showed decrease or loss of deep tendon reflexes.

The neurophysiologic evaluation showed that no changes in mean latency and sensory amplitude potentials of sural nerves occurred in the GSH and placebo arms after four cycles of chemotherapy. On the contrary, after eight cycles of chemotherapy in the GSH arm, no changes in mean latency and sensory amplitude potentials of the sural nerves had occurred; in the placebo arm, these parameters were significantly affected (Tables 3 and 4). Patients did not continue to receive GSH after oxaliplatin had been stopped, and none of the patients experienced a rebound of their neurologic symptoms.

The other chemotherapy toxicities are reported in Table 5. There were no chemotherapy-related deaths. The main toxicities were neutropenia, diarrhea, stomatitis,

Table 2. Clinical Evaluation of Neurotoxicity

Neurotoxicity NCI NCTC Grade	After 4 Cycles		After 8 Cycles		After 12 Cycles	
	Placebo (n = 26)*	GSH (n = 26)*	Placebo (n = 19)*	GSH (n = 21)*	Placebo (n = 8)*	GSH (n = 10)*
0	15	19	4	12	–	1
1	9	6	4	7	–	6
2	2	1	6	2	2	2
3	–	–	4	–	4	1
4	–	–	1	–	2	–
Score	–	–	1.68	0.52	3	1.3

*Number of assessable patients.

Table 3. Electrophysiologic Results in the Placebo Arm

Sural Nerve	Basal	After 4 Cycles	*P*	After 8 Cycles	*P*
Latency, msec	3.07 ± 0.33	2.90 ± 0.69	NS	3.19 ± 1.70	.03
SAP, μV	10.98 ± 6.92	9.80 ± 5.35	NS	7.20 ± 5.05	.05
CV, m/sec	45.91 ± 4.59	44.03 ± 10.19	NS	39.33 ± 11.66	.01

Abbreviations: SAP, sensory amplitude potential; CV, conduction velocity; NS, not significant.

nausea and vomiting, and transient hepatic failure. They were generally mild, and no statistically significant difference in incidence and severity of toxicities was found between the two groups (Table 5).

No complete response was observed in either arm. A partial response was observed in seven patients (26.9%; 95% CI, 9.8% to 43.9%) in the GSH group and in six patients (23.1%; 95% CI, 6.8% to 39.2%) in the placebo arm, for an overall response rate of 25.0% (95% CI, 13.2% to 36.7%) (Table 6).

After a median overall follow-up period of 11.5 months (range, 3 to 30 months), the median progression-free survival was 7 months (range, 2 to 12 months) for patients in the GSH arm and 7 months (range, 2 to 16 months) for those in the placebo arm. Median survival time was 16 months and 17 months in the GSH and placebo arms, respectively.

DISCUSSION

The mechanism of neurotoxicity induced by platinum drugs has been proposed to involve the accumulation of platinum within the peripheral nerve system.[11,12] The major site of damage seems to be the dorsal root ganglia, which is consistent with the platinum accumulation studies. In fact, biodistribution studies have shown that the platinum concentrations are greater in the dorsal root ganglia followed by the dorsal root and peripheral nerves.[12] Damage to the dorsal root ganglia seems to result in axonopathy of peripheral nerves, especially in the large myelinated fibers responsible for sensory nerve conduction. In a rat model, the sciatic nerves showed marked axonal atrophy and a decrease in the number of large sensory axons, whereas the motor axons remained unaffected.[21]

The neurotoxicity associated with oxaliplatin is similar in nature to that associated with cisplatin. However, unlike the case with cisplatin, the pathologic presence of oxaliplatin in the dorsal root ganglia is due to a relative slower clearance of the drug rather than to an increased accumulation.[13] These data suggest that an optimal strategy for reducing the neurotoxicity associated with oxaliplatin may be the use of agents such as GSH, which may be able to prevent the initial accumulation of platinum adducts in dorsal root ganglia.[13]

A major role of GSH in the prevention of platinum-induced neurotoxicity has been suggested by recent experimental findings. Park et al[22] showed that reactive oxygen species generated by platinum compounds play an important role in platinum-induced neuronal apoptotic cell death via activation of the p53 signaling pathway. Preincubation of nerves from a mouse dorsal root ganglion with *N*-acetylcysteine, a precursor of GSH, blocks or attenuate the accumulation of p53 protein in response to platinum, resulting in a block of platinum-induced apoptosis and in a neuroprotective effect.[22] Finally, preclinical and clinical experiences provided evidence that GSH was effective for the prevention of cisplatin-induced neurotoxicity without reducing the clinical activity of cisplatin.[14-17]

On the basis of these premises, we performed this double-blind, placebo-controlled, randomized trial using the same GSH schedule as reported in our previous work.[16] Our results indicate that GSH can exert a beneficial effect on oxaliplatin neurotoxicity. In fact, we have shown that GSH given concurrently with oxaliplatin is able to reduce the symptoms and signs of neuropathy significantly. In addition, neurophysiologic investigations based on the evaluation of latency and amplitude of the sensory nerve conduction, the most common indexes impaired in platinum neuropathy, supported the neuroprotective effects of GSH.

These findings may have important clinical implications. In fact, in several cases, despite good clinical activity, treatment with oxaliplatin must be discontinued because of the onset of neurotoxicity. The concomitant use of GSH

Table 4. Electrophysiologic Results in the GSH Arm

Sural Nerve	Basal	After 4 Cycles	*P*	After 8 Cycles	*P*
Latency, msec	2.98 ± 0.97	3.17 ± 0.76	NS	3.08 ± 0.99	NS
SAP, μV	9.09 ± 6.34	10.89 ± 7.89	NS	8.71 ± 5.50	NS
CV, m/sec	39.87 ± 13.0	39.48 ± 13.04	NS	39.13 ± 11.63	NS

Abbreviations: SAP, sensory amplitude potential; CV, conduction velocity; NS, not significant.

Table 5. Worst Grade of Toxicity by Each Patient (absolute numbers)

Toxicity (NCI CTC)/Grade	Placebo Arm	GSH Arm
Anemia		
1/2	2	5
3/4	0	0
Neutropenia		
1/2	7	7
3/4	4	1
Thrombocytopenia		
1/2	5	4
3/4	0	0
Nausea		
1/2	9	10
3	0	0
Vomiting		
1/2	7	10
3/4	0	0
Diarrhea		
1/2	6	6
3/4	0	2
Stomatitis		
1/2	6	6
3/4	0	0

may allow the administration of an effective treatment for a more prolonged time. In fact, in the placebo arm, none of the patients could receive further oxaliplatin treatment because of the development of neurotoxicity; in the GSH arm, seven patients did not develop any sign of clinical neurotoxicity and could continue on treatment. In the coming years, there will be an expanding use of oxaliplatin in several other cancers as well as in the adjuvant setting, as indicated by two ongoing randomized trials in colon cancer in Europe (Multicenter International Study of Oxaliplatin 5FU-LV in the Adjuvant Treatment of Colon Cancer [MOSAIC] trial) and the United States (National Surgical Adjuvant Breast and Bowel Project C-07), oxaliplatin-induced neuropathy will be a growing, relevant clinical problem.

Table 6. Tumor Response and Survival

	GSH Arm	Placebo Arm
Patients enrolled, n	26	26
CR	0	0
PR, %	26.9	23.1
SD, %	57.7	53.8
PD, %	15.4	23.1
OR, %	26.9	23.1
95% CI, %	9.8-43.9	6.8-39.2
PFS, months	7+	7
Survival, months	16	17

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; OR, overall response; PFS, progressive-free survival.

Regarding toxicity and possible interference with oxaliplatin antitumor activity by GSH, as previously reported in other studies on cisplatin, we did not observe either.

The results we achieved with this double-blind, placebo-controlled trial have provided evidence indicating that GSH is a promising drug for the prevention of oxaliplatin-induced neuropathy. Other attempts to reduce neurotoxicity associated with oxaliplatin included the development of regimens alternating the combination of oxaliplatin/5FU with 5FU alone in order to allow a long-term period of treatment but reducing the total cumulative dose of oxaliplatin, or the use of other possible chemoprotectants, such as gabapentin. Preliminary data with this drug seem to be promising.[23] In seven patients, neuropathy disappeared and did not recur with additional chemotherapeutic courses. However, in some patients, increased doses of gabapentin were needed; so far, a prolonged administration of this drug may be precluded because of its potential side effects. In contrast, the lack of toxicity and interference with oxaliplatin activity, as well as its low economic cost, makes GSH an ideal new drug for the prevention of oxaliplatin-induced neuropathy in colorectal cancer patients.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2013, Article ID 972913, 10 pages
http://dx.doi.org/10.1155/2013/972913

Review Article

Role of Glutathione in Cancer Progression and Chemoresistance

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Received 22 January 2013; Revised 26 April 2013; Accepted 29 April 2013

Academic Editor: Donald A. Vessey

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Glutathione (GSH) plays an important role in a multitude of cellular processes, including cell differentiation, proliferation, and apoptosis, and disturbances in GSH homeostasis are involved in the etiology and progression of many human diseases including cancer. While GSH deficiency, or a decrease in the GSH/glutathione disulphide (GSSG) ratio, leads to an increased susceptibility to oxidative stress implicated in the progression of cancer, elevated GSH levels increase the antioxidant capacity and the resistance to oxidative stress as observed in many cancer cells. The present review highlights the role of GSH and related cytoprotective effects in the susceptibility to carcinogenesis and in the sensitivity of tumors to the cytotoxic effects of anticancer agents.

1. Introduction

Reactive oxygen species (ROS) are physiologically produced by aerobic cells [1] and their production increases under conditions of cell injury [2]. Physiological levels of ROS mediate crucial intracellular signaling pathways and are essential for cell survival. However, an excess of ROS formation generates cell damage and death. To prevent the irreversible cell damage, the increase of ROS induces an adaptive response, consisting in a compensatory upregulation of antioxidant systems, aimed to restore the redox homeostasis [3].

Oxidative stress has long been implicated in cancer development and progression [4], suggesting that antioxidant treatment may provide protection from cancer [5]. On other hand, prooxidant therapies, including ionizing radiation and chemotherapeutic agents, are widely used in clinics, based on the rationale that a further oxidative stimulus added to the constitutive oxidative stress in tumor cells should, in fact, cause the collapse of the antioxidant systems, leading to cell death [6]. However, this latter approach has provided unsatisfactory results in that many primary tumors overexpress antioxidant enzymes at very high levels, leading to a resistance of cancer cells to drug doses [7].

Among the enzymatic systems involved in the maintenance of the intracellular redox balance, a main role is played by GSH [8] that participates, not only in antioxidant defense systems, but also in many metabolic processes [9].

Elevated GSH levels are observed in various types of tumors, and this makes the neoplastic tissues more resistant to chemotherapy [10, 11]. Moreover, the content of GSH in some tumor cells is typically associated with higher levels of GSH-related enzymes, such as γ-glutamylcysteine ligase (GCL) and γ-glutamyl-transpeptidase (GGT) activities, as well as a higher expression of GSH-transporting export pumps [11, 12]. Therefore, it is not surprising that the GSH system has attracted the attention of pharmacologists as a possible target for medical intervention against cancer progression and chemoresistance.

The main research in this field has been aimed at depleting GSH by a specific inhibition of GCL, a key enzyme of GSH biosynthesis. In this context, buthionine sulfoximine (BSO) is the most popular GSH-depleting agent studied in both preclinical and early clinical trials, but limitation on its availability has led to a search for alternatives [13, 14]. Recently, GSH analogues have been employed in order to sensitize tumors to cytotoxic effects of anticancer agents, by depleting GSH-related cytoprotective effects [15].

However, during the last decade, a new approach for the regulation of GSH-utilizing enzymes has emerged. It is also evident that many of the antioxidant enzymes are induced

by GSH depletion at the transcriptional level which involves the binding of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor to the antioxidant response element (ARE) in the promoter region of the genes encoding GCL and glutathione *S*-transferases [16].

2. GSH Biosynthesis

Glutathione (GSH) is a tripeptide formed by glutamic acid, cysteine, and glycine. The glutamic acid forms a particular gamma-peptic bond with cysteine by its gamma glutamyl group. Two forms of GSH are possible: the reduced form (GSH) which represents the majority of GSH, reaching millimolar concentration in the intracellular compartment, and the oxidized form (GSSG) that is estimated to be less than 1% of the total GSH. Intracellularly, the majority of GSH is found in the cytosol (about 90%), while mitochondria contain nearly 10% and the endoplasmic reticulum contains a very small percentage [17].

The synthesis of GSH from its constituent amino acids involves two ATP-requiring enzymatic steps: (1) the first step is rate-limiting and catalyzed by GCL which is composed of two subunits: one catalytic (GCLC) and one modifier (GCLM):

MgATP MgADP + Pi

L-Glu + L-Cys —GCL→ L-Glu L-Cys

γ-glutamylcysteine

(2) The second step is catalyzed by GSH synthetase (GS) [19]:

MgATP MgADP + Pi

L-Glu L-Cys + Gly —GS→ L-Glu L-Cys Gly

γ-glutamylcysteine GSH

Although GS is generally thought not to be important in the regulation of GSH synthesis, accumulating evidence suggests that GS may play an important role, at least in certain tissues and/or under stressful conditions [20].

However, under normal physiological conditions, the rate of GSH synthesis is largely determined by two factors, that is, cysteine availability and GCL activity. Cysteine is normally derived from the diet, protein breakdown and in the liver, from methionine via transsulfuration (conversion of homocysteine to cysteine). Cysteine differs from other amino acids because its sulfhydryl form, cysteine, is predominant inside the cell whereas its disulfide form, cystine, is predominant outside the cell [21].

3. Glutathione Functions

The chemical structure of GSH determines its potential functions, and its broad distribution among all living organisms reflects its important biological role. A major function of GSH is the detoxification of xenobiotics and some endogenous compounds. These substances are electrophiles and form conjugates with GSH, either spontaneously or enzymatically, in reactions catalyzed by GSH-*S*-transferases (GST) [22]. Human GSTs are divided into two distinct family members: the membrane-bound microsomal and cytosolic family members. The conjugates formed are usually excreted in the bile, but can also undergo modification to mercapturic acid.

Another important GSH function is the maintenance of the intracellular redox balance and the essential thiol status of proteins [21].

The reaction with the protein is as follows:

$$\text{Protein-SSG} + \text{GSH} \longleftrightarrow \text{Protein-SH} + \text{GSSG}. \quad (1)$$

The equilibrium of this reaction depends on the concentrations of GSH and GSSG. The reversible thiolation of proteins is known to regulate several metabolic processes including enzyme activity, transport activity, signal transduction and gene expression through redox-sensitive nuclear transcription factors such as AP-1, NF-kappaB (NF-kB) and p53 [21, 23]. In fact, DNA-binding activity of transcription factors often involves critical Cys residues, and the maintenance of these residues in a reduced form, at least in the nuclear compartment, is necessary [24]. AP-1 is a transcription factor related to tumor promotion [25], and its DNA-binding activity can be diminished if Cys-252 is oxidized [26]. Tumor suppressor p53, known as the "guardian of the genome," contains 12 Cys residues in its amino acid sequence [27], and oxidation of some of these inhibits p53 function [28]. Moreover, GSH performs an antioxidant function (Figure 1).

In addition, storage of cysteine is one of the most important functions of GSH because cysteine is extremely unstable extracellularly and rapidly autooxidizes to cystine in a process that produces potentially toxic oxygen-free radicals [29]. The γ-glutamyl cycle allows GSH to be the main source of cysteine (Figure 2).

4. Role of GSH in Regulating Cancer Development and Growth

In many normal and malignant cells, increased GSH level is associated with a proliferative response and is essential for cell cycle progression [30, 31]. The molecular mechanism of how GSH modulates cell proliferation remains largely speculative. A key mechanism for GSH's role in DNA synthesis relates to the maintenance of reduced glutaredoxin or thioredoxin, which is required for the activity of ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis [32].

Furthermore, in liver cancer and metastatic melanoma cells, GSH status is correlated with growth [33–35] and it has also been demonstrated that a direct correlation between GSH levels associated with cellular proliferation and metastatic activity exists [33]. In fact, intrasplenic inoculation of B16 melanoma (B16M) cells into C57BL/6J syngenic mice induced metastatic foci formation by colonizing different organs. However, the number and size of metastases were much higher when B16M cells with high GSH content were inoculated *in vivo* [33]. A high percentage of tumor cells with high GSH content were able to survive in the presence of

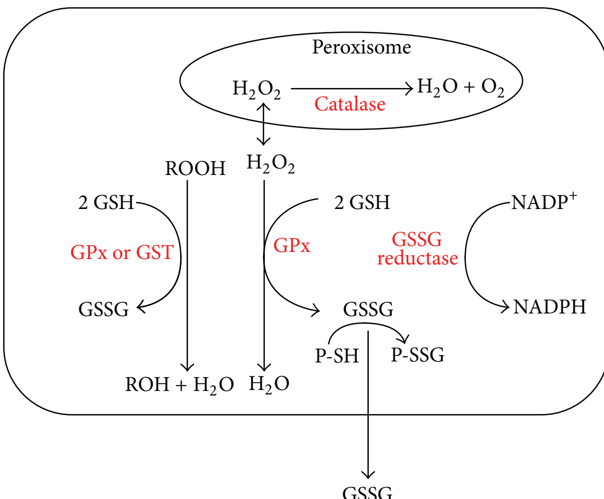

Figure 1: Antioxidant function of GSH. The hydrogen peroxide, produced during the aerobic metabolism, can be metabolized in the cytosol by GSH peroxidase (GPx) and catalase in peroxisomes. In order to prevent oxidative damage, the GSSG is reduced to GSH by GSSG reductase at the expense of NADPH, forming a redox cycle [17]. Organic peroxides can be reduced both by GPx and GSH-transferase (GST). In extreme conditions of oxidative stress, the ability of the cell to reduce GSSG to GSH may be less, inducing the accumulation of GSSG within the cytosol. In order to avoid a shift in the redox equilibrium, the GSSG can be actively transported out of the cell or react with protein sulfhydryl groups (PSH) and form mixed disulfides (PSSG).

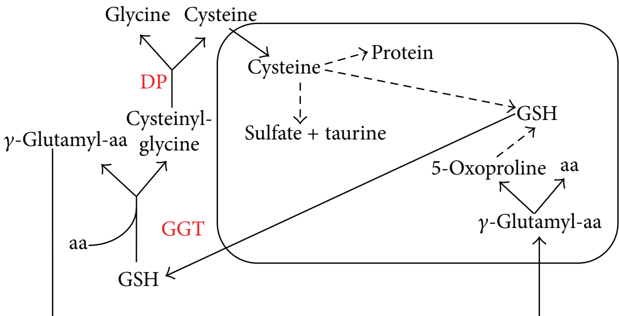

Figure 2: γ-Glutamyl cycle. In the γ-glutamyl cycle, GSH is released from the cell and the ectoenzyme GGT transfers the γ-glutamyl moiety of GSH to an amino acid (aa, the best acceptor being cysteine), forming γ-glutamyl-aa and cysteinyl-glycine. The γ-glutamyl-amino acid can then be transported back into the cell and once inside can be further metabolized to release the aa and 5-oxoproline, which can be converted to glutamate and used for GSH synthesis. Cysteinyl-glycine is broken down by dipeptidase (DP) to generate cysteine and glycine. Once inside the cell, the majority of cysteine is incorporated into GSH, some being incorporated into protein, and some degraded into sulfate and taurine [18].

the nitrosative and oxidative stress, thereby representing the main task force in the metastatic invasion [36]. Therefore, it is plausible that maintenance of high intracellular levels of GSH could be critical for the extravascular growth of metastatic cells. Moreover, maintenance of mitochondrial GSH homeostasis may be a limiting factor for the survival of metastatic cells in the immediate period following intra-sinusoidal arrest and interaction with activated vascular endothelial cells. Mitochondrial dysfunction is a common event in the mechanism leading to cell death [37], and, recently, it has been found to be an essential step for the killing of non-small-cell lung (NSCLC) carcinomas which are resistant to conventional treatments [38]. Thus, the impairment of GSH uptake by mitochondria may be important to sensitize invasive cancer cells to prooxidant compounds capable of activating the cell death mechanism.

As previously reported, GSH is effluxed by cells through GGT-mediated metabolism, allowing a "GSH-cycle" to take place, which is implicated in tumor development [39]. In fact, GGT-positive foci were found in animals exposed to prooxidant carcinogens, suggesting the hypothesis of GGT as an early marker of neoplastic transformation [40, 41]. Moreover, increased levels of GGT have been observed in cancers of the ovaries [42], colon [43], liver [44], melanoma [45], and leukemias [46]. In studies on melanoma cells *in vitro* and *in vivo*, elevated GGT activity has been found to accompany an increased invasive growth [45, 47, 48], and a positive correlation has been described between GGT expression and unfavourable prognostic signs in human breast cancer [49].

The prooxidant activity of GGT has also recently been shown to promote the iron-dependent oxidative damage of

DNA in GGT-transfected melanoma cells, thus potentially contributing to genomic instability and an increased mutation risk in cancer cells [50]. GGT/GSH-dependent prooxidant reactions has been shown to exert an antiproliferative action in ovarian cancer cells [51], while other studies in U937 lymphoma cells have shown that basal GGT-dependent production of hydrogen peroxide can represent an antiapoptotic signal [52]. The modulatory effects of GGT-mediated prooxidant reactions could contribute to the resistance phenotype of GGT-expressing cancer cells by regulating both signal transduction pathways involved in proliferation/apoptosis balance, as well as by inducing protective adaptations in the pool of intracellular antioxidants.

5. GSH Depletion as an Experimental Approach to Sensitize Tumor Cells to Therapy

Cancer cell lines containing low GSH levels have been demonstrated to be much more sensitive than control cells to the effect of irradiation [18]. In fact, GSH depletion obtained by BSO, the irreversible inhibitor of GCL, is the most frequently used approach and it is associated with many chemotherapeutic agents [53–57]. However, molecular signaling of BSO-induced apoptosis is poorly understood, and, recently, it has been demonstrated that in different leukaemia and lymphoma cells, the death receptor-mediated apoptotic pathway, induced by arsenic trioxide plus BSO, is triggered via JNK activation [58]. Moreover, in neuroblastoma cells susceptible to BSO treatment, DNA damage and apoptosis was triggered via PKC-δ activation and ROS production [59, 60].

In fact, BSO in combination with melphalan [14, 61], is currently undergoing clinical evaluation in children with neuroblastoma (NCT00002730; NCT00005835) and in patients with persistent or recurrent stage III malignant melanoma (NCT00661336). Recently, it has been demonstrated that a combination of azathioprine with BSO is useful for localized treatment of human hepatocellular carcinoma [62].

Therefore, BSO clinical use is restricted by its short half-life, with the consequent need for prolonged infusions resulting in its nonselective effect of GSH depletion on both normal and malignant cells [63].

6. Role of GSH in Chemoresistance

The increase in GSH levels, GCL activity and GCLC gene transcription is associated with drug resistance in tumor cells [64, 65].

The increase in GSH is a major contributing factor to drug resistance by binding to or reacting with, drugs, interacting with ROS, preventing damage to proteins or DNA, or by participating in DNA repair processes. In melanoma cells, GSH depletion and GGT inhibition significantly increased cytotoxicity via oxidative stress [66]. In addition, it has been demonstrated that GGT-overexpressing cells were more resistant to hydrogen peroxide [67] and chemotherapics, such as doxorubicin [68], cisplatin [64], and 5-fluorouracil [69].

Moreover, it has been found that the human multidrug resistance protein (MRP), a member of the superfamily of ATP-binding cassette membrane transporters, can lead to resistance to multiple classes of chemotherapeutic agents [70, 71]. Several studies have shown coordinated overexpression of GCLC and MRP in drug-resistant tumor cell lines, in human colorectal tumors and in human lung cancer specimens after platinum exposure [70, 71].

Three mechanisms have been proposed for the role of GSH in regulating cisplatin (CDDP) resistance: (i) GSH may serve as a cofactor in facilitating MRP2-mediated CDDP efflux in mammalian cells; (ii) GSH may serve as a redox-regulating cytoprotector based on the observations that many CDDP-resistant cells overexpress GSH and γ-GCS; and (iii) GSH may function as a copper (Cu) chelator.

Moreover, overexpression of specific GSTs can also affect chemoresistance, whereas polymorphisms that decrease GST activity are associated with a high risk of developing cancer [72]. An elevated expression of GSTs, combined with high GSH levels, can increase the rate of conjugation and detoxification of chemotherapy agents, thus reducing their effectiveness [73]. In addition to the transferase function, GSTs have been shown to form protein-protein interactions with members of the mitogen activated protein (MAP) kinases. By interacting directly with MAPKs, including c-Jun N-terminal kinase 1 (JNK1) and apoptosis signal-regulating kinase 1 (ASK1), GSTs bind the ligand in a complex structure, preventing interactions with their downstream targets [74]. Many anticancer agents induce apoptosis via activation of MAP kinases, in particular JNK and p38 [75, 76]. This novel, nonenzymatic role for GSTs has direct relevance to the GST overexpressing phenotypes of many drug-resistant tumors. As an endogenous switch for the control of intracellular signaling pathways, an elevated expression of GST can alter the balance of kinases during drug treatment, thereby causing a potential selective advantage for tumor growth.

The promoter regions encoding GSTs and γGCL possess binding sites for transcriptional regulators such as NF-kB, AP-1, AP-2, and the Nrf2/Kelch-like ECH-associated protein 1 (Keap1) system. After exposure to oxidative stimuli, Nrf2 dissociates from Keap1, its negative regulator, and translocates into the nucleus where it heterodimerizes with small Maf proteins [77] and binds to antioxidant responsive element (ARE) sequences, triggering a cytoprotective adaptative response. This response is characterised by upregulation of several cytoprotective and detoxification genes, including ferritin, GSH-*S*-reductase (GSR), GST, GCLM, and GCLC, phase-I drug oxidation enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1), MRP, and heme oxygenase-1 (HO-1) [78, 79].

However, in numerous types of cancer, Nrf2 is upregulated and takes on a protumoral identity since the above-cited cytoprotective genes, not only give tumors an advantage, but also lead to drug resistance [80–82].

To date, numerous mutations have been found of both Keap1 and Nrf2 in various human cancers resulting in the constitutive expression of prosurvival genes. Most of Nrf2

somatic mutations have led to the impairment of their recognition site for Keap1, which then has led to the continuous activation of Nrf2. Moreover, it has been observed that the prognosis of patients with either Nrf2 or Keap1 mutations is much lower than patients with no mutation [83].

Among Nrf2-regulated genes, HO-1 is the most well known as a stress protein that can have both antioxidative and anti-inflammatory effects [84]. It catalyzes the rate-limiting step in the catabolism of the prooxidant heme to carbon monoxide, biliverdin, and free iron [85].

Recent experimental evidence has shown the involvement of HO-1 in cancer cell biology. On one hand, HO-1 protects healthy cells from transformation into neoplastic cells by counteracting ROS-mediated carcinogenesis and, on the other hand, HO-1 protects cancer cells, enhancing their survival and their resistance to anticancer treatment [86]. In addition, high levels of HO-1 have been observed in various human solid tumors, such as renal [87], prostatic [88], and pancreatic cancers [89]. Moreover, HO-1 expression in tumor cells can be further increased by anticancer treatments (chemo-, radio-, and photodynamic therapy) [90], and it has been hypothesized that HO-1 and its products may have an important role in the development of a resistant phenotype. In this context, it has been recently demonstrated that BSO and/or the inhibition of the Nrf2/HO-1 axis is able to increase the sensitivity of neuroblastoma cells to etoposide [91, 92].

7. Therapeutic Potential of GSH and GSH-Modulating Agents

The modulation of the GSH-based antioxidant redox system (GRS), the major determinant of the cellular redox status [93], might represent a promising therapeutic strategy for overcoming cancer cell progression and chemoresistance.

However, GSH itself cannot be administered clinically with any effect, and for this reason, a variety of precursors or chemically modified analogues have been generated in order to mimic glutathione's various physiological or pharmacological effects. *N*-acetylcysteine (NAC; Mucomyst) represents the earlier GSH analogue, and YM737, a monoester of GSH, recently discovered, has been favourably compared to NAC [94]. Another GSH analogue approach is cysteine-substituted *S*-nitrosoglutathione [95].

Since abundant levels of GST [96] have been identified in resistant tumors, GSH analogues, that differentially inhibit GST isoforms, have been developed. Telcyta (TLK-286) is a GSH analogue utilized in combination with cytotoxic chemotherapies such as platinum, taxanes, and anthracyclines in a variety of tumors with very high levels of glutathione *S*-transferase pi-1-gene (GST-P1-1) [97]. Telintra (TLK199) is another small molecule inhibitor of GST-P1-1 developed for the potential prevention of myelosuppression in blood diseases, namely, myelodysplastic syndrome [98]. After a phase 1 clinical trial [99] and a phase 2 using an oral formulation, TLK199 appeared to be well tolerated and with some efficacy in the myeloplastic syndrome treatment [100].

In addition, the implication of GGT activity in the resistance phenotype of cancer cells suggests a potential use of GGT inhibitors associated with chemotherapeutics in order to deplete intracellular GSH and/or to inhibit extracellular drug detoxification. Different GGT inhibitors are known [101–103], but, unfortunately, these molecules are toxic and cannot be used in humans.

Moreover, drugs that target *S*-glutathionylation have direct anticancer effects since they act on a wide range of signalling pathways [57]. Among the agents that mediate their effects through *S*-glutathionylation, NOV-002 has been most extensively studied, with a phase III trial (NCT00347412) completed in advanced NSCLC [104], and data available from phase II trials in breast and ovarian cancers [105]. NOV-002 is a product containing oxidized glutathione that alter the GSH : GSSG ratio and induces *S*-glutathionylation [106]. NOV-002-induced *S*-glutathionylation has been shown to have inhibitory effects on proliferation, survival, and invasion of myeloid cell lines and significantly increased the efficacy of cyclophosphamide chemotherapy in a murine model of colon cancer [107].

In a randomized phase II trial, NOV-002 in combination with standard chemotherapy has shown promising effects in patients with stage IIIb/IV of NSCLC [108]. Positive results were also obtained from a phase II trial in patients with neoadjuvant breast cancer therapy [109].

Other therapeutic agents include phenolic antioxidants (α-napthoflavone, butylated hydroxyanisole, and *tert*-butyl hydroquinone), synthetic antioxidants (ethoxyquin, oltipraz, and phorbol esters), triterpenoid analogue (oleanolic acid derivatives, sesquiterpenes), and isothiocyanates (sulforaphane). Sulforaphane (SF) is the strongest natural inducer of Nrf2 and phase II detoxifying enzymes and it has a potent anticarcinogenic and chemopreventive effect by inducing apoptosis and cell cycle arrest [110].

On the other hand, the inhibition of Nrf2 signaling might be employed to enhance the sensitization of chemoresistant tumors to cytotoxic agents, and in this context, it has recently been reported that brusatol, a compound found in a plant extract, acts as inhibitor of this pathway and may exhibit therapeutic utility [111]. Another effective approach to increasing cancer cell sensitivity to chemotherapeutic drugs would be to silence both Nrf2 and Keap1 simultaneously [112]. Related to Nrf2, a potential target for redox chemotherapy is HO-1. HO-1 inhibitors, including zinc protoporphyrin and more soluble pegylated derivatives (PEG-ZnPP), have been successfully used to improve chemosensitization of cancer cells [113]. Moreover, HO-1 inhibitors administered intravenously, displayed cytotoxic activity in a murine hypoxic solid tumor model [114].

Moreover, disulfiram (DSF) does not cause depletion of total GSH, but shifts the ratio of GSH/GSSG towards the oxidized state. DSF induces apoptosis of human melanoma cells [115], and this apoptogenic effect has encouraged ongoing clinical phase I/II studies in human metastatic melanoma (NCT00256230).

Arsenic trioxide (As_2O_3) is a prooxidant chemotherapeutic compound combined with agents that deplete cellular GSH [116]. As_2O received FDA approval in 2000 for the treatment of nonacute promyelocytic leukemia and is used in patients who have relapsed or are refractory to first-line intervention using retinoid and anthracycline chemotherapy.

As_2O inhibits GPx and mitochondrial respiratory function that leads to increased ROS leakage contributing to antileukemia activity. Another piece of evidence suggests that the irreversible inhibition of thioredoxin reductase is the key mechanism underlying As_2O-induced breast cancer cell apoptosis [117]. Importantly, As_2O_3 sensitivity is associated with low levels of GSH in cancer cells and GSH depletion, obtained by BSO or ascorbate treatment, contributes to sensitizing cells toward apoptosis [118]. The potentiation of As_2O chemotherapeutic efficacy using BSO was demonstrated in an orthotopic model of prostate cancer metastasis [119].

8. Conclusions

The modulation of cellular GSH is a double-edged sword, both sides of which have been exploited for potential therapeutic benefits [120]. Enhancing the capacity of GSH and its associated enzymes, in order to protect cells from redox-related changes or environmental toxins, represents a persistent aim in the search for cytoprotective strategies against cancer. On the contrary, the strategy of depleting GSH and GSH-related detoxification pathways is aimed at sensitizing cancer cells to chemotherapy, the so-called chemosensitization [121]. In this context, it has been reported that GSH and GSH enzyme-linked system may be a determining factor for the sensitivity of some tumors to various chemotherapeutic agents. In particular, GST is a relevant parameter for chemotherapy response, and it may be utilized as a useful biomarker for selecting tumors potentially responsive to chemotherapeutic regimens.

However, the attempts to deplete GSH have been limited by the nonselective effects of BSO and have stimulated the research of new GCL inhibitors.

Since it is well known that GSH depletion leads to the upregulation of antioxidant genes, many of which are under Nrf2 control and, that in several types of tumors, Nrf2 is constitutively activated [122, 123], a new and indirect approach for cancer therapy may be used to modulate the Nrf2-ARE pathway. Based on this, Nrf2 creates a new paradigm in cytoprotection, cancer prevention, and drug resistance.

In summary, the involvement of GSH in the carcinogenesis and in the drug resistance of tumor cell is clear, but further studies, aimed at understanding the GSH-driven molecular pathways, might be crucial to design new therapeutic strategies to fight cancer progression and overcome chemoresistance.

Acknowledgments

This work was supported by Grants from PRIN 2009M8FKBB_002 and Genoa University, Italy. The authors would like to thank Ms. Suzanne Patten for language editing.

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Prog. Neuro-Psychopharmacol. & Biol. Psychiat. 1996, Vol. 20, pp. 1159-1170
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0278-5846/96 $32.00 + .00

PII S0278-5846(96)00103-0

REDUCED INTRAVENOUS GLUTATHIONE IN THE TREATMENT OF EARLY PARKINSON'S DISEASE

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(Final form, July 1996)

Abstract

Sechi GianPietro, Maria G. Deledda, Guido Bua, Wanda M. Satta, Giovanni A. Deiana, Giovanni M. Pes, and Giulio Rosati: Reduced intravenous glutathione in the treatment of early Parkinson's Disease. Prog. Neuro-Psychopharmacol & Biol. Psychiat. 1996. 20, pp. 1159-1170

1. Several studies have demonstrated a deficiency in reduced glutathione (GSH) in the nigra of patients with Parkinson's Disease (PD). In particular, the magnitude of reduction in GSH seems to parallel the severity of the disease. This finding may indicate a means by which the nigra cells could be therapeutically supported.
2. The authors studied the effects of GSH in nine patients with early, untreated PD. GSH was administered intravenous, 600 mg twice daily, for 30 days, in an open label fashion. Then, the drug was discontinued and a follow-up examination carried-out at 1-month interval for 2-4 months. Thereafter, the patients were treated with carbidopa-levodopa.
3. The clinical disability was assessed by using two different rating scale and the Webster Step-Second Test at baseline and at 1-month interval for 4-6 months. All patients improved significantly after GSH therapy, with a 42% decline in disability. Once GSH was stopped the therapeutic effect lasted for 2-4 months.
4. Our data indicate that in untreated PD patients GSH has symptomatic efficacy and possibly retards the progression of the disease.

Keywords: Parkinson's Disease; reduced glutathione.

Abbreviations: Columbia University Rating Scale (CURS); Parkinson's Disease (PD); Patients Global Impressions (PGI); reduced glutathione (GSH); resting tremor (RT); Webster Step-Second Test (W.S.S.T.).

Introduction

The mechanisms underlying dopamine cells death in the zona compacta of substantia nigra in Parkinson's disease (PD) remain unclear. However, current concepts of this process indicate that free radicals generated by oxidation reactions may play a key role (Jenner et al., 1992). Indeed, in postmortem tissues from patients with PD there is evidence for inhibition of complex 1 of the mitochondrial respiratory chain, altered iron metabolism and decreased levels of reduced glutathione (GSH) (Riederer et al., 1989, Jenner, 1993). Of these defence mechanisms implicated in the prevention of free-radical-induced tissue damage, only the reduction in the levels of GSH in substantia nigra appears to be specific to PD (Jenner, 1993, Sian et al., 1994) and, noteworthy, this reduction has been also found in cases of incidental Lewy body disease (presymptomatic PD) (Perry et al., 1982, Sian et al., 1992). In particular, the magnitude of reduction in GSH seems to parallel the severity of PD and, in advanced stages, in the nigra, GSH is virtually undetectable (Riederer et al., 1989). In addition, data from animal studies have shown that an induced GSH depletion in mice produces morphological changes in nigral dopamine neurons resembling those seen in normal aging and in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) neurotoxicity (McNeil et al., 1986). These observations have led us to determine the effect of intravenous (i.v.) GSH in patients with early, untreated PD.

Methods

Patients

After giving informed consent, 9 consecutive patients with idiopathic PD were enrolled in the study. There were 6 men and 3 women, age 66 ± 9 years (mean ± SD) (range, 49 to 77); Hoehn and Yahr stage of parkinsonism 2 ± 0.9 (mean ± SD) (range 1 to 4), with a disability duration of 13 ± 4.5 months (mean ± SD) (range, 8 to 24). The patients were considered eligible for the study, provided that they had not been treated previously with any antiparkinsonian drug or other agents active on the central nervous system including deprenyl or vitamin E. The patients with dementia (Mini-Mental State Examination), or depression (Hamilton Rating Scale) were excluded.

Procedures

GSH was administered i.v. 600 mg in 250 ml saline, as 1-hour infusion, twice daily, at 8.00 A.M. and 4.00 P.M, for 30 days, in an open label fashion. The patients were assessed at baseline and 30 days after the treatment. Then, GSH

was discontinued and, if the patient status improved, a follow-up examination was carried-out at 1-month interval, until the patient's clinical status returned to baseline, or when the patient felt he or she was worsened. Thereafter, the patients were treated with carbidopa-levodopa (25-250 mg), half-tablet three times daily, and a new examination carried-out after 30 days, about two hours after the intake of the drug.

Assessments

On each visit, the clinical disability was assessed according to a modified Columbia University Rating Scale (CURS) (Yahr et al., 1969), and to the Webster Step-Second Test (W.S.S.T.) (scoring method: time in seconds to stand and walk a prescribed course and sit again) (Webster, 1968).

The subscores evaluated at the modified CURS were: speech, hypomimia, tremor at rest, action or postural tremor of hands, rigidity, finger taps, hand movements, pronation and supination of hands, foot taping, arising from chair, posture, gait, balance and hypokinesia.

For the W.S.S.T., three sequential trials were performed for each patient, at baseline and at each control, with a fixed intertrial interval of 15 s. In the tabulation of the results the authors used the mean (± SD) of the three W.S.S.T. values obtained, for each patient, at the beginning of the experiment and after each of the trial periods.

Clinical response was also self assessed by patients according to Patients Global Impressions (PGI) (Guy, 1976). This scale (ranging from 1 = very much better, to 7 = very much worse) was used to assess the change in severity of the disease from the beginning of the study and from the previous visit.

All patients were evaluated by the same examiner throughout the study. On each examination, they were observed over two consecutive days. In addition, in patients with tremor, at baseline and at each examination, at approximately the same time of day, tremograms were recorded using an accelerometer transducer attached to the index finger of the hand and recorded on an EEG polygraph. Tremor frequency (in Hertz) and visual mean amplitude (mean value of tremor estimated visually in microvolts) were measured from the tracings.

Laboratory Assessments

The following laboratory tests were performed at entry and after 30 days of GSH therapy: routine blood chemistries, liver function tests, blood counts, urinalysis and ECG. A chest X-ray and a brain CT, performed without contrast, were conducted in all patients before study entry.

Data Analysis

A statistical analysis of W.S.S.T. values was made, for each patient, by Student's *t* test for paired samples. A nonparametric statistical method was used to compare clinical parkinsonian scores (Wilcoxon Matched-pairs Signed-ranks Test) and the PGI (McNemar's Test). In addition, the sums of clinical parkinsonian scores for each patient, at baseline, were correlated either with the percent improvement calculated through the same scale, or with the percent improvement at W.S.S.T. after GSH therapy. The percentage of change was calculated by the following formula:
prestudy value - treatment value / prestudy value x 100 = % change. The level of significance was $p < 0.05$.

Results

All 9 patients enrolled completed the study. There were no serious complications from i.v infusion of GSH. Two patients during the third week of i.v GSH treatment had fever (axillary, peak temperature, 38.2°C), erithemia of the skin, irritation and hardness at injection site, likely due to infusion thrombophlebitis. The irritation and fever cleared up after 5 days of antibiotic and antiphlogistic therapy. Patient 6 after completion of the wash-out period suffered a thigh-bone fracture. GSH did not induce clinically significant changes in any laboratory test compared with basal conditions. The brain CTs showed a mild cortical atrophy in two patients and no definite abnormalities in seven of them. The frequency of resting tremor (RT) was 5 to 6 Hz. In patient 1, the mean amplitude of RT, compared with the baseline period, was reduced, approximately, by 50% after GSH therapy, and by 25% after carbidopa-levodopa (Fig 1). No definite variations in the mean amplitude of RT were noted in the other patients, in the various sequences of treatment, with respect to baseline, or to the wash-out period. At W.S.S.T. all patients improved significantly after GSH, with respect to baseline (from $p < 0.05$, to $p < 0.01$); instead, after levodopa-carbidopa, only five patients improved significantly, with respect to the wash-out period (from $p < 0.02$, to $p < 0.01$). (Table 1). In our opinion, for the dosages of levodopa-carbidopa and GSH used, the transient improvement induced by these drugs was roughly comparable. The total scores for parkinsonian disability (modified CURS) were significantly lower either after GSH therapy, with respect to baseline ($p < 0.007$), or after levodopa-carbidopa with respect to the wash-out period ($p < 0.01$). (Table 2). A significant improvement after GSH therapy, with respect to baseline, of modified CURS subscores, was evidenced for speech, hypomimia, rigidity, pronation and supination of hands, foot taping, posture, gait, balance and hypokinesia (from $p < 0.02$, to $p < 0.007$).

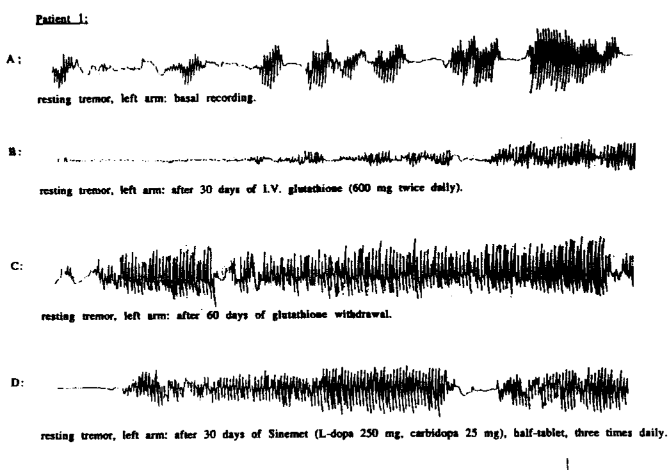

Fig 1. Representative resting tremor recordings in patient 1, at baseline (A); 30 days after therapy with reduced glutathione (B); 60 days after withdrawal of reduced glutathione (C); and 30 days after carbidopa-levodopa (D): Vertical calibration is 200 microvolts; horizontal scale is 1 second.

A similar improvement was noted after carbidopa-levodopa, with respect to the wash-out period (from $p < 0.04$, to $p < 0.01$). As seen from Tables, the values of the modified CURS scores and the values of W.S.S.T., after withdrawal of GSH therapy, reached the baseline values after 2.6 ± 0.7 months (range, 2 to 4 months). The correlation coefficient between total CURS scores at baseline, and the percent improvement calculated through the same scale, after GSH therapy, is shown in Fig 2. In Fig 3, is shown the correlation coefficient between total CURS scores at baseline and the percent improvement at the W.S.S.T. after GSH therapy. The slope of this late correlation is significantly different than zero ($r = 0.6813$; $p = 0.0433$), while the correlation shown in Fig 2 is non-significant.

Table 1

Webster Step-Second Test (W.S.S.T): Scoring Method: Time in Seconds to Stand and Walk a Prescribed Course and Sit Again (mean ± SD, of 3 sequential trials).

Patient	W.S.S.T. At Baseline	W.S.S.T. After GSH	% Improv.1	Wash-out (months)	W.S.S.T. After Wash-out	W.S.S.T. After Lev.+DCI	% Improv.2
1	46.8 ± 1.6	41.2 ± 0.3**	12	3	45.5 ± 0.86	43.0 ± 0.5**	5.5
2	58.7 ± 0.6	57.2 ± 0.6*	3	4	65.7 ± 1.1	63.7 ± 1.1	3
3	34.0 ± 0.1	33.0 ± 0.5*	3	3	37.7 ± 0.58	34.7 ± 0.57**	8
4	34.3 ± 0.5	33.3 ± 0.3*	3	2	42.7 ± 0.58	42.0 ± 0.5	2
5	59.0 ± 1.8	52.0 ± 1.8**	12	2	51.2 ± 0.3	50.0 ± 0.1**	2.5
6	32.9 ± 4.3	25.6 ± 1.5*	22	2	32.3 ± 2.6	-	-
7	44.3 ± 1.15	39.7 ± 0.58**	10	2	45.0 ± 1.2	39.6 ± 1.9***	12
8	51.7 ± 0.8	49.4 ± 1.0*	4.5	2	52.0 ± 1.2	48.2 ± 1.3***	7
9	25.0 ± 0.1	22.7 ± 1.1*	9	3	24.3 ± 0.6	24.0 ± 0.1	1

Lev.+DCI=Levodopa+Decarboxylase Inhibitor; W.S.S.T. values after GSH were compared with baseline; W.S.S.T. values after Lev.+DCI were compared with the wash-out period; *$p<0.05$; **$p<0.01$; ***$p<0.02$ (Student's t Test for paired samples).

Table 2

Modified Columbia University Rating Scale: Total Scores in 9 Patients with Parkinson's Disease Treated with GSH (600 mg/day, I.V.) or Levodopa (375 mg/day + DCI, per os).

Patient	Baseline T. Scores	T. Scores After GSH	% Improv.1	Wash-out (months)	T. Scores After Wash-out	T. Scores After Lev.+DCI	% Improv.2
1	25	15	40	3	24	14	42
2	12	3	75	4	16	3	81
3	29	20	31	3	27	20	26
4	15	7	53	2	18	7	61
5	35	17	51	2	30	14	53
6	42	26	38	2	42	-	-
7	20	15	25	2	21	14	33
8	31	19	39	2	32	21	34
9	34	25	27	3	35	26	26
Mean ± SD	27 ±10	16 ± 8*	42 ± 16	2.6 ± 0.7	27 ± 8	15 ± 7**	44.5 ± 19

Lev.+DCI=Levodopa+Decarboxylase Inhibitor; T. Scores=Total Scores; T. Scores values after GSH were compared with baseline; T. Scores values after Lev.+DCI were compared with the wash-out period; *$p<0.007$; **$p<0.01$; (Wilcoxon Matched-pair Signed-ranks Test).

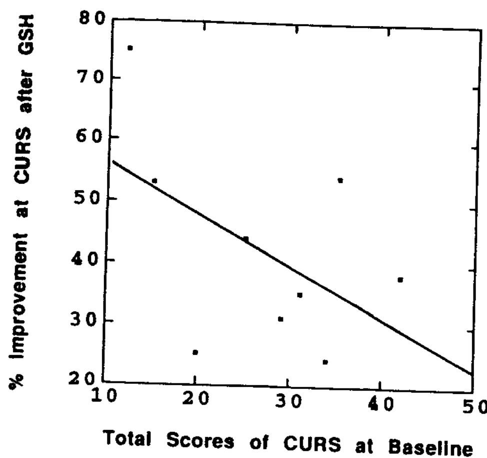

Fig 2. Correlation coefficient between total CURS scores at baseline and the percent improvement calculated through the same scale after therapy with reduced glutathione ($r= - 0.5027$; p=n.s.).

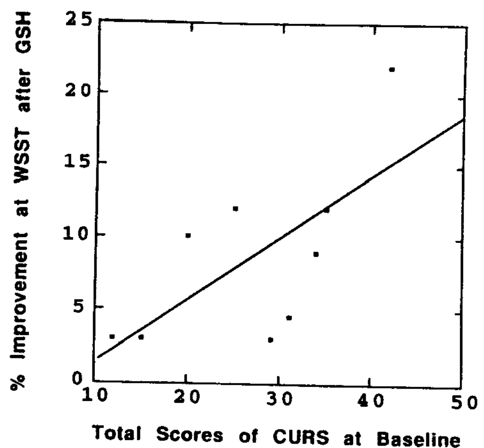

Fig 3. Correlation coefficient between total CURS scores at baseline and the percent improvement at the W.S.S.T. after therapy with reduced glutathione ($r=0.6813$; $p=0.0433$).

Total scores of PGI were significantly lower either after GSH therapy, with respect to wash-out period (or, baseline) ($p < 0.0039$) or after levodopa-carbidopa, with respect to the wash-out period ($p < 0.0039$), where, no significant difference was found at PGI between GSH therapy and levodopa-carbidopa. One patient (n.9) with a marked sialorrhea, reported the disappearance of this symptom after GSH therapy. After withdrawal of GSH the benefit lasted for about 3 months. Levodopa-carbidopa therapy was ineffective on sialorrhea in the same patient.

Discussion

The crucial observation, that in idiopathic PD the magnitude of reduction in GSH in substantia nigra seems to parellel the severity of the disease, may indicate a means by which the nigra cells could be therapeutically supported (Riederer et al., 1989).

GSH and the Blood-Brain Barrier

GSH is a tripeptide (gamma-glutamyl-cysteinyl-glycine) which in physiological conditions is believed to be extracted in minimal amount at the blood-brain barrier (Cornford et al., 1978). In addition, as it is a naturally occurring peptide, the possibility exists that there may be a breakdown of glutathione in plasma by peptidases, as in other tissues and at blood-brain barrier itself (Meister and Tate, 1976). Therefore, its clinical value as a therapeutic agent, if administered by a peripheral route, should be minimal. However, since recent investigations support the concept of a selective transcytosis for many peptides across an intact blood-brain barrier (Pardridge, 1986), and since the finding that in idiopathic PD the locus coeruleus, which helps to preserve the integrity of blood-brain barrier functions, is damaged (Tomonaga, 1983, Harik and McGunigal, 1984), the authors administered GSH as 1-hour infusion two times daily for 30 days in PD patients, to investigate a possible therapeutic effect of this peptide, after peripheral administration. Actually, recent experimental evidences have shown blood-brain extraction of circulating GSH in a brain perfusion model, and the transcytosis of intact GSH into the brain parenchyma without breakdown (Zlokovic et al., 1994).

Effects of Intravenous GSH in Parkinson's Disease

The results of our open study indicate that in PD this peptide given i.v. may reach its specific target in the brain (i.e, the nigra cells) and may have a significant beneficial effect on several parkinsonian signs. In particular, as

shown in Fig 3, the therapeutic effect of GSH on hypokinesia appears to be correlated to the severity of the symptom. This peptide was also effective in reducing the RT in one patient, but failed in other four. In this patient, GSH apparently improved the RT more than levodopa-carbidopa. In our opinion, since the dosages of levodopa-carbidopa and GSH used are not comparable, to draw this conclusion is incorrect. Once GSH was stopped the therapeutic effect lasted for 2-4 months. This finding, in our opinion, is a strong evidence against a placebo effect and this may indicate a protective effect of the drug on the rate of progression of PD. However, this does not necessarily exclude a symptomatic effect of GSH. These concepts are supported by the results of two double-blind studies on the use of selegine, or bromocriptine versus placebo in PD (Teychenne et al., 1982, Myllylä et al., 1992). Indeed, in these studies, the mean CURS scores in the placebo group returned to baseline after about 1-month, and the symptomatic effect of bromocriptine, once stopped, did not last for more than four weeks (Teychenne et al., 1982, Myllylä et al., 1992).

Hypothetical Mechanisms Underlying the Therapeutic Effect of GSH

The mechanism underlying the therapeutic effect of GSH in PD is unknown. According to the most basic neurochemical abnormality in the brain of PD patients (i.e., the marked loss of dopamine in the nigrostriatal neuron system) (Ehringer and Hornykiewiez, 1960), an action of GSH at dopaminergic synapses (presynaptically or postsynaptically) can be hypothesized. In particular, based on the theoretical notion that decreasing the oxidative load in substantia nigra may slow disease progression, it would seem that GSH, because of its antioxidant properties (e.g., reduced formation of hydrogen peroxide), may be able to protect the striatonigral cells and foster dopaminergic activity (Jenner, 1993). Recent evidences of glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes fit this hypothesis (Volterra et al., 1994). Another important biological function that has been ascribed to glutathione is the role in translocation of amino acids, and possibly also peptide, across cell membranes (Meister and Tate, 1976). This function may be important for the transport of substrate and specific proteic neurotrophic factors into the dopaminergic neurons of the substantia nigra (Tooyama et al., 1993). Given the reduction in the levels of GSH in cells of substantia nigra, in PD this function is likely impaired. A replacement therapy with exogenous GSH may contribute to its reinstatement. A controlled study of this peptide for the treatment of PD seems warranted.

Conclusion

The findings indicate that in PD GSH given i.v. may reach its specific target in the brain (i.e., the nigra cells), it has a significant beneficial effect on several parkinsonian signs and possibly retards the progression of the disease.

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Prog. Neuro-Psychopharmacol. & Biol. Psychiat. 1996, Vol. 20, pp. 1171-1180
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0278-5846/96 $32.00 + .00

PII S0278-5846(96)00104-2

ALCOHOL WITHDRAWAL AND DOPAMINE RECEPTOR SENSITIVITY AFTER PROLONGED ABSTINENCE

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(Final form, July 1996)

Abstract

Wiesbeck, Gerhard A., Eugen Davids, Norbert Wodarz, Johannes Thome, Gert Weijers, Franz Jakob and Jobst Boening: Alcohol Withdrawal and Dopamine Receptor Sensitivity after Prolonged Abstinence. Prog. Neuro-Psychopharmacol. & Biol. Psychiat. 1996, 20, pp. 1171–1180

1. Forty-four male inpatients suffering from moderate to severe alcohol dependence (DSM-III-R and ICD-10) as well as 14 healthy controls entered this study. Individuals were classified according to the severity of their withdrawal symptoms during detoxification i. e. group 1) no withdrawal, group 2) autonomic hyperactivity, group 3) withdrawal delirium and group 4) controls.
2. During the 6th week of treatment, that is, when all patients were recovered, controlled abstinent, and several weeks away from the end of their withdrawal syndrome, dopamine receptor sensitivity was neuroendocrinologically assessed by stimulating human growth hormone (HGH) with apomorphine (APO).
3. In a repeated measures model ANOVA, the four groups differed significantly in their HGH release. However, when excluding the controls from the analysis and focusing on alcoholics only (group 1 - 3), the significant difference disappeared. Covariates such as age, weight, quantity of drinking and duration of dependence were not related to the dependent variable.
4. In conclusion, the first significant result (with controls) reflects a blunted HGH response in alcoholics. It confirms earlier reports. The second, non significant result with the alcohol dependents only, suggests that the severity of withdrawal is not reflected by the amount of HGH released. Therefore, in alcoholics, a reduced dopamine receptor function after six weeks of abstinence, as neuro-endocrinologically assessed with apomorphine, seems to be related to alcohol dependence rather than to the severity of alcohol withdrawal.

Keywords: alcohol dependence, alcohol withdrawal, apomorphine, dopamine receptor sensitivity.

Abbreviations: apomorphine (APO), analysis of variance (ANOVA), body mass index (BMI), cerebrospinal fluid (CSF), Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R), homovanillic acid (HVA), human growth hormone (HGH), International Classification of Diseases (ICD-10), Muenchner Alkoholismus Test (MALT), Statistical Package for the Social Sciences (SPSS).

Movement Disorders
Vol. 24, No. 7, 2009, pp. 979–983
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Randomized, Double-Blind, Pilot Evaluation of Intravenous Glutathione in Parkinson's Disease

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Abstract: The objective of this study was to evaluate the safety, tolerability, and preliminary efficacy of intravenous glutathione in Parkinson's disease (PD) patients. This was a randomized, placebo-controlled, double-blind, pilot trial in subjects with PD whose motor symptoms were not adequately controlled with their current medication regimen. Subjects were randomly assigned to receive intravenous glutathione 1,400 mg or placebo administered three times a week for 4 weeks. Twenty-one subjects were randomly assigned, 11 to glutathione and 10 to placebo. One subject who was assigned to glutathione withdrew from the study for personal reasons prior to undergoing any postrandomization efficacy assessments. Glutathione was well tolerated and there were no withdrawals because of adverse events in either group. Reported adverse events were similar in the two groups. There were no significant differences in changes in Unified Parkinson's Disease Rating Scale (UPDRS) scores. Over the 4 weeks of study medication administration, UPDRS ADL + motor scores improved by a mean of 2.8 units more in the glutathione group ($P = 0.32$), and over the subsequent 8 weeks worsened by a mean of 3.5 units more in the glutathione group ($P = 0.54$). Glutathione was well tolerated and no safety concerns were identified. Preliminary efficacy data suggest the possibility of a mild symptomatic effect, but this remains to be evaluated in a larger study. © 2009 Movement Disorder Society

Key words: glutathione; Parkinson's disease; treatment; antioxidant; neuroprotection; UPDRS

Glutathione is a tripeptide of glutamate, cysteine, and glycine, that plays multiple roles in the brain.[1] It serves as an important central nervous system antioxidant, clears free radicals including superoxide radicals, hydroxyl radicals, nitric oxide, and carbon radicals,[2,3] and helps clear hydrogen peroxide.[4] In addition, glutathione helps maintain the cellular redox state of protein thiols and low-molecular-weight antioxidants such as vitamin E and ascorbate.[5] Recent evidence suggests that glutathione can also act as a neurotransmitter and neurohormone.[6]

In Parkinson's disease (PD), glutathione is reduced by 40–50%.[7–11] This reduction is specific within the brain to the substantia nigra[9] and correlates with

Additional Supporting Information may be found in the online version of this article.

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Potential conflict of interest: All authors have indicated that they have nothing to disclose regarding Wellness Health and Pharmaceuticals or glutathione. Dr. Robert Hauser has received honoraria or consulting fees from Bayer Schering Pharma AG, Bertek, Boehringer Ingelheim, Centopharm, Eisai, Genzyme, GlaxoSmithKline, Impax, Kyowa Pharmaceutical, Merck KgaA, Ortho McNeil, Novartis, Pfizer, Prestwick, Schwarz Pharma, Schering, Solvay, Synosia, Teva Neuroscience, Valeant, and Vernalis. Dr. Kelly Lyons has received honoraria or consulting fees from Advanced Neuromodulation Systems, GlaxoSmithKline, Novartis, Teva Neuroscience, UCB Pharma, and Valeant Pharmaceuticals. Terry McClain has received honoraria or consulting fees from Eisai, GlaxoSmithKline, Kyowa Pharmaceutical, Solvay, Teva Neuroscience, Serono, and Vernalis. Summer Carter has received honoraria or consulting fees from Solvay and Asubio Pharmaceuticals. Dr. David Perlmutter reports nothing to disclose.

Received 8 July 2008; Revised 20 August 2008; Accepted 24 October 2008

Published online 19 February 2009 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/mds.22401

disease severity.[10] In contrast to PD, glutathione is not decreased in multiple system atrophy or progressive supranuclear palsy, despite nigral neuronal loss.[8,9] In incidental Lewy body disease, glutathione is substantially decreased even as nigral cell loss is modest, and complex I activity is not significantly decreased,[12] suggesting that the reduction of glutathione is a very early event in PD.

In vitro experiments indicate that prolonged depletion of glutathione results in selective impairment of mitochondrial complex I activity.[13] To the extent that mitochondrial dysfunction impairs dopaminergic function, it is possible that glutathione replacement could provide symptomatic benefit in PD. There is also interest in glutathione as a possible neuroprotective agent. In a genetically engineered mouse line, glutathione depletion resulted in mitochondrial complex I inhibition, followed by age-related nigrostriatal neurodegeneration.[14] In addition, deficiency of glutathione peroxidase enhanced MPTP toxicity in a mouse knockout model,[15] whereas pretreatment with glutathione reduced MPTP toxicity in mouse brain slices.[16]

There have been few studies of glutathione as a treatment for PD. Sechi et al.[17] reported a 42% decline in PD disability with 600 mg glutathione administered intravenously twice daily for 30 days. The therapeutic effect was observed to last for 2 to 4 months. We performed a randomized, placebo-controlled, double-blind, pilot evaluation of the tolerability, safety, and preliminary efficacy of intravenous glutathione (1,400 mg) administered three times a week for 4 weeks compared with placebo in PD patients. This regimen was chosen because of its anecdotal use in clinical practice.

PATIENTS AND METHODS

This was a 12-week, randomized, parallel group, double-blind, placebo-controlled pilot study. Eligible patients were those with a diagnosis of PD (with at least two of three cardinal signs—rest tremor, bradykinesia, and rigidity) whose motor symptoms were not adequately controlled on their current medication regimen. Inclusion criteria included Hoehn and Yahr Stage II–IV and Mini Mental State Examination score of >24. Subjects were on a stable regimen of antiparkinsonian medications for at least 1 month prior to study entry. Exclusion criteria included prior exposure to glutathione, atypical parkinsonism, use of neuroleptics, history of a seizure, or drug addiction. All subjects provided written informed consent prior to study participation.

After screening and baseline evaluations, subjects were randomly assigned 1:1 to receive 1,400 mg glutathione or placebo diluted in 10 mL of normal saline and administered by intravenous push over 10 minutes on Monday, Wednesday, and Friday of each week for a 4-week period. Attempts were made to administer study medication at the same time of day for each subject, usually in the morning. Glutathione and placebo were supplied by Wellness Health and Pharmaceuticals (Birmingham, Alabama). Antiparkinsonian medications were kept stable throughout the study.

All adverse events were recorded. Safety assessments included vital signs and ECGs. Supine and standing blood pressures were obtained at baseline and 10 minutes after the completion of each intravenous infusion. ECGs were obtained before the first infusion and ~10 minutes after the completion of each infusion. Efficacy assessments were performed at baseline and at the end of weeks 1, 2, 3, 4, 8, and 12. Assessments at the end of weeks 1, 2, 3, and 4 (treatment phase) were performed 1 hour following study medication infusion. Assessments at weeks 8 and 12 were performed at approximately the same time of day as during the treatment phase to evaluate changes after study medication discontinuation. Assessments included Unified Parkinson's Disease Rating Scale (UPDRS, parts I–III), timed walking, tapping, and clinical global impression of change (CGI-C). For patients with motor fluctuations, motor assessments were obtained when subjects were in the "ON" state. The predefined efficacy outcome measure of greatest interest was the change in UPDRS ADL + motor scores from baseline to week 4.

Randomization was performed using a computer-generated randomization schedule. The sample size selected for the study was 20 subjects. Because this was a pilot study, no efficacy power analysis was performed. Adverse events and discontinuations were to be reported descriptively. Efficacy measures were compared across groups using Wilcoxon-signed rank comparisons and analysis of variance for nonparametric data, with no correction for multiple analyses and significance set at $P < 0.05$. Total levodopa (L-dopa) dose equivalents were calculated as follows: (regular L-dopa dose × 1) + (L-dopa controlled-release dose × 0.75) + (pramipexole dose × 67) + (ropinirole dose × 16.67) + (pergolide dose × 100) + (bromocriptine dose × 10) + ([regular L-dopa dose + (L-dopa controlled-release dose × 0.75)] × 0.25 if taking tolcapone) + ([regular L-dopa dose + (L-dopa controlled-release dose × 0.75)] × 0.1 if taking entacapone).[18]

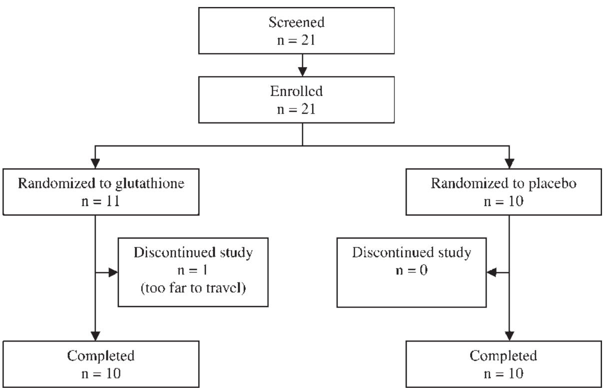

FIG. 1. Subject flowchart.

RESULTS

Subjects were enrolled in the study from September 2003 to January 2007 (Fig. 1). One subject enrolled in the study underwent baseline evaluations, was randomized, received two doses of glutathione, and withdrew prior to any post–dose efficacy assessments, because he felt it was too difficult to continue to drive to the study site. This subject was a 69-year-old man with a 7-yr duration of PD. He did not report any adverse events and is not considered here further. For the remaining 20 subjects, mean age (SD) was 64.3 (10.4) years, with a range of 43 to 84 years. Mean disease duration was 7.6 (4.6) years, with a range of 2 to 18 years. Eleven subjects were men and nine were women. There were no significant differences in baseline characteristics between the two groups (Table 1).

Mean daily L-dopa equivalents in the placebo group were 483.7 (256.2) mg and in the glutathione group were 611.0 (317.4) mg ($P = 0.34$). Individual daily L-dopa equivalents, daily L-dopa dosages, and other medications taken are delineated in Supporting Table 1. Of the 10 subjects in the placebo group, nine were receiving L-dopa at a mean dose of 467 mg/day (203 mg/day). The one subject in this group not receiving L-dopa was receiving pramipexole 1.5 mg/day. Of 10 subjects in the glutathione group, 7 were receiving L-dopa at a mean dose of 629 mg/day (263 mg/day). Of 3 subjects not receiving L-dopa in this group, 1 was receiving ropinirole 22.5 mg/day and selegiline 10 mg/day, 1 was receiving pergolide 4 mg/day, pramipexole 6 mg/day, amantadine 400 mg/day, and trihexyphenidyl 8 mg/day, and 1 was receiving pramipexole 2.5 mg/day. Five of ten subjects who were assigned to placebo were experiencing motor fluctuations and 7 of 10 who were assigned to glutathione were experiencing motor fluctuations.

Adverse events reported during the 4 weeks of study medication administration are listed in Table 2. The adverse events reported by more than one subject in the glutathione group were headache (three glutathione, two placebo), muscle soreness/cramps (three glutathione, three placebo), nausea (two glutathione, 0 placebo), and dizziness/lightheaded (two glutathione, two placebo). One subject in the glutathione group reported mild nausea 2 days after her fourth medication administration, and another reported mild nausea 1 day after her third medication administration. Five subjects in the placebo group reported fatigue/tiredness compared with one in the glutathione group, and three subjects in the placebo group reported cold symptoms/cough compared with none in the glutathione group. No subjects in either group withdrew from the study because of adverse events and there were no serious adverse events. Analysis of vital signs and ECGs revealed no significant differences between groups.

TABLE 1. *Baseline characteristics*

	Placebo (n = 10) [mean (SD), range]	Drug (n = 10) [mean (SD), range]	*P*
Age (yr)	65.9 (12.6), 43–84	62.6 (7.9), 49–73	0.49
Disease duration (yr)	5.8 (3.1), 2–10	9.4 (5.2), 3–18	0.08
Gender	6 M, 4 F	5 M, 5 F	
Levodopa equivalents (mg)	483.7 (256.2), 50–825	611.0 (317.4), 168–1200	0.34
UPDRS mentation	2.2 (1.6), 0–4	1.8 (1.7), 0–5	0.59
UPDRS ADL	11.7 (6.4), 3–27	12.1 (3.9), 7–19	0.87
UPDRS motor	23.4 (9.5), 8–40	25.5 (6.2), 17–39	0.57
UPDRS ADL + motor	35.1 (14.8),13–67	37.6 (9.5), 28–58	0.66
UPDRS total	37.3 (16.0),13–71	39.4 (9.5), 28–58	0.73
HY	2.0 (0), 2.0	2.1 (0.3), 2.0–3.0	0.33
SE	81.5 (15.3), 40–90	83.0 (9.5), 60–90	0.80
MMSE	29.7 (0.5), 29–30	29.6 (0.7), 28–30	0.71

UPDRS, Unified Parkinson's Disease Rating Scale; ADL, activities of daily living; HY, Hoehn and Yahr staging; SE, Schwab and England; MMSE, Mini Mental State Examination.

TABLE 2. *Adverse events during treatment phase (baseline through week 4)*

	Glutathione (n = 10)	Placebo (n = 10)
Headache	3	2
Muscle soreness/cramps	3	3
Dizziness/orthostatic hypotension	2	2
Nausea	2	0
Erythema at infusion site	1	0
Falling/unbalanced gait	1	2
Fatigue/tired	1	5
Hair loss	1	0
Pain (knee, foot)	1	2
Sleep difficulties	1	0
Sweating increased	1	0
Upper respiratory infection	1	0
Vivid dreaming	1	0
Cold symptoms/cough	0	3
Difficulty breathing	0	1
Dyskinesia increased	0	1
Edema	0	1
Hot flashes	0	1
Mouth tremor increased	0	1
Numbness	0	2
Strep throat	0	1
Vision problems worsening	0	1

There was no significant difference between groups in change in UPDRS ADL + motor scores from baseline to week 4 ($P = 0.32$; Fig. 2, Supp. Table 2). UPDRS ADL + motor scores improved from 37.6 (9.5) at baseline to 34.8 (8.1) at week 4 in the glutathione group and were unchanged in the placebo group, 35.1 (14.8) at baseline and 35.1 (14.2) at week 4. Also, there was no significant difference between groups in change in UPDRS ADL + motor scores from weeks 4 to 12 (evaluating possible loss of symptomatic benefit; $P = 0.54$). UPDRS ADL + motor scores worsened from 34.8 (8.1) at week 4 to 36.3 (8.3) at week 12 in the glutathione group and improved in the placebo group from 35.1 (14.2) at week 4 to 33.1 (15.2) at week 12. Significant differences across groups were also not observed for other outcome measures (Supp. Table 2). In the glutathione group, CGI-C indicated that at 4 weeks (end of treatment phase) compared with baseline, four were mildly improved and six were unchanged. At 12 weeks (8 weeks after study medication discontinuation) compared with baseline, seven were unchanged and three were mildly worse. In the placebo group, at week 4, 1 subject was moderately improved, 7 were unchanged, and 2 were mildly worse. At week 12, 1 was still moderately improved, 4 were unchanged, 4 were mildly worse, and 1 was moderately worse compared with baseline.

DISCUSSION

In this pilot study, glutathione was well tolerated and there were no withdrawals because of adverse events. Reported adverse events were similar in the glutathione and placebo groups, with the possible exception of nausea, which was reported in 2 of 10 glutathione patients and 0 of 10 placebo patients. In both cases, nausea occurred once, did not occur on the same day as medication administration, and was mild and transient. No safety concerns were identified regarding vital signs or ECGs.

We did not observe a significant improvement in parkinsonian signs and symptoms in the glutathione group when compared with the placebo group. However, this was a pilot study and was not powered to definitively evaluate efficacy. In addition, efficacy observations may be further limited by the heterogeneity of the study population. Nonetheless, during the 4-week course of study medication administration, the glutathione group was observed to improve a mean of 2.8 units more than the placebo group as assessed by UPDRS ADL + motor scores. In the 8 weeks following study medication discontinuation, the glutathione group was observed to worsen a mean of 3.5 units more than the placebo group as assessed by UPDRS ADL + motor scores. These observations suggest the possibility that glutathione may provide a mild symptomatic benefit, but this would have to be evaluated in a larger clinical trial.

In an open-label study of nine PD subjects, Sechi et al.[17] reported that intravenously administered glutathione (600 mg) twice daily for 30 days reduced disability by 42% as assessed using a modified Columbia University Rating Scale. We did not observe improvement of this magnitude. This could be because their study was open label whereas ours was placebo-

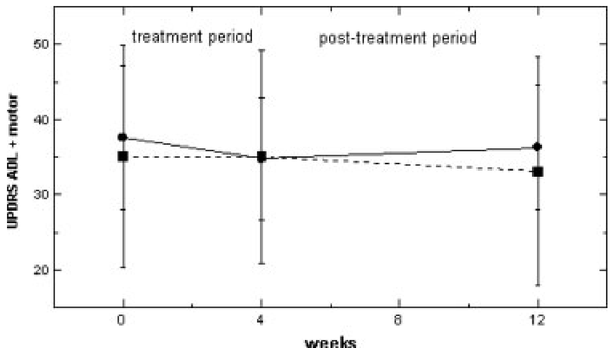

FIG. 2. Mean UPDRS ADL + motor scores for placebo (squares, dashed lines) and glutathione groups (circles, solid lines) at baseline, week 4, and week 12.

controlled and double-blind. However, their patients received a total of 36,000 mg of glutathione over 1 month, whereas our patients received 16,800 mg over 1 month and we cannot exclude the possibility that higher or more frequent doses might provide greater benefit. In addition, patients in their study had early PD and were otherwise untreated, whereas ours were all receiving antiparkinsonian medications and most were receiving L-dopa.

A critical question regarding glutathione as a potential treatment for PD is whether it crosses the blood-brain barrier (BBB). Studies have demonstrated that glutathione is transported across the BBB in the rat and guinea pig.[19,20] More recently, human cerebrovascular endothelial cells were demonstrated to exhibit glutathione uptake and efflux.[21] Both sodium-dependent and sodium-independent uptake mechanisms were identified. Sodium-dependent transport was localized to the luminal membrane consistent with the notion that this transporter effects uphill glutathione transport from low plasma to higher endothelial cellular concentrations. There was also evidence for the presence of an efflux mechanism from the abluminal surface of the endothelial cells. These observations suggest that there is a BBB transporter system for glutathione. The degree to which administered glutathione actually crosses the BBB in humans remains to be determined.

Our pilot study addressed the short-term tolerability, safety, and preliminary efficacy of glutathione. It is important to recognize that our study was not designed to evaluate a potential neuroprotective effect of glutathione. During 4 weeks of administration, glutathione appeared to be well tolerated and we did not identify any safety concerns. This suggests that glutathione might be considered for longer trials to evaluate potential slowing of clinical disease progression.

Acknowledgments: This study was conducted with a grant from Wellness Health and Pharmaceuticals (Birmingham, Alabama) and was supported, in part, by the Florida Coalition to Cure PD.

Author Roles: Robert Hauser participated in study design, acquisition of data, and interpretation of data, wrote the first draft and revised it based on critical review by coauthors. Kelly Lyons participated in study design, performed statistical analyses, participated in interpretation of data, and critically revised the draft. Terry McClain participated in data acquisition and critically revised the draft. Summer Carter participated in data acquisition and critically revised the draft. David Perlmutter participated in study design and critically revised the draft. There were no ghost writers and this manuscript complies with the *Movement Disorders* policy on ghost writing.

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Comments:

Journal Title: **European journal of clinical investigation**

Volume: **21** Issue: **1**
Month: **Feb** Year: **1991** Pages: **103-10**
Article Author: **Aebi, S**
Article Title: **High-dose intravenous glutathione in man. Pharmacokinetics and effects on cyst(e)ine in plasma and urine**
Imprint: Entrez:PubMed

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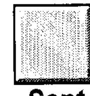

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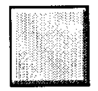
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European Journal of Clinical Investigation (1991) **21**, 103–110 ADONIS 001429729100017U

High-dose intravenous glutathione in man. Pharmacokinetics and effects on cyst(e)ine in plasma and urine

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Received 17 January 1990 and in revised form 19 April 1990

Abstract. Parenteral glutathione has therapeutic potential for targeted delivery of cysteine equivalents. Thus, high doses of reduced glutathione (GSH) protect from the nephrotoxic and urotoxic effects of cisplatinum and oxazaphosphorines. In order to elucidate the underlying mechanisms the kinetics and the effect of glutathione on plasma and urine sulphydryls were studied in 10 healthy volunteers. Following the intravenous infusion of 2 g m^{-2} of glutathione the concentration of total glutathione in plasma increased from $17{\cdot}5 \pm 13{\cdot}4$ μmol l^{-1} (mean $\pm$ SD) to 823 ± 326 μmol l^{-1}. The volume of distribution of exogenous glutathione was 176 ± 107 ml kg^{-1} and the elimination rate constant was $0{\cdot}063 \pm 0{\cdot}027$ min^{-1} corresponding to a half-life of $14{\cdot}1 \pm 9{\cdot}2$ min. Cysteine in plasma increased from $8{\cdot}9 \pm 3{\cdot}5$ μmol l^{-1} to 114 ± 45 μmol l^{-1} after the infusion. In spite of the increase in cysteine, the plasma concentration of total cyst(e)ine (i.e. cysteine, cystine, and mixed disulphides) decreased, suggesting an increased uptake of cysteine from plasma into cells. Urinary excretion of glutathione and of cyst(e)ine was increased 300-fold and 10-fold, respectively, in the 90 min following the infusion. The present data suggest that the concentration of sulphydryls in the urinary tract and, more importantly, the intracellular availability of cysteine increase markedly following parenteral glutathione. The high intracellular concentration of cysteine may protect against cisplatinum and oxazaphosphorine toxicity either directly or indirectly by supporting the synthesis of glutathione.

Keywords. Cysteine, cystine, glutathione, kinetics, mixed disulphides, plasma concentration.

Introduction

Glutathione, an endogenous tripeptide, plays an important role in the regulation of numerous enzymatic reactions and in the protection of cells from oxidant injury and alkylating agents [1,2]. Depletion of glutathione renders cells more susceptible to the toxic effects of certain cytostatic drugs and radiation [2–4] and might be involved in the pathogenesis of the acquired immunodeficiency syndrome [5]. Although circulating glutathione is not taken up by cells [6] and total glutathione is not depleted in the kidneys of animals treated with cisplatinum [7,8], exogenous administration of high-dose reduced glutathione (GSH) has repeatedly been reported to protect from cisplatinum nephrotoxicity and from oxazaphosphorine (cyclophosphamide, ifosphamide) urotoxicity in animals [9–11] without interfering with the antitumoral efficacy of the drugs. These findings have recently been confirmed in humans. Pretreatment with $1{\cdot}5$ g m^{-2} GSH protected from the nephrotoxic effects of 90 mg m^{-2} cisplatinum in patients with advanced ovarian cancer [12–14]. Dose schedules for the combination of glutathione with cisplatinum, the pharmacokinetics of which are well characterized [15], have been empirically chosen based on animal data [9,10,16]. In order to better understand the protective mechanism and to design a rational dosing strategy, a better understanding of the fate and disposition of high dose GSH in man is required. Therefore, the pharmacokinetics of high-dose reduced glutathione and its effect on sulphydryls in plasma and urine were studied in healthy volunteers.

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Subjects and methods

Subjects

Ten healthy volunteers (Table 1) were chosen from coworkers of the department and from students of the local medical school. Informed consent was obtained from each of the participants. Screening laboratory tests including aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, albumin, creatinine, haemoglobin and the mean erythrocyte volume were normal except in subject no. 8 who had an alanine aminotransferase of 108 IU l^{-1} (normal up to 37 IU l^{-1}). The study was approved by the ethics committee of the local medical school.

Table 1. Clinical characteristics of the volunteers

Volunteer	Initials	Sex	Age (years)	Weight (kg)	Height (cm)	Surface area (m^2)	Dose* (mmol)
1	B.L.	M	45	69	178	1·88	12·2
2	U.S.	F	27	48	148	1·30	8·5
3	I.J.	F	29	51	160	1·50	9·8
4	R.B.	F	24	60	180	1·74	11·3
5	F.W.	M	28	83	190	2·11	13·7
6	J.F.	M	26	65	170	1·78	11·6
7	D.R.	M	27	77	192	2·05	13·4
8	C.S.	M	23	105	190	2·35	15·3
9	L.C.	M	32	90	188	2·16	14·1
10	R.S.	M	27	76	178	2·00	13·0

* Intravenous dose of reduced glutathione.

Protocol

All studies were performed in the morning after an overnight fast. No medication had been taken during the preceding 24 h. The body surface area was estimated according to Gehan & George [17]. An indwelling intravenous catheter was placed into an antecubital vein of both arms. Thereafter 2 g m^{-2} (6·5 mmol m^{-2}) sterile and pyrogen-free GSH (Boehringer Biochemia Robin, Milan, Italy) dissolved in 50 ml of normal saline were infused over 15 min by a motor-driven infusion pump. Blood samples were drawn from the contralateral vein before infusion. After the infusion was completed, blood samples were obtained at 5-min intervals for 25 min and at 10-min intervals for another 40 min. A last sample was obtained 120 min after the start of the infusion in four volunteers. Urine was collected before and 90 min after the infusion. Part of the samples of two subjects were lost accidentally during sample preparation.

Analytical methods

Blood was collected into 5-ml heparinized tubes and was immediately mixed with L-serine/Na-borate (final concentration 20 mml l^{-1}) to inhibit the degradation of GSH by gamma-glutamyl transferase [18]. The samples were centrifuged at 3500 × ***g*** for 2 min and the supernatant plasma was removed immediately.

Measurement of sulphydryls in plasma. After addition of penicillamine as an internal standard an aliquot of 0·1 ml of plasma was derivatized with monobromobimane (10 μl of a solution of 18·5 mmol l^{-1} acetonitrile; Thiolite reagent, Calbiochem, La Jolla, USA) within 5 min from collection [19]. After 5 min at room temperature 20 μl of perchloric acid (20%) were added to precipitate the proteins and to stabilize the sulphydryl-monobromobimane adducts. After centrifugation the samples were stored at −20°C until analysis by HPLC.

Measurement of total glutathione in plasma and urine. Total glutathione (i.e. GSH, glutathione disulphide (GSSG), and glutathione in mixed disulphides, including protein mixed disulphides) was measured after reduction of disulphides with tributylphosphine. To 0·5 ml of plasma penicillamine was added as an internal standard. Disulphide bonds were reduced by adding 0·1 ml tributylphosphine (50 μl in 1 ml 1-propanol). The samples were incubated for one hour at room temperature under nitrogen with constant vortexing. Twenty μl of sulphosalicylic acid (50%) were then added and the samples were centrifuged. The clear supernatant was put on a C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA), and the sulphydryls were eluted by washing with 2 ml 0·1 mol l^{-1} phosphate buffer (pH 7·4). To 0·2 ml of the eluate monobromobimane (25 μl of a solution of 18·5 mmol l^{-1} in acetonitrile) was added. After 5 min at room temperature, 25 μl of perchloric acid (20%) were added and the samples were kept at −20°C until analysed by HPLC. Recovery of glutathione disulphide (GSSG) added to plasma was 85±9·4% (mean±standard deviation, $n = 6$).

Chromatography. The sulphydryls were separated on a LiChisorb RP-18 column, 7 μm particle size (E. Merck, Darmstadt, FRG), using a mobile phase consisting of 4·3 mmol l^{-1} octanesulphonic acid containing 1% acetic acid and acetonitrile 125 ml l^{-1}. Sulphydryl-monobromobimane adducts were detected by means of a Perkin-Elmer 204 fluorescence spectrophotometer (exciter wavelength 385 nm, analyser wavelength 490 nm) [19,20]. The ratios of the peaks of interest to the area of the internal standard were used for quantification. Individual standard curves in plasma were established daily by adding known amounts of GSH and cysteine to plasma samples.

Measurement of total cyst(e)ine in plasma and urine. Total cyst(e)ine (cysteine, cystine, and cysteine in mixed disulphides) in plasma and in urine was measured after reduction with dithiothreitol (Cleland's reagent, Calbiochem, La Jolla, USA) by a colorimetric reaction with ninhydrin in acidic solution [21,22].

Calibration curves, determined individually by adding known amounts of cysteine, were linear in a concentration range of 20 to 500 μmol l^{-1}. Recovery of cystine added to plasma was 105±7·5% (mean±standard deviation, $n=6$). Total cyst(e)ine could not be measured in one subject because of a technical mishap.

Calculation of pharmacokinetic parameters

The concentration–time curve of glutathione in a one-compartment model following the intravenous infusion of GSH in the presence of a continuous input of endogenous GSH is described by the following equation:

$$c(t) = c_{basal} + c_0 \times e^{-K \times t}$$

where c_{basal} = basal concentration of glutathione, c_0 = extrapolated concentration of glutathione at $t=0$, and K = elimination rate constant. The latter two parameters were estimated from the individual concentration–time curves by non-linear regression analysis. The area under the curve was estimated by the trapezoidal rule after subtraction of the basal concentration. The clearance was calculated by dividing the dose of GSH by the area under the concentration–time curve. The volume of distribution was obtained by dividing the clearance by the elimination rate constant.

Statistics

The results are given as mean ± standard deviation. In each case the goodness of fit for a normal distribution was evaluated by means of a Komolgorov–Smirnov test.

Results

None of the subjects experienced important adverse side effects during the infusion of GSH.

Table 2. Pharmacokinetic parameters of i.v. high-dose reduced glutathione

Volunteer	C_{basal}* (μmol l^{-1})	C_{max}† (μmol l^{-1})	AUC‡ (μmol l^{-1} min)	K§ (min^{-1})	$t_{\frac{1}{2}}$¶ (min)	Cl** (ml min^{-1} m^{-2})	V_d corr.†† (l kg^{-1})
Reduced Glutathione							
1	12	478	10612	0·050	13·7	613	0·331
2	9	323	5257	0·108	6·4	1312	0·310
3	6	301	9022	0·062	11·2	766	0·343
4	7	647	12989	0·123	5·6	520	0·118
5	4	512	8017	0·201	3·4	813	0·103
6	8	351	7910	0·072	9·7	822	0·314
7	6	211	5242	0·102	6·8	1240	0·324
8	8	534	7100	0·208	3·3	928	0·099
9	5	541	10432	0·152	4·6	725	0·098
10	4	538	7067	0·140	4·9	758	0·173
Mean	6·9	444	8365	0·122	7·0	850	0·221
Standard deviation	2·5	138	2453	0·055	3·5	252	0·111
95% confidence interval lower limit	5·1	345	6609	0·083	4·5	670	0·142
95% confidence interval upper limit	8·7	542	10120	0·161	9·5	1030	0·301
Total Glutathione							
1	20	882	24218	0·037	18·8	269	0·199
2	45	1117	26823	0·067	10·3	243	0·098
3	9	533	29414	0·018	37·9	221	0·356
4	20	1030	26484	0·061	11·3	246	0·117
5	7	1399	33867	0·091	7·6	192	0·054
6	11	560	14442	0·057	12·1	451	0·216
7	6	534	15078	0·053	13·1	432	0·216
8	37	972	16336	0·112	6·2	398	0·080
9	12	862	19926	0·084	8·2	327	0·093
10	8	340	11013	0·046	15·0	591	0·336
Mean	17·5	823	21760	0·063	14·1	337	0·176
Standard deviation	13·4	326	7495	0·027	9·2	128	0·107
95% confidence interval lower limit	7·9	589	16397	0·043	7·5	246	0·100
95% confidence interval upper limit	27·1	1056	27124	0·082	20·6	428	0·253

* C_{basal}, basal concentration of glutathione in plasma.
† C_{max}, maximum concentration of glutathione following infusion.
‡ AUC, area under the concentration–time curve.
§ k, elimination rate constant.
¶ $t_{\frac{1}{2}}$, apparent half-life in plasma.
** Cl, systemic clearance corrected for the body surface area.
†† V_d corr., estimated volume of distribution corrected for the body weight.

Glutathione in plasma

Basal plasma concentrations of GSH and total glutathione were 6·9 ± 2·5 μmol l^{-1} and 17·5 ± 13·4 μmol l^{-1} (Table 2), respectively. Peak plasma concentrations of 444 ± 138 μmol l^{-1} GSH (Fig. 1) and 823 ± 326 μmol l^{-1} total glutathione were achieved following the infusion of GSH (Fig. 2). Semilogarithmic plots of the concentration vs. time after subtraction of the basal concentration showed a monoexponential decline (data not shown). Therefore, the kinetics were considered to follow a first-order one compartment model. The elimination rate constants for GSH and total glutatione were 0·122 ± 0·055 min^{-1} and 0·063 ± 0·027 min^{-1}, corresponding to an elimination half-life of 7·0 ± 3·5 and 14·1 ± 9·2 min, respectively. There was a statistically significant negative linear correlation (Fig. 3) between the dose per kilogram body weight and the elimination rate constant of GSH ($r^2 = 0·71$, $P = 0·002$) and total glutathione ($r^2 = 0·59$, $P = 0·01$). The dose per kilogram was not significantly correlated with the peak plasma concentration, the area under the concentration–time curve, nor the volume of distribution (data not shown). The apparent volume of distribution was 15·0 ± 6·4 l for GSH and 11·9 ± 6·7 l for total glutathione. Corrected for body weight the volume of distribution amounted to 0·221 ± 0·111 l kg^{-1} and 0·176 ± 0·107 l kg^{-1}. Thus, the estimated volume of distribution corresponds approximately to the extracellular space. Volumes of distribution calculated by model independent methods [23,24] yielded similar results (GSH: 0·243 ± 0·091 l kg^{-1}, total glutathione: 0·141 ± 0·069 l kg^{-1}). The systemic clearance was 850 ± 252 ml min^{-1} m^{-2} for GSH and 337 ± 128 ml min^{-1} m^{-2} for total glutathione, respectively.

Cysteine in plasma

The basal concentrations of cysteine and total cyst(e)ine in plasma (Table 3) were 8·9 ± 3·5 μmol l^{-1} and 289 ± 50 μmol l^{-1}, respectively. Cysteine increased over tenfold to a maximum of 114 ± 45 μmol l^{-1}, peaking 5–15 min after the end of the infusion, whereas total cyst(e)ine transiently decreased to 248 ± 59 μmol l^{-1} (Fig. 4). Cysteine returned to basal levels within 90 min. In contrast, total cyst(e)ine increased to a maximal value of 415 ± 31 μmol l^{-1}, the peak being registered 60 to 90 min after the beginning of the infusion.

Urinary excretion of glutathione and metabolites

During the 90 min following the infusion the excretion of glutathione in urine increased from 0·3 ± 0·1 to 100 ± 182 nmol μmol^{-1} creatinine (Fig. 5). Similarly, urinary cyst(e)ine increased from 32·4 ± 11·1 to 434 ± 299 nmol μmol^{-1} creatinine. Although the 300-fold increase in urinary glutathione is impressive only an average of 0·82 ± 1·03% of the administered dose appeared in urine as unchanged GSH or as GSSG within 90 min.

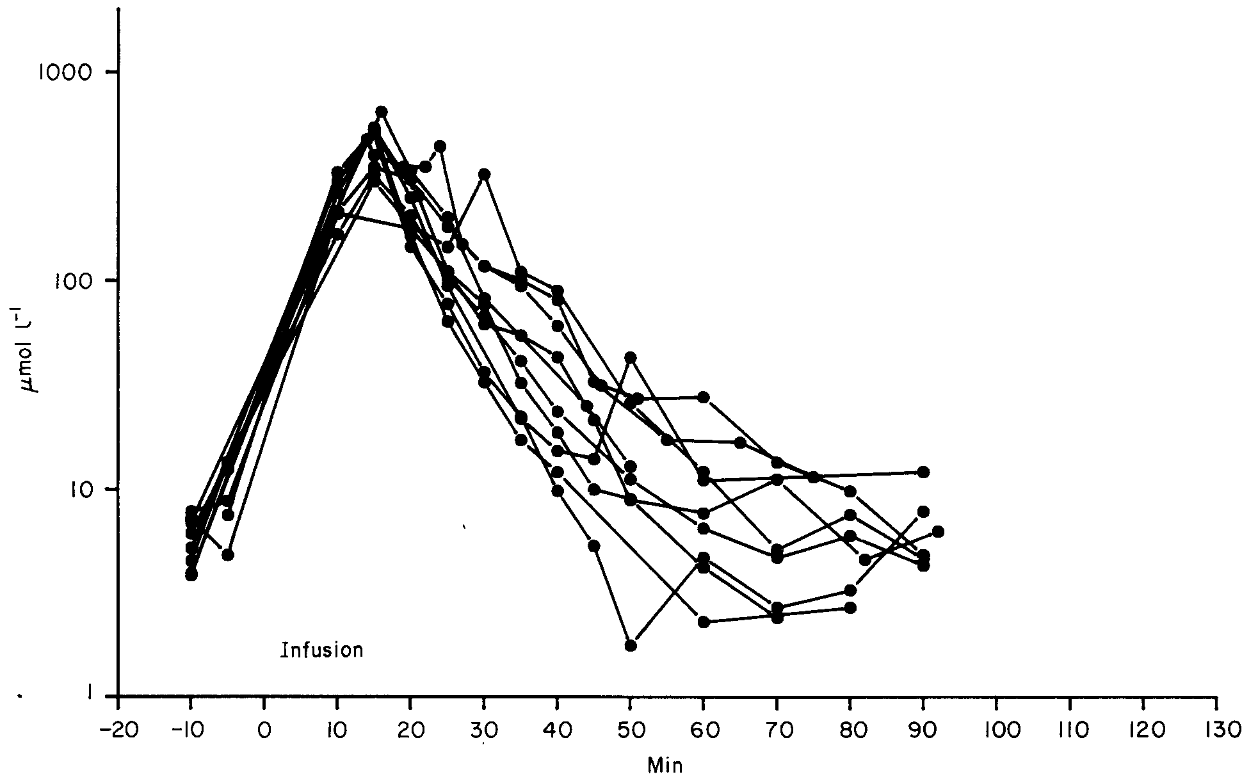

Figure 1. Plasma concentrations of GSH following the intravenous infusion of 2 g m^{-2} of GSH.

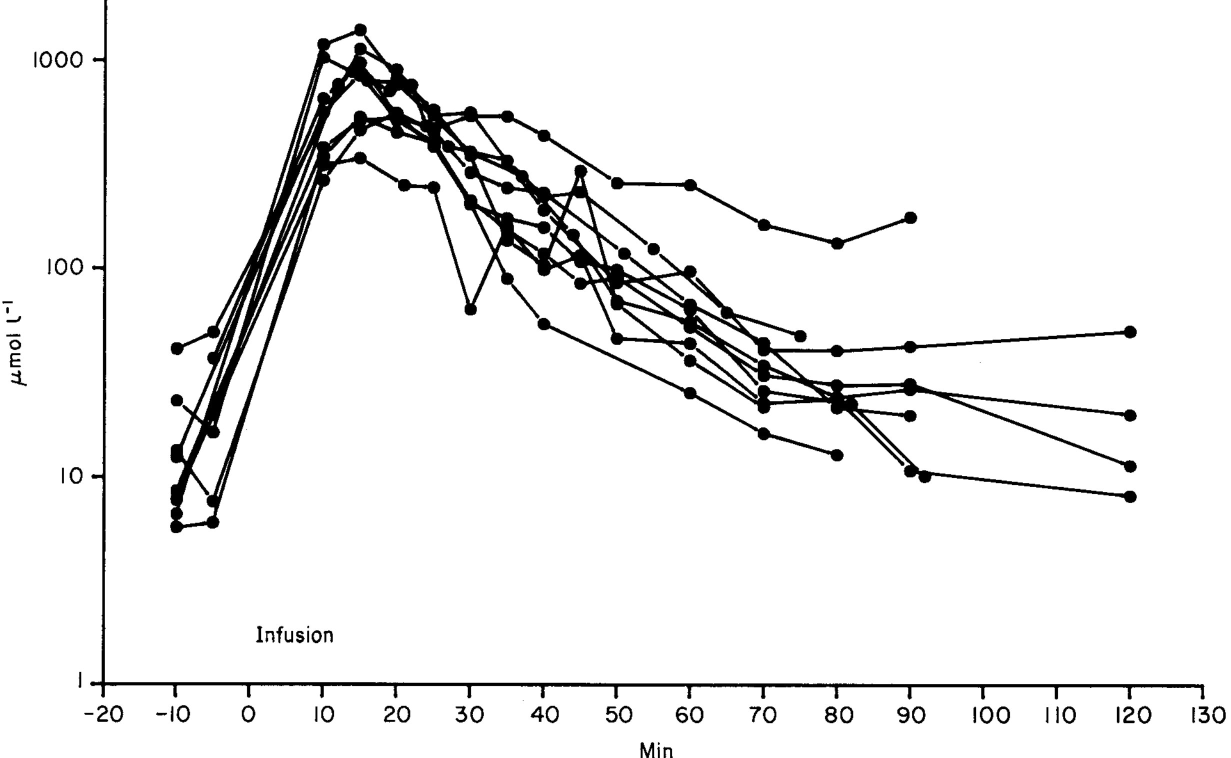

Figure 2. Plasma concentrations of total glutathione following the intravenous infusion of 2 g m^{-2} of GSH.

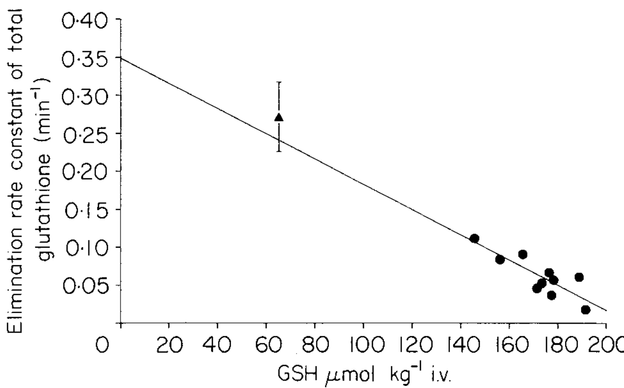

Figure 3. Correlation between the dose of GSH per kilogram body weight and the elimination rate constant (*K*) of total glutathione (filled circles). Triangle: elimination rate constant for 65 μmol GSH kg^{-1} reported in reference 25 (mean ± 95% confidence interval).

Discussion

The present study demonstrates that high-dose intravenous GSH (2 g m^{-2}) disappears from the circulation following single compartment monoexponential kinetics. The rate of disappearance of GSH was substantially faster than the disappearance of total glutathione. This is most likely due to the fact that GSH is not only metabolized by gamma-glutamyl transferase but also forms mixed disulphides. The estimated volume of distribution corresponds approximately to the extracellular space. This can be expected from the fact that glutathione does probably not enter cells [6]. The same model with a similar volume of distribution was found to apply after the administration of a much smaller intravenous bolus dose of GSH (65 μmol kg^{-1}). However, in that study the calculated elimination rate constant for total glutathione was substantially larger, averaging 0·272 min^{-1} compared with 0·063 min^{-1} following the large dose of GSH in the present study [25]. Thus, the disposition of glutathione appears to be dose dependent and subject to saturation kinetics. Even in the small range of doses per kilogram body weight resulting from dosing based on surface area in the present study a significant negative correlation between elimination rate constant and dose per kilogram was observed. Estimation of the elimination rate constant for a dose of 65 μmol kg^{-1} based on this correlation yielded a value of 0·241 min^{-1}, which agrees well with the measured value for this dose [25] (Fig. 3, triangle: mean ± 95% confidence interval). Attempts to model the disposition of glutathione to Michaelis–Menten kinetics did not result in better fits than the linear one-compartment model.

The large increase in cysteine which paralleled the increase in GSH most likely results from the reduction of circulating cystine or cysteine-mixed disulphides by high concentrations of GSH. Since cysteine, in contrast to cystine, is readily taken up by cells [26,27] this

Table 3. Cysteine in plasma

Volunteer	Cysteine C_{basal}* (μmol l^{-1})	Cysteine C_{max}† (μmol l^{-1})	Total Cyst(e)ine C_{basal}* (μmol l^{-1})	Total Cyst(e)ine C_{20}‡ (μmol l^{-1})	Total Cyst(e)ine C_{max}† (μmol l^{-1})
1	7	102			
2	9	64	266	216	432
3	10	134	372		424
4	9	213	277	222	426
5	7	132	201	381	452
6	7	107	314	205	392
7	8	67	306	218	457
8	9	102	250	226	372
9	18	142	332	282	403
10	5	74	281	232	373
Mean	8·9	114	289	248	415
Standard deviation	3·5	45	50	59	31
95% confidence interval lower limit	6·4	82	251	199	390
95% confidence interval upper limit	11·4	146	327	297	439

* C_{basal}, basal concentration of cysteine in plasma.
† C_{max}, maximum concentration of cysteine.
‡ C_{20}, concentration of cyst(e)ine in plasma 20 min after the beginning of the infusion.

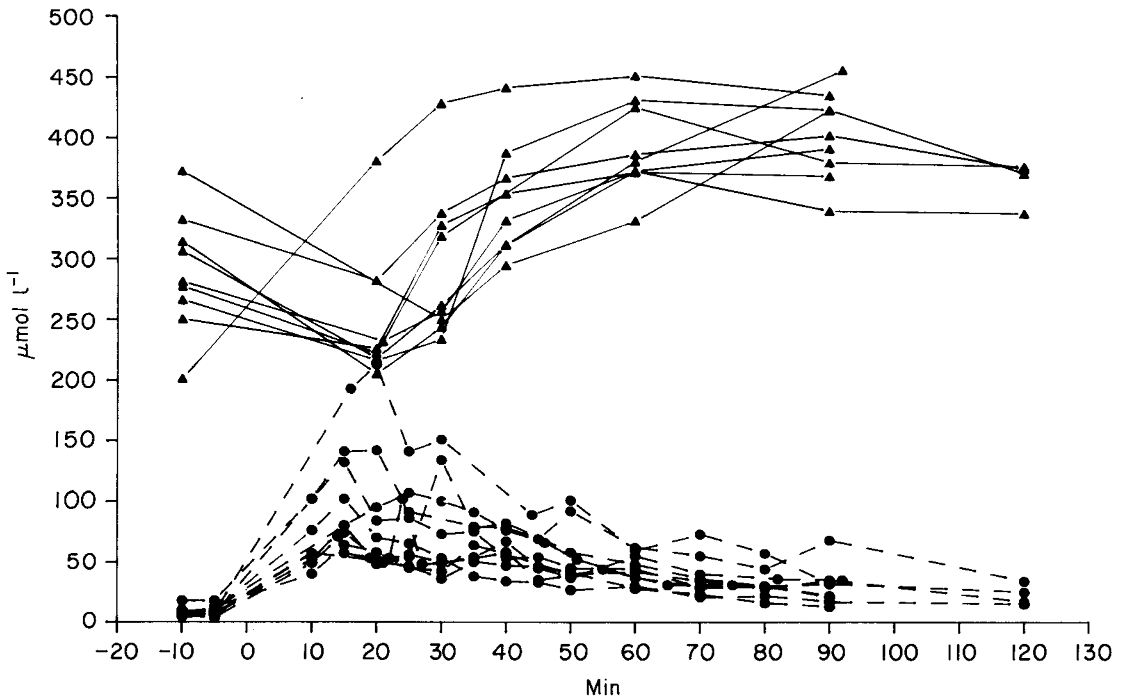

Figure 4. Plasma concentration of cysteine (filled circles) and total cyst(e)ine (triangles) following the intravenous infusion of 2 g m^{-2} of reduced glutathione.

GSH-induced increase of cysteine in plasma would be expected to enhance the uptake of cysteine into cells. Such a shift of cysteine from the extra-cellular to the intracellular compartment is indeed supported by the observed transient decrease in total plasma cyst(e)ine (Fig. 4). Alternatively, the increase in cysteine could originate from the catabolism of GSH by gamma-glutamyl transferase [28]. Although the generation of cysteine by this mechanism could explain the rise in circulating cysteine it could, however, not explain the transient decrease in total cyst(e)ine. The time-lag between the peak concentrations of GSH and total glutathione on one side and the increase in total cyst(e)ine on the other suggests that the cysteine

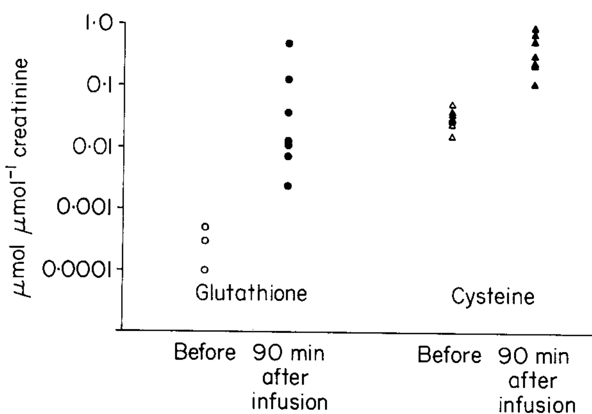

Figure 5. Urinary excretion of total glutathione and cyst(e)ine (corrected for the excreted creatinine) before and following the intravenous infusion of 2 g m^{-2} of GSH. The concentration of glutathione in urine before infusion was below the detection limit in four volunteers.

responsible for this delayed increase has passed through the intracellular compartment. Cysteine is probably taken up, in part used for glutathione synthesis, and then again released by the cell either in form of cyst(e)ine, or glutathione which will be catabolized to cyst(e)ine by extracellular gamma-glutamyl transferase.

The excretion of glutathione in urine increased dramatically after infusion of GSH. However, only a small fraction of the administered GSH appeared unchanged in urine, reflecting the capability of the kidneys to handle high loads of this tripeptide. Glutathione is hydrolysed by the action of gamma-glutamyl transferase and cysteinylglycine dipeptidase in tubular cells on both, the luminal and the basolateral domain of the cell membrane [6,28]. The amount of cyst(e)ine formed in the renal tubuli apparently exceeds the maximum reabsorptive transport capacity and results in an important renal excretion of cyst(e)ine. Cysteine is rapidly reabsorbed from the tubular lumen [6] and could stimulate intracellular *de novo* synthesis of glutathione.

In summary, high-dose intravenous reduced glutathione distributes in the extracellular compartment and is cleared from the circulation with a half-life of approximately 15 min. Plasma cysteine concentration and urinary excretion of total cyst(e)ine are markedly increased. Whether the protective effect of exogenous glutathione against cisplatinum and oxazaphosphorine toxicity is related to glutathione itself or to the increased availability of cysteine in plasma, in urine, and most likely also in the intracellular compartment, requires further investigation.

Acknowledgments

Supported by grant 3.812-0.87 from the Swiss National Foundation for Scientific Research and a grant from Boehringer Biochemia Robin.

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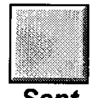

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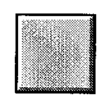
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Clinical Study

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Oncology 1999;57:115–120

Dose Intensification of Platinum Compounds with Glutathione Protection as Induction Chemotherapy for Advanced Ovarian Carcinoma

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Key Words
Ovarian carcinoma · Cisplatin · Glutathione

Abstract
Based on previous clinical experience indicating the tolerability and efficacy of high-dose cisplatin with glutathione protection in the treatment of advanced ovarian cancer, this study was undertaken to explore the efficacy and feasibility of an alternative high-dose, platinum-based approach including a combination of high-dose cisplatin plus carboplatin as induction chemotherapy of advanced ovarian carcinoma and intervention surgery. Fifty consecutive eligible patients with untreated stage III or IV epithelial ovarian cancer received 40 mg/m^2 cisplatin daily on days 1–4 and 160 mg/m^2 carboplatin on day 5. The cycle was repeated after 28 days. Patients received glutathione (2,500 mg) before each cisplatin or carboplatin administration and standard intravenous hydration. After 2 courses of induction chemotherapy, the patients underwent surgical reevaluation with debulking, when possible, followed by a further 3 cycles of 120 mg/m^2 cisplatin (i.e. 40 mg/m^2 daily for 3 consecutive days plus 600 mg/m^2 cyclophosphamide on day 3) except in instances of lack of response. All eligible patients were assessed for response and toxicity. The toxicity was moderate with lack of significant nephrotoxicity. Neurotoxicity and ototoxicity were acceptable and in no patient was treatment discontinued for those toxic effects. Myelotoxicity was somewhat more severe than that observed with our previous study with high-dose cisplatin and probably related to the addition of carboplatin. Of the 40 responsive patients, 23 (46%) had a pathological complete response and 4 (8%) had a clinical complete response (without second-look laparotomy). The efficacy of the present protocol was also documented by overall survival (median survival >48 months), which appeared to be better than expected with the current therapy in this group with advanced/bulky disease. The impressive efficacy suggests a possible contribution of reduced glutathione itself in improving the outcome, as supported by preclinical studies. The results of this study should be placed in context with current platinum-based therapy including paclitaxel.

Introduction

Cytoreductive surgery followed by combination chemotherapy has become the standard treatment for patients with advanced ovarian cancer. Although the value

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of cytoreductive surgery is still a matter of debate [1, 2], the size of residual tumor is widely recognized to be an important prognostic factor [3]. Platinum compounds have proved to be the most active agents. Thus, platinum-based chemotherapy, usually cisplatin with cyclophosphamide, is generally accepted as a standard regimen [4]. However, a number of issues related to optimal dose and total dose of platinum compounds, drugs to be used in combination (alkylating agents or taxanes), number of cycles and route of administration remain somewhat unresolved. In particular, there is controversy about whether the efficacy of platinum compounds can be improved by the use of high-dose regimens. Clinical experience with high-dose cisplatin (>100 mg/m^2) is still limited by substantial toxicity, including nephrotoxicity, neurotoxicity and ototoxicity [5, 6]. We have recently reported that the use of reduced glutathione (GSH) allows dose escalation of cisplatin with acceptable toxicity [7]. Using this new approach, a high-dose regimen (160 mg/m^2/course) appeared very promising. Indeed, the therapeutic outcome of the study compared favorably in terms of response rate and survival with the results of regimens considered standard therapy for advanced ovarian carcinoma [4, 5, 8]. As an alternative approach to increasing dose intensity of platinum, the combination of cisplatin and carboplatin has been explored with encouraging results [9]. In our previous study, a striking response has been observed following 2–3 courses of high-dose cisplatin [7], thus suggesting that the impact of dose intensification is more critical in responsive patients during the first cycles. The present study was undertaken to examine the feasibility and outcome of an aggressive approach, as a first-line treatment of advanced ovarian carcinoma, including platinum dose intensification in induction therapy and intervention surgery. Based on the information derived from previous studies, a further dose escalation of platinum compounds was attempted with a combination of high-dose cisplatin with carboplatin as induction therapy.

Patients and Methods

Eligibility

Untreated patients with histologically confirmed ovarian carcinoma were eligible for this study provided they met the following criteria: (a) no prior therapy other than surgery; (b) advanced disease (FIGO stage III or IV); (c) performance status >70% on the Karnofsky scale and life expectancy >3 months; (d) normal bone marrow, liver and kidney functions as indicated by leukocyte and platelet counts of at least 4,000 and 150,000/mm^3, respectively; normal serum bilirubin, SGOT, SGPT and serum creatinine levels and creatinine clearance; (e) informed consent. This study was approved by the Institutional Human Investigations Committee. The patient characteristics are presented in table 1.

Treatment Plan

All the patients underwent initial cytoreductive surgery and staging. Two courses of induction chemotherapy consisting of cisplatin (40 mg/m^2 given daily for 4 days as a 30-min infusion in 250 ml normal saline) and carboplatin (160 mg/m^2 given on day 5 as a 15-min infusion in 250 ml normal saline) were administered with an interval of 4 weeks. GSH 2,500 mg was given intravenously in 100 ml of normal saline over 15 min before each platinum administration. Hydration schedule of 2,000 ml of fluid without diuretic was used, as previously reported [7]. Unless there was progression, second surgery was performed after the 2 courses of chemotherapy to assess the response in order to attempt further cytoreduction. Responsive patients received three additional courses of chemotherapy with cisplatin (40 mg/m^2 given daily for 3 consecutive days, as previously described) and cyclophosphamide (600 mg/m^2 given as intravenous bolus on day 3 only). An antiemetic regimen consisting of metoclopramide and ondansetron was employed. Whenever possible, the response was evaluated by explorative laparotomy, as specified below.

Response

To assess the pathological response, second-look laparotomy included multiple biopsies from areas of macroscopic disease, from sites of previous disease, and from any suspicious or high-risk area (diaphragm, paracolic gutters and pelvic peritoneum). A complete response was considered to be pathologically documented if all the biopsies and washings were negative. A clinically complete response was defined as the complete disappearance of disease detectable by physical and/or radiological examinations. A partial response was defined as a reduction of >50% in all measurable lesions at second surgery. Objective tumor response to treatment was defined clinically in patients with clinical progression.

Toxicity

Toxicity was evaluated according to the National Cancer Institute common toxicity criteria. Myelotoxicity, nephrotoxicity and hepatotoxicity were assessed on the basis of routine hematological and biochemical parameters. Ototoxicity was assessed clinically and by audiography before treatment and at the end of the chemotherapeutic program. Peripheral neuropathy was assessed by clinical examination and by neurophysiological parameters, including assessment of sensory action potentials, motor conduction velocities. Neurological tests were performed before therapy, after 2 courses and after completion of the therapy. Only clinical tests were employed during follow-up. In 12 patients visual-evoked potentials were recorded serially, before starting chemotherapy, after 2 courses and after 5 courses. Checkerboard stimulation at 30 and 15 min spatial frequency was employed to record visual-evoked potentials. The latency and amplitude of the P100 component were considered in evaluating potential therapy effects. Brain stem responses were recorded with monaural acoustic stimulation per each ear using 70-dB above threshold stimulation and the same schedule of visual-evoked potential recordings.

Table 1. Patients characteristics

Eligible patients	50
Patients evaluated	50
Median age (range)	55 (26–74)
Histology	
Serous	33
Endometrioid	5
Mucinous	5
Undifferentiated	2
Mixed	2
Clear cells	1
Unclassified	2
Tube	1
Grade	
1	3
2	13
3	32
Nonspecified	2
Stage	
III optimal	12
III suboptimal	26
IV	12

Table 2. Response to therapy

Response	At second surgery (after 2 courses)	At second-look laparotomy (after 5 courses)
Complete		
Pathological	8	23
Clinical (without second-look)		4
Partial		
Macroscopic residual disease	28 (8)[a]	8 (1)[a]
Microscopic residual disease	4	5
Minimal	3	3
No response	7	
Total	50	43

[a] In parenthesis, NED following surgery.

Results

Patient Characteristics

From April 1992 to May 1995, 51 patients with advanced-stage epithelial ovarian cancer were entered into the prospective clinical study. One patient was excluded for hematological disorders. None of the patients had received prior chemotherapy or radiation therapy. All 50 eligible patients underwent a surgical tumor-reductive procedure. Only 12 of 38 patients with stage III disease could be optimally debulked at initial laparotomy. All treated patients were assessable for toxicity and response to therapy.

Response

The treatment program included additional debulking surgery after 2 courses of induction chemotherapy with high-dose, platinum-based therapy. Such an aggressive approach was selected in an attempt to remove residual disease, since most patients had bulky and extensive disease after initial surgery. Since the procedure was expected to provide detailed information on the response status of the disease, the same surgical approach was employed also for patients with optimal stage III disease. Treatment was discontinued in cases who did not have any significant response (7 patients). After second surgery, responsive patients received 3 additional courses of consolidation therapy. Second-look laparotomy was performed in all responsive patients with persistent disease at the second debulking surgery and no clinical evidence of disease at completion of the chemotherapy. The results regarding response to chemotherapy are summarized in table 2. Of 50 patients, 23 (46%) had achieved a pathological complete response at the conclusion of therapy. Eight patients were found negative at the intervention surgery after the 2 courses of induction surgery (including 4 patients with extensive residual disease after initial surgery). Of the 23 patients who achieved a complete response, 15 had bulky residual disease and 8 had optimal cytoreduction after initial surgery. Four patients were clinically disease free at the completion of chemotherapy without pathological documentation. Thirteen patients had a partial response. Therefore, the overall response rate was 80%. Among patients who achieved a partial response after two courses, macroscopically residual tumor was completely removed in 8 cases, in whom surgical complete response was possible as a consequence of a dramatic tumor shrinkage. In another 4 patients, only microscopic residual tumor could be detected. At the time of analysis (with a median follow-up of 54 months), 13 of the complete responders (26% of the total treated patients) were alive and disease free (table 3). Twenty-two patients of the overall series died of progressive disease. By actuarial analysis of survival for all patients who entered the study, the estimated 4-year survival rate was still >50%.

Toxicity

In no patient was treatment discontinued for toxicity. Indeed, 40 patients received the planned 5 courses of the

Table 3. Current status[a]

Response[b]	Total	NED	Recurrence	Dead
pCR	23	11	12	5
cCR	4	2	2	1
Other	23			16
Total	50	13		22

[a] Median follow-up, 54 months (range 28–74).
[b] The responsiveness status is referred to response at treatment completion (pCR, pathological complete response; cCR, clinical complete response).

Table 4. Hematologic toxicity

Leukopenia, /mm^2	
3,000–3,999	4
2,000–2,999	16
1,000–1,999	22
<1,000	1
Thrombocytopenia, cells/mm^2	
75,000–150,000	20
50,000–75,000	3
25,000–50,000	3
<25,000	3
Anemia (hemoglobin, g/100 ml)	
10–11	6
8–9.9	23
6.5–7.9	9
<6.5	0

Maximum toxicities experienced by indicated patients.

Table 5. Nonhematologic toxicity

Toxicity	Worst grade[a]			
	1	2	3	4
Nausea or vomiting	6	13	15	0
↑ Creatinine[b]	2	0	0	0
↓ Mg^{2+}	1	4	2	0
Peripheral neuropathy	9	5	0	0
Ototoxicity	12	1	0	0
↑ Transaminases	5	2	1	0

[a] According to NCI common toxicity criteria.
[b] Pretreatment values never exceeded 1.2 mg/dl.

protocol. Seven nonresponsive patients were treated with only 2 courses of induction therapy and then proposed for a second-line therapy. Protocol violations occurred in 2 cases after 1 and 4 cycles, respectively (both patients had appropriate treatment elsewhere). Chemotherapy was stopped in only 1 case after 3 cycles for postsurgical complications. Toxicity data are presented in tables 4 and 5. Hematologic toxicity was moderate and manageable and was generally more pronounced after 2–3 cycles of therapy. No symptoms of neutropenia or bleeding episodes were reported. In general, myelosuppression was of short duration and never required reduction of drug dosage. Granulocyte colony-stimulating factor support was required for persistent neutropenia in 9 patients to avoid delay between cycles. Nonhematologic toxicity was minimal (table 5). The commonest toxicity was nausea and/or vomiting, which was in part controllable with the use of appropriate antiemetic therapy. Nephrotoxicity was uncommon: only 2 patients experienced a marginal increase in serum creatinine. Hypomagnesemia (<1.4 mEq/l) occurred in 7 patients. Since peripheral neuropathy and ototoxicity are reported to be the most relevant dose-limiting toxic effects of platinum compounds [5–7], neuropathy was initially assessed by clinical and neurophysiological parameters. Since evidence of tolerability clearly emerged in the first 20 patients, the study was amended to monitor neurotoxicity only with clinical parameters. In particular, sensory symptoms were found in 13 patients (26%), and in only 1 patient was grade 3 toxicity, involving motor impairment, detected. No delayed neurotoxicity was found and a partial reversion of the sensory symptoms was evident in all patients. Ototoxicity was detected in 13 patients (26%) and was generally asymptomatic, with high-frequency hearing loss documented by conventional audiometry. Among the first 20 evaluated patients, a significant change in auditory-evoked potentials could be recorded only in 1 patient and consisted of the disappearance of component I of the brain stem response after 5 courses of therapy that was still evident at 6 months of follow-up; the click threshold was also increased. Visual changes were seen in 7 patients. In 2 of them, a clear-cut reduction of P100 amplitude (>50%) was seen together with an increase in latency. The reduction was present in both eye recordings, but it was often asymmetrical. It was most evident after 5 courses of treatment and the patient did not recover after 6 months. No patient reported visual symptoms. As already noted in our previous study with high-dose cisplatin [7], a significant but transient elevation of liver enzymes was detected in 16% of the patients.

Discussion

The platinum compounds, cisplatin and carboplatin, are currently the most effective drugs in the treatment of ovarian carcinoma [10]. The role of high-dose chemotherapy remains to be defined [11]. Some studies suggest that platinum dose intensity may be an important determinant of drug efficacy [4, 12]. Thus, a number of dose intensification approaches have been proposed. Based on its relatively different cytotoxic potential, cisplatin could be preferred for dose escalation. Dose intensification can be achieved by increasing doses given per cycle or decreasing the interval between standard-dose administrations. Both approaches have been tested for cisplatin in ovarian carcinoma. Unfortunately, high-dose cisplatin regimens, using conventional administration modalities, are accompanied by significant (and in some cases unacceptable) renal and neurologic toxicity [5, 6]. Based on these limitations, only carboplatin is now considered for adequate intensification in high-dose trials with autologous bone marrow transplantation and/or peripheral blood stem cell support, since the analog offers the potential advantage of a different toxicity profile (i.e., mainly hematologic toxicity). The inherent expenses and increased toxicity of current approaches of intensive therapy still represent important limitations.

We have reported a novel modality of cisplatin administration with the use of GSH as a chemoprotector to allow safe delivery of high doses of the drug (160–200 mg/m^2). The improvement of the therapeutic index of cisplatin using this approach is documented by the finding that increased dose intensity was not associated with unacceptable toxicity. The rationale for using GSH was to ameliorate some of the known toxicities of platinum chemotherapy without reducing the efficacy by any rescue of the tumor. The present study with the cisplatin and carboplatin combination confirmed the tolerability of the new approach of dose intensification. Indeed, the lack of renal toxicity is consistent with the well-known nephroprotective potential of GSH [13, 14]. Moreover, the present regimen was associated with apparently reduced neurotoxic manifestations compared to the previous protocol with 5 courses of 160 mg/m^2/course of cisplatin [7]. It is likely that the better tolerability is related to a lower cumulative dose of cisplatin (i.e., 680 mg/m^2). In contrast, a somewhat increased hematologic toxicity of the regimen could be consistent with the addition of carboplatin. However, such toxicity remained manageable.

The clinical and pathological response rates observed in our study support a marked antitumor efficacy of the high-dose regimen and confirm the clinical interest of dose intensification with GSH protection in induction therapy of advanced ovarian carcinoma. The efficacy of the cisplatin and carboplatin combination is also consistent with the clinical results of other studies with such a combination [9]. However, our study design favors cisplatin over carboplatin, an approach that appears feasible due to the protective effect of GSH against organ-specific toxicity [7]. It is possible that a balance between doses of cisplatin and carboplatin may be critical to optimize activity and minimize toxicity. The efficacy and tolerability of the present regimen are comparable to those of the previous protocol [7]. Dose intensification in induction therapy seems to have the potential advantage of permitting early identification of responsive patients. Both pathological response rate and survival compare favorably with outcome in most series treated with standard regimens or with very high-dose chemotherapy [8, 15]. Such improvement is also emphasized by the observation that most patients (76%) had extensive disease after initial surgery. It is conceivable that the therapeutic outcome may be attributed, at least in part, to the aggressive surgery attempted in the study design.

The pathological complete response rate in this subgroup (15/38, i.e. 39%) was comparable to that of a previous study in which only patients with stage III suboptimal or stage IV disease were considered [7]. The difficulty to overcome drug resistance in around 50% of the patients (i.e., patients who did not achieve complete response) in both protocols may be related to intrinsic resistance. Thus, it is possible that only a subgroup may derive a therapeutic benefit from high-dose cisplatin chemotherapy as suggested by the limited efficacy of a similar protocol in p53 mutant tumors [16]. Emerging evidence of a superior activity of taxol over cyclophosphamide in combination with cisplatin suggests a more rational combination for future approaches of high-dose therapy [17, 18]. Our approach apparently possesses sufficiently improved efficacy to warrant comparative randomized trials to determine the role of high-dose chemotherapy.

In conclusion, the present study confirms the marked efficacy of high-dose, platinum-based regimens with GSH. The efficacy and tolerability of this high-dose regimen further support the clinical interest of GSH as a safe and selective protector against organ-specific toxicities of cisplatin [19]. GSH protection allows administration of the full planned dose of platinum compounds in contrast to conventional modalities of administration [20]. The selectivity of GSH has been related to its peculiar pharmacokinetic behavior (i.e., rapid removal from plasma

and lack of significant uptake by most tumors) and to a slow rate of interaction of GSH-cisplatin in the extracellular space (i.e. before the formation of reactive species) [21]. Surprisingly, recent preclinical studies indicate that GSH itself may have a potential antiproliferative activity against ovarian carcinoma [22].

Potential advantages of the alternative approach including carboplatin are: (a) a reduced neurotoxicity (presumably related to lower cumulative dose of cisplatin; (b) the possibility to precociously define the responsivity status of the disease, thus allowing a more rational approach of second-line treatment based on clinical and molecular parameters of drug response (e.g., taxol for resistant p53-mutant tumors); (c) the possible contribution of an aggressive surgical approach as suggested by recent studies [2]. The significant pathological complete response rate and the impressive median survival achieved by the treatment approach including dose intensification of platinum compounds with GSH protection as induction chemotherapy and intervention surgery warrant future evaluation to identify the relative contribution of dose intensification, aggressive surgery and GSH itself. The promising results of this study may have implications for current therapy including taxanes.

Acknowledgments

The study was supported in part by the Associazione Italiana Ricerca sul Cancro and by the Ministero della Sanitá. We are grateful to L. Zanesi for editorial assistance.

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[CANCER RESEARCH 54, 4313–4320, August 15, 1994]

Perspectives in Cancer Research

Glutathione-associated Enzymes in Anticancer Drug Resistance[1]

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Abstract

The importance of thiol-mediated detoxification of anticancer drugs that produce toxic electrophiles has been of considerable interest to many investigators. Glutathione and glutathione *S*-transferases (GST) are the focus of much attention in characterizing drug resistant cells. However, ambiguous and sometimes conflicting data have complicated the field. This article attempts to clarify some of the confusion. The following observations are well established: (*a*) tumors express high levels of GST, especially GSTπ, although the isozyme components vary quite markedly between tissues and the isozymes are inducible; (*b*) nitrogen mustards are good substrates for the GSTα family of isozymes which are frequently overexpressed in cells with acquired resistance to these drugs; (*c*) most drugs of the multidrug-resistant phenotype have not been shown to be GST substrates and although GSTπ is frequently overexpressed in multidrug-resistant cells, most indications are that this is an accompaniment to, rather than a cause of, the resistant phenotype; (*d*) transfection of GST complementary DNAs has produced some lines with increased resistance to alkylating agents. Most studies of the relationships between GST and resistance have overlooked the potential importance of other enzymes involved in the maintenance of cellular glutathione homeostasis, and this has complicated data interpretation. Translational research aimed at applying our knowledge of glutathione pathways has produced preclinical and clinical testing of some glutathione and GST inhibitors, with some encouraging preliminary results. In brief, GSTs are important determinants of drug response for some, not all, anticancer drugs. Caution should be encouraged in assessing cause/effect relationships between GST overexpression and resistance mechanisms.

Introduction

Enzymes involved in the metabolism of drugs and xenobiotics are subdivided into two major categories. Phase I, exemplified by the P-450 isozyme family, renders "small" changes onto drugs, sometimes creating reactive sites which can covalently bind with other intracellular molecules. Phase II reactions, exemplified by the GST[2] isozyme family, involve the addition of relatively "bulky" groups onto the drug, most often creating a more water-soluble conjugate, which may be less toxic and more readily excretable. Thus, phase II reactions are generally cytoprotective. GSH, first described in 1888 as philothion, is the most ubiquitous and abundant non-protein thiol in mammalian cells and serves as a necessary nucleophile in a number of detoxification reactions. Normal levels of GSH in humans are 10–30 μM (plasma), 1–3 μM (urine), 3 mM (kidney proximal tubule), and 1–10 mM (tumors of various organ sites) (1). Maintenance of a homeostatic GSH content is achieved by both *de novo* synthesis and salvage synthesis and a number of interrelated pathways are involved (Fig. 1).

The suggestion that GSH is a universal protectant against electrophilic challenge is supported by evolutionary considerations. Most indications point to the emergence of life on Earth in the pre-Cambrian period some 4 billion years ago. Given that the standard rules of natural selection have been in place for this period, time and success have honed many biological traits. Arguably, one of the most critical adaptations has been the capacity to survive a hostile environment. Environmental stresses can take many forms and even humans, despite, or perhaps because of, our propensity to manipulate the forces of natural selection, are subject to a multitude of toxic challenges. Paradoxically, molecular oxygen, while providing an efficient energy production from ingested food, results in free radical and peroxide by-products which have high intrinsic toxicity. Indeed, the emergence of oxygen-liberating phytoplankton and blue-green algae began an era in Earth's development that must have severely stressed existing and emerging life forms. In more contemporary times, especially post-industrial revolution, environmental stresses have multiplied disproportionately. The air of the planet is suffused with both natural and man-made chemicals. Plants, even those propagated by humans, produce toxic components as a strategy to reduce grazing; many of the by-products of food production are mutagenic or carcinogenic. Even natural radiation and sunlight have the capacity to produce toxic insults.

It is likely that such hostile and competitive surroundings provided the need for the buildup and refinement of intracellular protective arsenals. Molecular evolution has contributed to the conveyor belt of selected chemical detoxification systems. At the present time, mammals have a cellular detoxification system of protean proportions, with considerable redundancy of function. Through divergent and convergent evolution, organisms are constantly "refining" the form and function of detoxification gene products. The MDR P-glycoprotein is simply part of a large family of so-called ATP-binding cassette "membrane pumps." Moreover, specific GST isozymes serve detoxification, structural, and transport functions in many phyla. For example, certain bacteria overexpress GST to develop resistance to the antibiotic fosfomycin (2); many insects and their larvae use GST to express resistance to insecticides (3); squid and other cephalopods utilize GST as structural lens crystallins (4); mammals use GST for detoxification purposes and to bind and transport molecules like heme and bilirubin (hence the early GST nomenclature of ligandin). Indeed, there is ample evidence that species from every documented phylum utilize GST-mediated detoxification mechanisms.

One of the distinguishing properties of both the P-glycoprotein and GST-mediated detoxification systems is their capacity to recognize diverse chemical structures. From a teleological standpoint this would seem to make sense. For example, an organism may not be able to predict when, which, or how much of a toxin it will encounter. Thus, the question arises, "What would constitute the most energy-efficient response to an acute stress stimulus?" If exposure to a specific toxin recurs in a chronic fashion, there may be value in the development of an enzyme detoxification system specific for that molecule. However, given the vagaries of exposure, natural selection would more likely favor a broad-based recognition system, where comparatively low avidity, recognition, or binding is compensated by the potential to detoxify a wide range of chemical structures. For an acute toxin exposure to remain sublethal, a cell or organism may have but one chance to prevent terminal damage. Therefore, the evolution of systems such as the GST isozyme families, in which relatively low

Received 4/6/94; accepted 6/14/94.

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[1] This work was supported by National Cancer Institute Grant R35 CA 53893.

[2] The abbreviations used are: GST, glutathione *S*-transferase; BSO, buthionine sulfoximine; γ-GCS, γ-glutamylcysteine synthetase; γ-GGT, γ-glutamyl transpeptidase; GSH, glutathione; MDR, multidrug resistance; cDNA, complementary DNA; CLL, chronic lymphocytic leukemia.

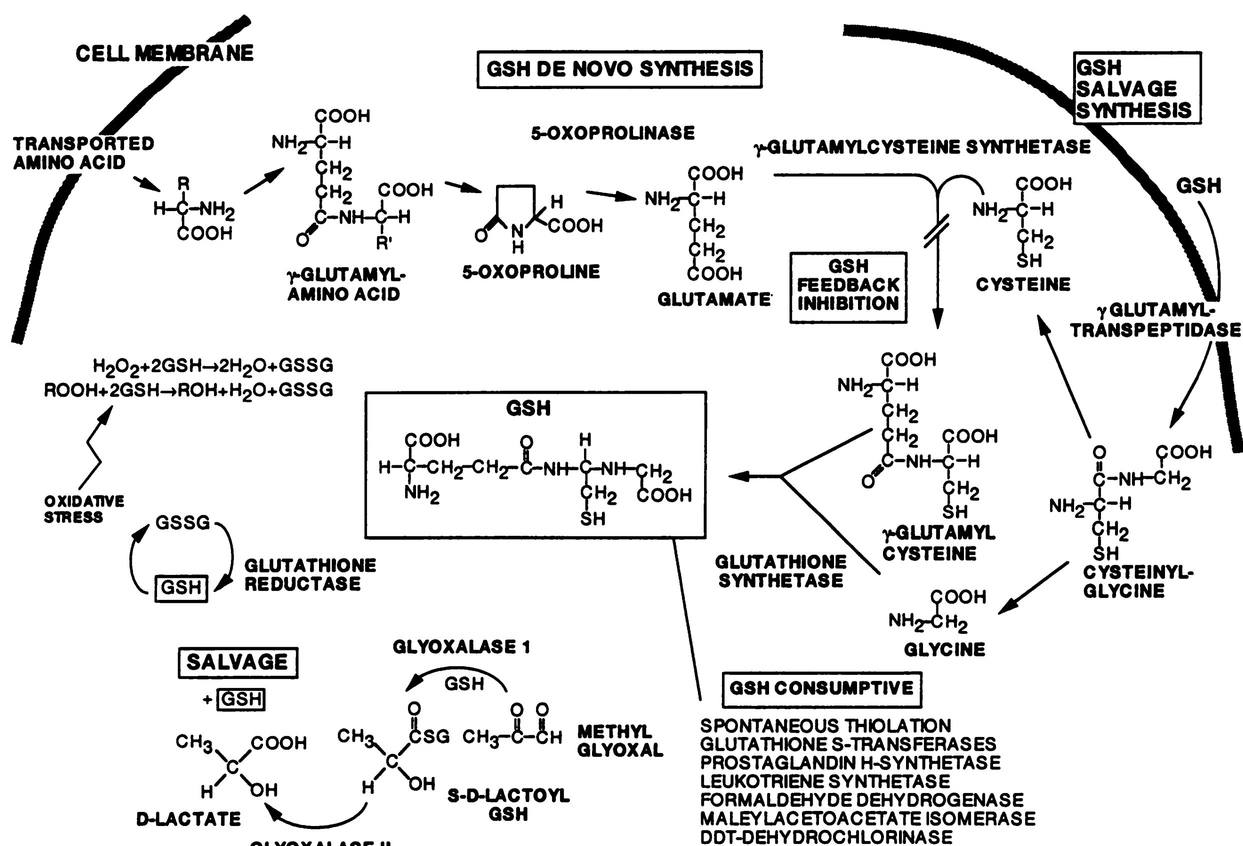

Fig. 1. Summary schema of interrelated GSH-producing and -utilizing pathways. *GSSG*, reduced glutathione; *DDT*, bis(*P*-chlorophenyl)-trichloroethane.

binding affinities are offset by broad substrate specificities would seem to make biological sense.

With this background, there appears to be a sound rationale for considering the role that GSH-associated enzymes play in determining sensitivity to anticancer drugs. While most investigators accept that GSH has a role in drug resistance, the involvement of GSH-associated enzymes (especially the GSTs) has been more contentious. Recent reports have helped to clarify the situation. At least to some extent, the confusion has resulted from the merging of a number of overlapping research endeavors. This is especially true with studies of electrophile-producing alkylating agents and those drugs which constitute the MDR phenotype. Over the last 10 years, a large proportion of the resistance literature has focused on the MDR phenotype. By using the term multidrug, the original definition was intended to include (almost exclusively) natural product drugs with hydrophobic and cationic properties. Only in rare instances have electrophile-producing anticancer drugs been implicated in the MDR phenotype. Thus, while some quinone-producing agents, especially the anthracyclines, are part of this rubric, the classical alkylating agents and cisplatin are exempt. Generally, resistance to these groups of drugs involves separate mechanisms. Because changes which accompany the acquisition of resistance are frequently multiple, they do not always distinguish cause and effect. There are numerous literature examples of optimistic correlative associations which link biochemical changes with resistance. In particular, increased expression of GSTπ in MDR cell lines has frequently been cited as a causal resistance mechanism. In fact, there is little compelling evidence that GSTπ catalyzes the detoxification of MDR drugs through conjugation with GSH. The most likely link between GSTπ and MDR would seem to be isozyme-mediated detoxification of hydroxyalkenal products of lipid peroxidation. Since anthracyclines are especially likely to cause peroxidation of membrane lipids, there is a possibility that increased GSTπ levels and activity could contribute to protection from toxicity caused by these agents. It is, however, unlikely that increased GSTπ is a major cause of resistance to drugs such as Adriamycin, just as increased expression of the *MDR-1* gene product is frequently not the sole cause of resistance to natural products.

GSTπ is the most prevalent isozyme in cell lines and this has disproportionately focused attention on this area, overshadowing other reports where GST overexpression can be linked with drug resistance. The remainder of this article will present the substantial evidence favoring the direct involvement of GSH and GST in resistance to a number of cancer drugs used in clinical treatment today.

Enzymes of GSH Homeostasis

As shown in Fig. 1, a number of enzymes contribute to the maintenance of intracellular levels of reduced glutathione. The recent cloning and characterization of γ-GCS, the rate-limiting enzyme in *de novo* synthesis of GSH via the γ-glutamyl cycle, has permitted studies which show that increased expression of this enzyme along with γ-GGT may be causally linked to resistance to alkylating agents and platinum-based anticancer drugs (5). Increasing the intracellular capacity for *de novo* synthesis of GSH could presumably impact upon other enzymes (including GST) which use GSH as a cofactor. Intracellular GSH levels are most often within a range of 0.1 to 10 mM. Micromolar concentrations of BSO can produce a 90% or greater reduction of intracellular GSH over a 24-h period, thus producing intracellular concentrations in the high micromolar range. The K_m values for most of the GST-catalyzed conjugations of alkylating agents with GSH are in the low micromolar range. However, direct extrapolation must be applied with caution, since intracellular com-

partmentalization and a variety of endogenous GSH functions may restrict the availability of GSH for detoxification reactions.

The capacity of a tumor cell to maintain GSH is determined by a number of interacting pathways which are shown in Fig. 1. Many of the enzymes involved in these pathways have been targeted for therapeutic intervention by modulators of anticancer drug resistance. Perhaps the most widely studied is BSO, an inhibitor of γ-GCS, the rate-limiting step of GSH synthesis via the γ-glutamyl cycle. A large number of *in vitro* and *in vivo* reports have shown that BSO can enhance tumor cell susceptibility to alkylating agents and reverse some acquired resistant phenotypes. Because BSO is a specific inhibitor of γ-GCS, reversal of resistance can reasonably be attributed to the resultant depletion of intracellular GSH. In addition, a number of drugs can cause an increased expression of mRNA for γ-GCS transcript, illustrating the propensity of the cell to provide a compensatory response to a drug-induced depletion. Thus, interference with *de novo* synthesis of GSH appears to be an effective means of modulating resistance to certain drugs and regulation of γ-GCS is a critical control point for cellular protection. It will be of considerable interest to determine if cotransfection of γ-GCS with other GSH-utilizing enzymes such as GST can confer greater levels of resistance to alkylating agents. Relevant to this is a recent observation that life span extension in *Drosophila melanogaster* is achieved only when cDNAs for both superoxide dismutase and catalase are cotransfected, not with either alone (6). This suggests a cooperativity of the free radical detoxification systems in this species. Such a principle could readily be extrapolated to the GSH-based detoxification pathways. With so many interacting and mutually dependent biochemical pathways, the question arises as to how critical coregulation of the enzymes of GSH homeostasis may be in controlling the overall cellular protective thiol environment. In addition to the many reports of elevated GST, increased expression of both γ-GGT and glutathione peroxidase have been implicated in drug resistance (7). The fact that at least three distinct human peroxidase genes have been identified suggests that care will be needed in analyzing differential expression and regulation of these enzymes and their potential role in drug resistance.

Since recycling of the constituent amino acids of GSH is an important factor in GSH homeostasis, membrane-associated γ-GGT might logically be expected to be critical in allowing the "salvage synthesis" of the tripeptide. Whether or not enhanced γ-GGT will produce an overall increase in intracellular GSH is, once again, dependent upon GSH-mediated feedback responses, most of which have yet to be elucidated. In addition to γ-GGT, glutathione reductase and the glyoxalase system function to recycle GSH (Fig. 1). Under conditions of extreme oxidative stress, it is possible that glutathione reductase could become saturated. By extrapolation, increased reductase activity could enhance the ability of a cell to survive such conditions. In mammalian cells, this correlation has not been reported, perhaps reflecting either the relative uninducibility of the gene or the efficient extrusion of oxidized glutathione from the cellular milieu. Often overlooked in terms of GSH metabolism is the glyoxalase system. Methylglyoxal is a by-product of intermediary metabolism and can be present in millimolar concentrations in rapidly growing tissues. Indeed, increased glyoxalase I protein and transcript levels are found in tumor tissue compared to the normal tissue counterpart (8). Since methylglyoxal is toxic, its rapid and continual conversion to D-lactate is a necessary requisite for cell survival. Glyoxalase II serves to recycle the GSH used in the initial thioether reaction (Fig. 1). Thus, the overall net use of GSH should be zero. However, with such potentially high levels of methylglyoxal, the net commitment and flux of GSH through this pathway may be quite significant. Mammalian glyoxalase II has yet to be cloned and little is presently known of the regulation of the gene or its coordination with glyoxalase I. Future investigations should probably consider how much of a "GSH sink" this pathway represents.

While GST represents a primary focus for many drug resistance studies, numerous other enzymes which either produce, recycle, or use GSH can also be involved in determining cellular response to certain anticancer drugs. The cloning and characterization of coding and regulatory sequences of many of the genes, the products of which are illustrated in Fig. 1, lag behind those of GST genes. The next few years may help to establish the importance to the resistant phenotype of coordinated expression and regulation of these enzymes.

Glutathione *S*-Transferases

Many excellent review articles have detailed the nomenclature and biological importance of the GST supergene family (*e.g.*, Ref. 9) An in depth discussion of GST nomenclature is outside of the scope of this article. However, in mammalian species the four major cytosolic families have been designated α, μ, π, and θ. At least one microsomal form has also been described. The cytosolic forms share homology and probably have evolved from a common ancestral gene. Most of the published material linking GST to anticancer drug resistance has focused on the cytosolic families, although this does not preclude a role for microsomal GST. Indeed, the importance of membrane-associated GST in drug metabolism has not been studied in any great detail and could prove to be a fertile area for future research.

The cytosolic GSTs exist as monomeric subunits which have catalytic activities either as homo- or heterodimers. Xenobiotics, including anticancer drugs, can influence the transcriptional regulation of GST genes, primarily through effects mediated by AP1-like regulatory elements, which for the rat α GST family have been characterized as antioxidant response elements (10). GSTπ is also inducible and frequently overexpressed in neoplastic tissues when compared to their normal counterparts. Indeed, GSTπ is a tumor marker for a number of different cancers. The fact that it is usually the most common of the GST in both tumor tissues and cell lines and therefore the easiest to detect experimentally contributes to the prevalence of literature references for this isozyme. GSTμ is variably expressed in human tissues or cell lines. This is because approximately 40–50% of the human population has a null phenotype for GSTμ and thus have no constitutive expression of the isozyme. This has elicited interest in the relationship between cancer incidence and GST expression and large scale population studies are currently under way to correlate susceptibility to lung cancer in smokers who have the GSTμ null phenotype (11). These studies are predicated on the hypothesis that lack of expression of GST is related to increased DNA damage by mutagens in smoke. This rationale parallels the hypothesis of altered drug metabolism by GST in resistant cells and illustrates one example of potential overlap between drug resistance and chemoprevention.

GST Studies in Cell Lines

Of most significance to this article are the following general associations between increased GST expression and drug resistance: (a) GSTα isozymes and nitrogen mustards; (b) GST μ and nitrosoureas; (c) GSTπ and the MDR phenotype. The evidence for a causal link for point a and point b is good. For point c, most of the published data would support the principle that increased GSTπ is more likely a consequence of a pleiotropic stress response. As discussed below, if GSTπ is to be unambiguously defined as a causative factor in MDR, better evidence is required.

Empiric assessment of GST involvement in anticancer drug resistance has come primarily from work with cultured mammalian cell lines. It has been standard operating procedure to develop a resistant cell line by selection with the drug of choice and then to analyze a

Fig. 2. GST-catalyzed conjugation of glutathione with chlorambucil. *Inset*, increase in rate and extent of conjugate formation (chlorambucil equivalents) in the presence or absence of GSTα isozyme catalysis. The comparative catalytic efficiencies (K_m/V_{max}) indicate that human GSTα is more proficient than GSTπ in catalyzing the reaction.

number of cellular properties to achieve a correlation between a particular trait and drug resistance. Such studies have generated extensive correlative data and have provided many tumor cell lines with comparatively high levels of GST expression. Inherently disadvantageous in this approach is the natural tendency to overinterpret the cause/effect relationship of GST overexpression and drug response (a problem not unique to GST). This is especially true for GSTπ. In most instances, the data which link GSTπ to enhanced cellular detoxification of anticancer drugs, and by extrapolation to drug resistance, is not completely convincing. For example, chlorambucil conjugation with GSH can be catalyzed by human GSTπ, but the catalytic efficiency is some 40- to 50-fold less than for human GSTα (Fig. 2; Ref. 12). In a cell line with high GSTπ and no GSTα, it has not been conclusively demonstrated that GST catalysis would be a major detoxification pathway. As will be discussed, there is presently little direct evidence that any anticancer drugs are direct substrates for GSTπ.

Selected cells with an acquired resistance phenotype are often not cloned from a single progenitor and are represented by a heterogeneous population; this may serve to compound interpretation problems. In some instances, authors may state that because cell line A has 10-fold the GST activity of cell line B, it is 10-fold resistant to a particular drug. Notwithstanding the heterogeneity problem, the relationship between steady-state GST levels and drug response may be linear, but this must be demonstrated, not assumed. GST activity measurements in cell lines vary from as low as 4 nmol/min/mg (MCF7 human breast) to >800 (numerous hepatomas). Because GSTs have numerous endogenous metabolic roles (*i.e.*, ligand binding/transport, eicosanoid synthesis, etc.; see Ref. 13) in addition to their detoxification functions, there is no straightforward way to make a direct quantitative comparison between enzyme amount or activity and drug response. In addition, there are other factors which must also be considered in such an analysis. For example, the turnover of both the enzyme and transcript will influence the ability of a cell to adjust the steady-state levels. There is evidence in drug-resistant cells that the half-lives of GSTπ and its mRNA are doubled in comparison to wild type cells (14)[3]. This propensity to "recognize" a toxic challenge by altering protein or transcript half-life has also been identified for other cellular proteins such as tubulin, where cells resistant to antimitotic drugs accumulate higher levels of transcript for β-tubulin (15).

Thus, the intrinsic steady-state levels of GST, the transcriptional and translational rates, and the stability and turnover of both protein and transcript should all be factored into any analysis of response to chemotherapy. Moreover, binding affinities and relative amounts of different GST isozymes, as well as competing reactions and relative intracellular content of substrate and substrate-GSH conjugate, will be important. Since GSTs use GSH as a cofactor in the formation of a thioether bond with its drug substrate, these same concerns will relate to the enzymes of GSH biosynthesis. As indicated above, GSH homeostasis is itself influenced by a multiplicity of cellular controls and feedback mechanisms. While concentrations of reduced glutathione in tumor cells often approach millimolar, this does not automatically imply a surplus of available GSH. Compartmentalization and competing reactions will play an important role in determining availability. In addition, there is now significant evidence that drug-GSH conjugates are removed from the interior of the cell by an energy-dependent membrane transporter. Although the precise identity of the pump in tumor cells is not known, a potentially important report has recently appeared which identifies a M_r 38,000 membrane protein which can efflux both GSH conjugates and drugs such as daunomycin, daunorubicin, and vinblastine (16). Partial homology with other members of the ATP-binding cassette family members is reported. Thus, it may be that certain membrane proteins can serve to recognize both hydrophobic drugs and other phase I metabolites such as GSH conjugates. This could provide a possible link between MDR and GSH metabolism. It appears likely that other GSH-conjugate membrane pumps will eventually be identified. Obviously, differences in expression of such proteins may influence the apparent intracellular concentrations of drug metabolites and this could produce potential differences in cytotoxicity. There would seem to be value in pursuing this avenue of research investigation.

Any listing of drug-resistant tumor cells with elevated GST will frequently include a number of MDR lines. With the exception of the mouse Friend erythroleukemia cell line, the MDR lines (or more specifically those selected with drugs which are part of the MDR

[3] H. Shen, S. Ranganathan, S. Kuzmich, and K. D. Tew, submitted for publication.

phenotype) primarily overexpress GSTπ (1). The prevalence of GSTπ as a marker of transformation and carcinogen exposure suggests that the enhanced enzyme levels may be an effect of the selection process, rather than a cause of the MDR-resistant phenotype. Indeed, there is presently little indication that any of the classical MDR drugs are substrates for this isozyme. Most do not form reactive electrophilic species. One potential caveat is Adriamycin. While the debate over the precise cytotoxic mechanism of the anthracyclines has become less contentious since the identification of topoisomerase II as a drug target, there is little doubt that quinone-mediated free radicals formed from Adriamycin have the capacity to cause oxidative damage and cytotoxicity (7). Lipid peroxidation is a frequent cellular result of Adriamycin exposure. Such peroxides may further break down to yield hydroxyalkenals, which have been shown to be substrates for GST isozymes (17). It is possible to hypothesize that these hydroxyalkenals can induce, or select for, increased GST expression and that their detoxification by GST can act as a cellular protective effect. Such a mechanism has been invoked previously for the low level of resistance to Adriamycin in a GSTπ-transfected pT 22-3 cell line (18). Because of the extensive data to support the role of the P-glycoprotein and more recently the *MRP* gene product in resistance to MDR drugs, it would seem reasonable to conclude that if GSTπ has a role in determining the MDR response it may be minor. Increased levels of GSTπ in MDR cells may prove merely to be the consequence of a coordinate up-regulation of a number of stress-inducible genes.

Anticancer Drugs as Substrates for GST

When designing antimetabolite anticancer drugs, tight binding to the target enzyme (frequently involved in some aspect of DNA synthesis) is desirable. For GST to function as efficient detoxifiers of a wide range of chemical structures, such tight binding could prove to be problematic and indeed for those alkylating agents which have been shown to be substrates, the binding constants suggest affinities in the micromolar range. What seems to be gained from the reduced affinity for an individual chemical is promiscuity of substrate recognition. Those anticancer drugs which have been definitively identified as GST substrates are listed in Table 1. Perhaps of equal significance are those drugs for which there is no convincing evidence of GST-mediated catalysis (Table 1). It is apparent that many, if not all, of the MDR drugs fall into this latter category. An additional consideration is that while no positive substrate specificity results have been published, no specific negative data have been generated for the drugs in this column. Anthracyclines are known to produce reactive quinone

Table 1 *Anticancer drugs as substrates for glutathione S-transferases*[a]

Convincing substrate/ kinetic data exist	No definitive proof of catalysis exists	Indirect evidence[b] exists
Chlorambucil[c]	Antimetabolites	Bleomycin
Melphalan[c]	Antimicrotubule drugs[d]	Hepsulfam
Nitrogen mustard[c]	Topoisomerase I and II inhibitors	Mitomycin C
Phosphoramide mustard[c,e]		Adriamycin
Acrolein[e]		Cisplatin
BCNU[f]		Carboplatin
Hydroxyalkenals[g]		
Ethacrynic acid		
Steroids[h]		

[a] The catalyzed reaction is assumed to involve conjugation with GSH through thioether bond formation.
[b] Indirect evidence can include low levels of resistance conveyed by transfection.
[c] Aziridinium intermediate of the nitrogen mustards is the main GST substrate.
[d] The antimicrotubule drug estramustine is an inhibitor of GST, but there is no direct evidence that it is a substrate.
[e] Metabolites of cyclophosphamide.
[f] GST catalyzes a denitrosation of BCNU.
[g] Most electrophilic anticancer drugs produce lipid peroxidation, degradation of which produces a variety of hydroxyalkenals.
[h] GST can act as transporter ligands for some steroids.

moieties, as does the antibiotic fosfomycin (2). Since this antibiotic does undergo GST-mediated catalysis, it would not be unreasonable to suggest that the quinone species of Adriamycin may be subject to catalyzed conjugation with GSH. Furthermore, mitomycin C toxicity is reduced by the presence of increased cellular GSH, although it is not clear if this is a function of an altered reducing environment or the propensity of the drug to form GSH conjugates. Even if the latter is true, the value of GST in enhancing the spontaneous reaction has not yet been determined. It may also be possible that glutathione peroxidases may mediate detoxification of Adriamycin and associated free radical species (7).

A review of the chemical structures of those drugs which are GST substrates shows that a common characteristic is the electrophilic nature of their active cytotoxic moieties. In every case the drug can interact with the thiol of reduced glutathione in a spontaneous manner, creating a thioether product which is characteristically less toxic and more water soluble. The nitrogen mustards have bifunctionality as a result of the two chloroethyl arms. Because of this, there is the possibility that a second reactive site could produce a diglutathionyl product. This bifunctionality is important to the overall cytotoxic properties of the drug, since there is potential to form cross-links within DNA. Such lesions can make the drug 1 to 2 logs more cytotoxic than an equivalent monofunctional mustard. Thus, in a simplistic way, the initial conjugation with GSH serves to reduce the potential cytotoxic threat by turning the bifunctional property into monofunctionality (see Fig. 2). A similar principle can be applied to emphasize the importance of GST catalysis in determining the rate of GSH conjugation. The spontaneous reaction of the nitrogen mustard aziridinium ion with the sulfhydryl of GSH is determined by the potential energy states of the drug electrophile and the macromolecular target nucleophile, which have been defined as "hard" or "soft" based upon the polarization of their reactive centers (19). The aziridinium ion of an activated nitrogen mustard is highly polarized, carrying a high positive charge density at the electrophilic center and is thus defined as a hard electrophile. These will most favorably react with hard nucleophiles, where the high energy transition state of the reaction is most favorable. Within these guidelines, the alkylating species of, *e.g.*, chlorambucil will react with nucleophiles in the following order: oxygen of phosphates > oxygen of bases > amino groups of purines > amino groups of proteins > thiol group of methionine > thiol group of the cysteine in GSH. The less favorable reactions still occur spontaneously, but nucleophilic selectivity may not favor such reactions in a mixed nucleophile environment. To this end, GST catalyzes the conjugation of mustards with GSH by bringing the two into close proximity, creating a conducive hydrophobic environment and reducing the apparent pK of the cysteine thiol from 9.6 to a more neutral value. All of these factors serve to increase both the rate and extent of the conjugation when compared to the spontaneous reaction.

Because nitrogen mustards undergo spontaneous decomposition to produce active alkylating species, it is particularly significant that the aziridinium intermediate of melphalan has been reported as the true substrate for the GST-catalyzed reaction (20). Since all bifunctional nitrogen mustards have the potential to form two aziridinium ions, a significant role for GST-mediated catalysis may be possible. Intracellular chloride concentrations of 8 mM predict that aziridinium ion formation is essentially irreversible. The reactivity of this species with cellular nucleophiles such as phosphate and water is approximately equivalent and assuming GSH concentrations of 2.5–5.0 mM and a pH of 7.4, Bolton *et al.* (20) calculated that the reaction of GSH with the aziridinium will be 5.5 times greater than hydrolysis. However, tumor microenvironments frequently produce a more acidic pH. At pH 6.5 the contribution of GSH to melphalan detoxification was only equivalent to water. Because of this, the spontaneous GSH reaction may prove to be limiting in terms of how much and how quickly detoxi-

fication can be achieved. The glutathionylation of melphalan catalyzed by GSTα was 2.5- and 9-fold (pH 7.4 and 6.5, respectively) greater than the spontaneous rate, values which correspond well with the comparative rates and extents of chlorambucil conjugation shown in Fig. 2. Thus, the GST-catalyzed rate of both chlorambucil and melphalan conjugation with GSH will be significantly greater than the spontaneous rate (12, 20). The cytoprotective consequences of this enhancement could prove to be the difference between drug-induced sublethal and lethal damage.

Transfection of GST

It is often considered that enhanced resistance to a particular drug must be shown in a transfected cell line in order for the gene product to be causatively linked to the resistant phenotype. This one area has provided substantial controversy for the GST field. A number of transfection studies have been reported and some disparity is apparent. The lack of anticancer drug resistance in MCF7 cells transfected with GSTπ, -α, or -μ isozymes (21) has led many to conclude that the link between enhanced GST and anticancer drug resistance is tenuous. This concept was given further credence by data showing that only low levels of resistance to alkylating agents and anthracyclines were found in NIH 3T3, C3H 10T½, or monkey COS cells transfected with rat or human GSTs (1). The significance of low levels of resistance has been the subject of much debate. Degrees of selected resistance in MDR cell lines frequently reach the 1,000–10,000-fold range. Yet when *MDR-1* recipient transfectant cells are tested, they usually display 5- to 20-fold resistance, with maximal degrees of resistance approaching only 30-fold. For alkylating agent-resistant cell lines, the degree of selected resistance rarely exceeds 20-fold; therefore, it could be argued that the 1.5- to 3-fold resistance reported in the GST transfectants is relatively consistent when comparing the ratio of selected:transfected resistance for alkylating agents/GST and MDR drugs/P-glycoprotein.

Of further relevance is the potential clinical meaning of low levels of resistance. There is little doubt that such levels are experimentally less easy to assay and to analyze statistically. A number of GST transfection studies have characterized low levels of resistance (*i.e.*, 1.5- to 3-fold) to numerous anticancer drugs (reviewed in Ref. 1). Understandably, these levels of resistance have not been convincing. Alkylating agents and electrophile-producing drugs as a group tend to have survival curves characterized by a sublethal damage shoulder and steep kill curve. Given the nature of drug dose escalations in clinical protocols, a 2-fold shift in *in vitro* sensitivity may translate into a meaningful difference in therapeutic efficacy and patient response. However, it is likely that the debate over how to measure response and what constitutes a "meaningful" degree of resistance will continue. Notwithstanding these quantitative issues, a number of possible factors could help to explain why GST transfection in some cell lines does not produce significantly enhanced drug resistance: (*a*) intrinsic GSH/GST levels; (*b*) transcriptional and/or translational rate and turnover of GSH/GST synthesis (enzyme or transcript half-life, etc.); (*c*) feedback control mechanisms; (*d*) presence and/or efficiency of a GSH-conjugate membrane efflux pump; (*e*) effective compartmentalization of GSH/GST; (*f*) competing detoxification reactions; (*g*) proven substrate specificity of the transfected GST for the anticancer drug(s) tested.

The uncertainty of these earlier data in proving a direct role for GST in determining drug sensitivity has been largely alleviated by several recent reports. The first is in *Saccharomyces cerevisiae* where significant resistance to chlorambucil and Adriamycin (maximum resistance, 8- and 16-fold, respectively) was reported in cells transfected with mammalian GST isozymes (22). Because of the yeast cell wall, high concentrations of drug were required to achieve cell kill, leading to some debate as to the relevance of yeast as a test organism. Since yeasts do express endogenous GST that have detoxification functions, the addition of heterologous GST would seem to be a legitimate, reasonable approach to answering specific functional questions.

In mammalian cells, Schecter *et al.* (23) recently transfected the rat Y_c gene (α family) into rat mammary tumor cells and showed high levels of induced resistance to a number of nitrogen mustards. The degree of resistance conferred ranged from 6- to 30-fold for chlorambucil, melphalan, and nitrogen mustard. These resistance factors are equal to, or exceed, those of most acquired resistant cell lines. The results are perhaps more convincing, since overexpression of the Y_c GST subunit has been circumstantially linked to mustard resistance by nature of increased expression in rat cell lines (1, 24). In addition, the mouse and human α-GST family show the best catalytic constants for the conversion of chlorambucil and melphalan to their respective monoglutathionyl derivatives (12, 20).

Two other studies have transfected Chinese hamster ovary cells with GSTπ (25) or GSTα (26). GSTπ increased resistance to cisplatin and carboplatin by 2- to 3-fold, while GSTα afforded protection against bleomycin. The latter study also quantitatively correlated GSTα expression with bleomycin resistance. Although both papers implicate GST-catalyzed detoxification of the parent drugs or their metabolites as a mechanism of increased resistance, neither of them included such data. These drugs are known to form conjugates with GSH and it is therefore feasible that they may be substrates for GST catalysis. They are included in the category of "indirect evidence" in Table 1, indicating that they are implicated as but not proved to be GST substrates. One feasible explanation for the occurrence of low levels of resistance in transfectants is that the increased GST provides additional thiol groups to act as a drug sink. Some drugs [*e.g.*, ethacrynic acid (27)] can bind directly to GST isozymes. The physical presence of additional GST in transfected cells could subsequently reduce the effective intracellular concentrations of some drugs.

It would seem reasonable to suggest that the positive transfection data should satisfy the question as to whether GSTs have a direct role in some types of drug resistance. However, it is of importance to interpret the clearly positive data in the context of the more equivocal or negative results. There are several possible explanations for the less convincing or negative data. One is that GSTs are not in of themselves sufficient to convey resistance. This would require the existence of putative complimentary "factors" in cells which yielded positive correlates. For example, the requirement for GSH as a cosubstrate may be limiting in some target cells. As discussed elsewhere in this article, the maintenance of intracellular levels of reduced glutathione is controlled by a complex, interrelated series of pathways. The transfection and ultimate overexpression of one enzyme within this pathway may not be the optimal way to achieve or maintain a GSH homeostasis. Interestingly, the negative transfection data have primarily come from MCF7 cells. The wild type cells have a GST specific activity of 4 nmol/min/mg protein; one of the lowest activities, if not the lowest activity, measured in tumor cell lines. In addition, transcript levels for γ-GCS are also extremely low in MCF7 cells,[4] suggesting a limited capacity for *de novo* GSH synthesis via the γ-glutamyl cycle. Perhaps MCF7 cells, with such low intrinsic steady-state levels of GST and capacity for GSH synthesis, utilize other detoxification pathways more efficiently.

[4] Unpublished observation.

Therapeutic Correlates of GST Expression

Despite a profusion of recent literature reports cataloguing GST expression in many tumor and normal tissues from human biopsies, actual attempts at therapeutic manipulation through drug intervention have been more limited. Much effort has been focused on seeking correlates between patient treatment failures and enhanced expression of genes involved in drug resistance. To date such correlations have been detailed in only a portion of trials involving *MDR-1*, O^6-alkylguanine transferase (nitrosourea resistance), GSH, and GST (1). For all of these systems, negative results may have been compromised by the limited sensitivity of the methodology used and the fact that low levels of resistance may be clinically important but difficult to show experimentally. Limited availability of tissue (especially tumor) that is amenable to sequential biopsy during and/or following chemotherapy also limits the feasibility of this approach. However, there are at least two examples in which enhanced expression of GST has been found in tumor samples from patients who have become refractory to drug treatment.

In the first, two cell lines were established from an ovarian cancer patient with abdominal ascites (28). PEO1 was established from cells that were harvested before starting treatment. PEO4 was established following the acquisition of resistance to a regimen of *cis*-platinum, chlorambucil, and 5-fluorouracil. When comparing a variety of parameters of glutathione metabolism, increases in γ-GGT (6.5-fold) glutathione peroxidase (2.3), and GST (2.9) were found. The issues which were of most concern in this study were the fact that the tumor cells were cultured (albeit a short time) prior to analysis, perhaps allowing drift in sensitivity. Furthermore, a drug combination was used in the patient with the result that definitive proof that chlorambucil was the causative agent in the overexpression of these enzymes could not be shown. Because of the easy access to sequential tumor biopsy, CLL provides a good model system to study clinical resistance. Analysis of lymphocytes from CLL patients using the general GST substrate 1,2-chlorodinitrobenzene showed a statistically significant 2-fold increase in cells from chlorambucil-resistant patients compared to untreated patients or normal individuals (29). Furthermore, chlorambucil therapy caused a 1.5-fold elevation in enzyme activity in three previously drug-naive patients. These two clinical reports have provided inferential correlative data for patient response and drug sensitivity. Clearly, such studies are not conclusive and more work will be required; but in an area beset by practical limitations, these correlations are encouraging.

The emergence of ethacrynic acid as an effective inhibitor of GST has led to its consideration as a modulator of GST-mediated drug resistance. Preclinical studies have demonstrated that reversal of resistance in cell lines may be achieved at low concentrations of ethacrynic acid. A Phase I trial suggested that drug-induced diuresis with resultant fluid imbalance was the major dose-limiting toxicity (30), but this was not serious enough to prevent the formulation of a Phase II study of ethacrynic acid and chlorambucil in drug-resistant CLL. Because of the low number of such patients and the emergence of fludarabine as a viable treatment for chlorambucil-refractory CLL, the protocol is accruing patients at a slow rate in the United States. Its potential, therefore, remains largely unknown. However, one case report (31) details a partial reversal of chlorambucil resistance in a B-CLL patient treated simultaneously with ethacrynic acid and chlorambucil. Thus, while the approach has merit, large scale studies are needed. Design, synthesis, and testing of novel GST inhibitors are presently in progress (32) and may well yield new compounds worthy of clinical testing.

Assessment of the quantitative aspects of drug resistance is also critical in appreciating the value of a resistance reversal approach. The measurement of drug synergy is usually achieved by isobologram analysis. Although there is some debate over the best mathematical approach to define synergy, it is generally agreed that their combined effects must be greater than their composite additive cytotoxicities. For modulators of resistance, there is frequently little or no intrinsic toxicity at the concentrations required to reverse resistance; therefore synergy can be readily shown. More problematic, however, is the extrapolation of preclinical synergy to human trials. For example in MDR cell lines, conversion of a 1000-fold resistant phenotype to 20-fold would apparently represent a gain worthy of clinical study based upon published and ongoing clinical trials with *MDR-1* modulators. For alkylating agents, such a quantitative *in vitro* enhancement may be difficult to achieve experimentally, since 10–20-fold resistance is apparently maximal for this class of drugs. These comparisons are further confounded by the clinical consideration that low level resistance may be quite critical in determining success or failure to therapy. It may be necessary to apply a dual standard for judging efficacy. Modulators of alkylating agents may not need to be as quantitatively effective *in vitro* as those of MDR yet may still be therapeutically valuable.

Conclusions and Perspectives

There is a growing body of evidence that the phenotype of drug resistance may result from reduction in the ability of a cell to recognize and/or instigate programmed cell death as a consequence of exposure to drug. For example, the adenovirus gene *E1A* has been shown to sensitize cells to a range of anticancer drugs. In this system a functional *p53* tumor suppressor gene is required to allow programmed cell death (33). Similarly, *bcl-2* seems to prevent cell death by protecting cells from oxidative stress. Moreover, if the *bcl-2* gene expression is suppressed, free radical damage accumulates sufficiently to activate the apoptotic pathway (34). These findings could prove to have enormous significance with regard to how drug resistance is viewed and perhaps manipulated. However, in the absence of genetic changes which could create an "apoptotic null phenotype," they may be of more limited relevance to the role of detoxification in determining cell response. As stated earlier, cells have a characteristic survival pattern when treated with alkylating agents. The short half-life, electrophilic reactivity, and covalent nature of these drugs ensures that a damage threshold is reached in an acute, rather than a chronic, manner. Most clinically used alkylating agents have half-lives of less than 1 h. Although there may be latent damage which can lead to cellular toxicity, generally if a cell can minimize the early lesions and maintain damage below a specific threshold, it will survive. The quantitative definition of this threshold is offered in an arbitrary fashion for the purpose of this discussion. However, there is reason to believe that this value will vary both within and between tumor cell populations. In any case, the importance of GSH and associated enzymes lies in the provision of an enhanced rate and extent of detoxification, helping to maintain the amount of damage below that which may trigger any apoptotic cascade. Even in the absence of definitive information as to which drug lesions (and how many) may induce this pathway, the principle of detoxification maintaining damage control would be of significance. Although there is some debate as to the precise role of free radical damage in mediating the initiation of apoptosis, it could be argued that an enhanced cellular capacity to maintain a reduced thiol status may even contribute directly to an interference in the early stages of this process. Indeed, free radical-mediated damage appears to be of primary significance in aging, cancer, and many other human conditions.

What are the future therapeutic possibilities involving GSH/GST? Presently, serum GST levels are being tested as potential markers for cancer detection and even as markers for organ rejection in liver

transplants. GSTπ is up-regulated in a large proportion of the human malignancies thus far analyzed. Certain prodrugs are under development to take advantage of this observation. An inactive drug which upon catalysis by GSTπ becomes cytotoxic, offers a nonempiric rationale for gaining an enhanced therapeutic index. Use of inhibitors of GST have, to this time, focused upon small chemical moieties with little tissue or isozyme specificity. Current and future endeavours are aimed at targeting the inhibitor against specific isozymes. If successful, this could achieve a directed inhibition of GSTα which would yield the best chance of reversing alkylating agent resistance (32). The obverse of this approach is exemplified by ongoing chemoprevention trials with oltipraz. Chronic low level doses of oltipraz are known to induce GST (and other Phase II detoxification enzymes) expression in colon epithelium. The efficacy of this approach in preventing cancer incidence in high risk populations is under intensive study.

One of the more recent in vogue efforts at therapeutic management involves the transfer of drug resistance genes or cDNAs into normal tissues, such as bone marrow. Theoretically, this approach should increase the normal tissue tolerance and enhance the therapeutic index of a drug for which marrow is the dose-limiting tissue. This paradigm if applied to alkylating agents and GSH and GST enzymes may allow protection of bone marrow stem cells at drug levels sufficient to kill tumor cells. Some consideration of existing data may suggest at least two adaptations which may enhance the chances for success: (*a*) transfer γ-GCS together with a GSTα family member. This will reduce the chances that GSH may be limiting as a cofactor for GST; (*b*) the rat Y_c is the most inducible of all the α isozymes by nitrogen mustards (human GST α genes do not have the same 5′ regulatory elements as those of the rat). Transfection of the Y_c cDNA has yielded the most mustard-resistant clones. Preliminary substrate specificity data indicate that Y_c isozymes have meaningful catalytic constants for detoxification of the nitrogen mustards (data not shown). These observations suggest that in humans, transgenic Y_c transfers could be more effective than using human α isozyme constructs. Whether this is combined with a rat or human γ-GCS is less important, since there is no present indication that the two enzymes differ significantly in their capacity for *de novo* synthesis of GSH.

For as long as toxic chemicals with electrophilic centers are used in cancer chemotherapy, there will be a sound rationale for studying GSH and the enzymes that produce it and use it. At least for the short term, such drugs should continue to play a significant role in patient treatment.

Acknowledgments

The critical evaluations of Drs. Gary Kruh, Tom Hamilton, and Chuck Smith are gratefully acknowledged.

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Cancer Treatment Reviews (2008) **34**, 505–520

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ANTI TUMOUR TREATMENT

Combined action and regulation of phase II enzymes and multidrug resistance proteins in multidrug resistance in cancer

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Received 6 December 2007; received in revised form 11 February 2008; accepted 1 March 2008

KEYWORDS
Multidrug resistance;
Oncolytic drugs;
Multidrug resistance proteins;
Phase II enzymes;
Nrf2

Summary A major limitation in the treatment of cancer patients is the ability of cancer cells to become resistant to chemotherapeutic drugs, a phenomenon known as multidrug resistance (MDR). Two important mechanisms involved in multidrug resistance are the increased activity of efflux pumps, such as those of the multidrug resistance proteins (MRPs) and the detoxification by phase II conjugating enzymes, like glutathione *S*-transferases and UDP-glucuronosyltransferases. A synergistic interaction between these two mechanisms, MRPs and phase II enzymes, in conferring MDR has been shown for multiple anticancer drugs. In addition, there is substantial evidence of a coordinate regulation of the expression of phase II enzymes and MRPs, most likely mediated by the nuclear factor-erythroid 2 p45-related factor (Nrf2) and antioxidant response elements. Further elucidation of the combined action and regulation of phase II enzymes and MRPs in MDR will be an aid in the improvement of the chemotherapeutic treatment of cancer patients.
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Introduction

Cytotoxic agents are still one of the most often used and successful treatments in cancer. In addition, there are many new promising anticancer agents with different types of mechanisms, such as the tyrosine kinase inhibitors erlotinib and gefitinib. A major problem is, however, the

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doi:10.1016/j.ctrv.2008.03.002

Nomenclature

γGCS	γ-glutamylcysteine synthase	Keap1	Kelch-like ECH-associated protein
β-NF	β-naphtoflavone	MAPK	mitogen-active protein kinase
ABC	ATP-binding cassette family	MDR	multidrug resistance
Ahr	aryl hydrocarbon receptor	MNNG	1-methyl-2-nitro-1-nitrosoguanidine
ARE	antioxidant response element	MRP	multidrug resistance protein
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea	Nrf2	nuclear factor-erythroid 2 p45-related factor
BHA	butylated hydroxyanisole	Pgp	P-glycoprotein
BSO	buthionine sulfoximine	PI3K	phosphatidylinositol 3-kinase
CAR	constitutive androstane receptor	PKC	protein kinase C
CYP	cytochrome P450	PPAR	peroxisome proliferator-activated receptor
ERCC-1	Excision Repair Cross Complementation Group 1	PXR	pregnane X receptor
GSH	glutathione	RXR	retinoid X receptor
GSHS	GSH synthase	SN38	7-ethyl-1-hydroxycamptothecin
GST	glutathione *S*-transferase	SULT	sulfotransferases
GSX	glutathione *S*-conjugates	t-BHQ	*tert*-butylhydroquinone
JNK1	c-Jun N-terminal kinase I	UGT	UDP-glucuronosyltransferase

unpredictability of the treatment effect due to the ability of cancer cells to acquire resistance to different drugs, a phenomenon known as multidrug resistance (MDR), and the capacity of the body to protect itself against xenobiotic drugs.

Several mechanisms of drug resistance of tumour cells have been suggested, which can be linked to the pharmacokinetics and pharmacodynamics of cytostatic drugs (Table 1). Of these mechanisms one of the most encountered ways of tumour cells to acquire resistance is the induction and activation of efflux transporter proteins, such as those of the ATP-binding cassette (ABC) transporter family. The increased expression of these transporters leads to a decrease in drug accumulation by the cell. So far, the ABC transporter P-glycoprotein (Pgp), multidrug resistance associated-protein 1 (MRP1 or ABCC1) and breast cancer resistance-protein (BCRP or ABCG2) have been observed to be involved in MDR. However, several studies have shown that cells resistant to oncolytic drugs express additional, sometimes multiple, transporters[1] (reviewed by Szakacs et al.[2]). The efflux transporters, especially Pgp, have been the main focus of many researchers to find strategies to prevent MDR, like identifying peptides and antibodies that inhibit Pgp, finding ways to downregulate MDR genes and the design of anticancer drugs that are not substrate of these transporters.[2]

The second way by which tumour cells can circumvent the cytotoxic action of oncolytic drugs are cellular changes that influence the activity of oncolytic drugs, like the increased detoxification by metabolizing enzymes. In tumour cells, often overexpression of drug metabolizing enzymes such as cytochrome P450 enzymes (CYP) and glutathione *S*-transferases is found.[3–6]

Another way of obtaining MDR is alterations in target molecules. Some chemotherapeutic drugs, like methotrexate,[7,8] inhibit key enzymes in pathways that control the proliferation of cells, and MDR can arise when there is an increased transcription of the key protein in the cell. Other examples are the loss of oestrogen receptors in patients with breast cancer treated with anti-oestrogen therapy,[9] and mutations in topoisomerase II to gain MDR against drugs that block the activity of topoisomerase II, like etoposide.[10]

Tumour cells can also become resistant due to the enhanced repair of DNA. Drugs like platinum derivatives react with DNA to form DNA-adducts, leading to DNA lesions. An important enzyme involved in the DNA-repair of cisplatin adducts, is excision repair cross-complementing protein (ERCC-1) and the expression of this enzyme was shown to

Table 1 Mechanisms of multidrug resistance of tumour cells

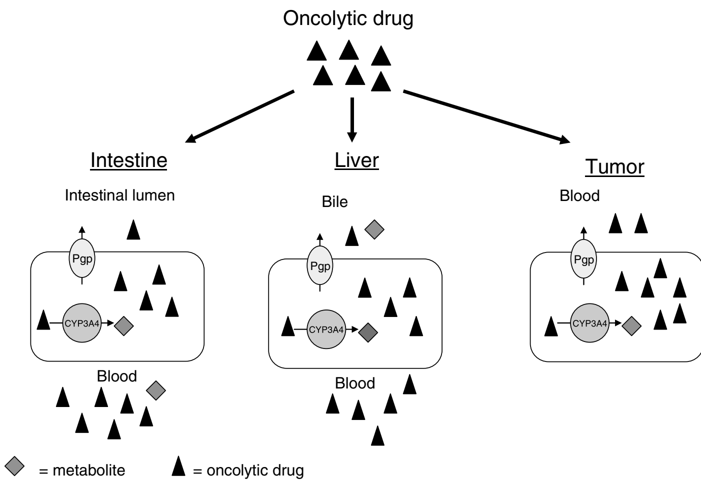
Mechanism	Examples
Increased expression and activity of efflux transporter proteins	P-glycoprotein, multidrug resistance associated-proteins (MRP/ABCC1) and breast cancer resistance protein (BCRP/ABCG2)
Increased detoxification by metabolizing enzymes	Cytochrome P450 enzymes Glutathione *S*-transferases
Alterations in target molecules	Increased transcription target proteins Decreased expression target proteins Mutation target proteins
Enhanced DNA repair	Excision repair cross-complementing protein (ERCC1)
Alteration in genes that control cell cycle and apoptosis	p53, Bcl-2

Based on reviews by[2,18–21].

be increased in cells and tumours insensitive to cisplatin and carboplatin.[11–13]

Finally, changes in genes that are critical for proliferation or apoptosis can lead to the abrogation of apoptosis or cell cycle arrest. The p53 protein is an important protein in the regulation of the cell cycle and the induction of apoptosis in response to DNA damage. Deletion of p53, and mutations that cause a loss of function of p53, have been observed in several MDR tumour cell lines.[14] Other apoptotic genes, like Bcl-2/Bax have also been implicated in MDR.[15–17] As it is beyond the scope of this review, for more detailed information on the background of MDR the reader is referred to some excellent reviews.[2,18–21]

A synergistic interaction between the processes that cause MDR is also possible, as has been observed for the metabolizing CYP3A4 and Pgp. In colon cancer cells,[22–24] primary hepatocytes[25] and liver cell lines,[24] Pgp and CYP3A4 show a coordinated co-expression, regulated by nuclear receptors like the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR). This co-regulation of Pgp and CYP3A4 forms an integral part of the defence system to protect the body against xenobiotics, like oncolytic drugs (Fig. 1). Exposure to drugs that can activate PXR or CAR, like the anti-cancer drugs tamoxifen and paclitaxel, will lead to an increased expression of Pgp and CYP3A4 (reviewed by[26]). The increased levels of Pgp will regulate the intracellular concentrations of the oncolytic drugs, so that they will stay within the linear range of the detoxifying capacity of CYP3A4.[23,27] In the intestine this coordinate action between Pgp and CYP3A4 leads to a decrease in the oral uptake of drugs, while in the liver, drugs will be more rapidly excreted and detoxified. Although CYP3A4, Pgp, PXR and CAR are



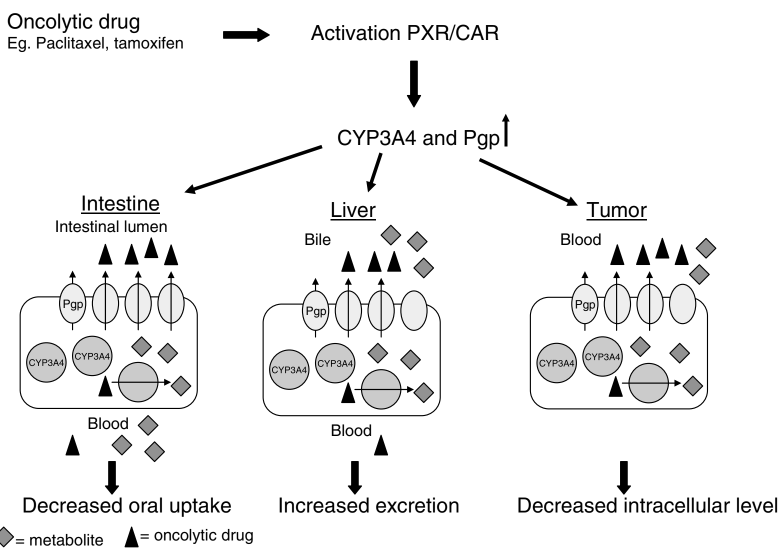


Figure 1 Multidrug resistance by the coordinate regulation of CYP3A4 and Pgp. In the presence of basal levels of CYP3A4 and Pgp, the detoxifying capacity of CYP3A4 is insufficient, and the drug is taken up in the blood and only partly removed by the liver. Intracellular levels of the drug in the tumour will be high (A). The coordinate increase of the expression of CYP3A4 and Pgp, mediated by PXR or CAR, will cause multidrug resistance by an increased detoxification by CYP3A4 and increased excretion of the oncolytic drug. Intracellular levels of the drug in the tumour will be low (B).

mainly expressed in the liver and the intestine, cancer cells also have shown to posses PXR and CAR mediated increased Pgp and CYP3A4 expression, leading to MDR.[28,29] The common regulators of the expression of Pgp and CYP3A4, PXR and CAR, therefore provide an excellent synergistic protection system of the body and cancer cells in the response to increased levels of oncolytic drugs (Fig. 1).

In addition to Pgp, other ABC transporters responsible for MDR have been identified, like the multidrug resistance (associated) proteins (MRPs). These transporters are mainly involved in the extrusion of organic anions, including the conjugative metabolite(s) that arise from detoxification reactions by phase II conjugating enzymes, like glutathione *S*-transferases (GST), sulfotransferases (SULT) and UDP-glucuronosyltransferases (UGT). Besides the efficient transport of conjugated drugs or their metabolites, MRPs also export glutathione (GSH),[30] and co-transport with GSH was shown to be required for the MRP mediated extrusion of oncolytic drugs.[30,31]A synergistic interaction between MRPs and phase II enzymes in MDR is therefore expected. This review will provide an overview of the current knowledge of the coordinate function of MRP and phase II enzymes in MDR, and will discuss the clinical consequences thereof for the treatment of cancer patients. As most information is available on GSTs and UGTs this review will focus on these two phase II enzymes.

Multidrug resistance proteins

The MRP (ABCC) subfamily of ATP-dependent membrane transporters includes thirtheen members: MRP1 (ABCC1), MRP2 (ABCC2), MRP3 (ABCC3), MRP4 (ABCC4), MRP5 (ABCC5), MRP6 (ABCC6), MRP7 (ABCC10), MRP8 (ABCC11), MRP9 (ABCC12), CFTR (ABCC7), the sulfonylurea receptors SUR1 (ABCC8) and SUR2 (ABCC9),[32] and the recently discovered pseudogene MRP10 (ABCC13).[33,34] All the members of the ABC-family of drug transporters share an extensive sequence homology and domain organisation; a core structure consisting of two nucleotide-binding domains and two membrane spanning domains, each containing 6 transmembrane helices. Exceptions to this structure are found in the MRP subfamily, as MRP1-3 and MRP6-7 contain and extra amino (N)-terminal membrane-bound region of five transmembrane regions that are N-glycosylated near the N-terminus and at the sixth extracellular loop. MRP4-5 and MRP8-9 lack the N-terminal transmembrane domain of MRP1-3 and are N-glycosylated at the fourth extracellular loop (reviewed by[2,35–38]).

The MRPs have been shown to transport exogenous and endogenous organic anions, like conjugated metabolites such as glutathion *S*-conjugates (GSX) and compounds conjugated with glucuronic acid.[39,40] Substrates of MRP include several oncolytic drugs and their metabolites (Table 2). Because MRPs differ in substrate specificity, tissue distribution and intracellular location, the MRPs have a specific, sometimes overlapping, ability to confer resistance to oncolytic drugs.[38]

Several in vitro and in vivo studies have confirmed the involvement of MRP 1 and 2 in MDR to oncolytic drugs (Table 3). The MDR pattern of MRP3 is somewhat distinct as it was shown to confer resistance to etoposide and vincristine, but not to anthracylines and taxol.[41] The other members of the MRP family are less well characterized at the moment, however, based on the fact that the disposition of several oncolytic drugs has already been identified to be modulated by these transporters (Table 1) it is very likely that they are also involved in MDR to oncolytic drugs.

Phase II enzymes

Glutathione *S*-transferase enzymes

Many drugs or their reactive electrophilic metabolites, formed in phase I metabolism reactions by CYP enzymes, are detoxified by phase II conjugating enzymes, like GST and UGT. Although, until know, only a few studies have examined the actual conjugation of chemotherapeutic drugs by GSTs, indirect evidence in the form of MDR studies, clearly implicate the involvement of GSTs in the metabolism of a wide variety of electrophilic endogenous and exogenous compounds, among which are several oncolytic drugs (Table 4). The effectiveness of GSTs is dependent on the supply of GSH, which is mainly determined by γ-glutamylcysteine synthase (γ-GCS) and GSH synthase (GSHS), the rate limiting enzymes of GSH biosynthesis[39] (Fig. 2). Because of their capacity to react with electrophiles, radicals and reactive oxygen species, GSTs, together with GSH, have a major role in the protection against oxidative stress.

There are three distinct super-family members of GSTs; the membrane microsomal, mitochondrial and cytosolic GSTs. While the microsomal GSTs are mainly involved in the metabolism of endogenous compounds, like leukotrienes and prostaglandines, the cytosolic GSTs also conjugate exogenous compounds. The cytosolic GSTs are divided into seven classes, Alpha (A), Mu (M), Omega (O), Pi (P), Sigma (S), Teta (T) and Zeta (Z) which have a promiscuous substrate specificity and are localized in different tissues with organ specific expression patterns.[42–45]

The expression of GSTs has been shown to be linked with the development and expression of MDR (Table 5). First, increased GST and γ-GCS levels have been observed in MDR tumours and cell lines. In addition, cells transfected with different isoenzymes of GST express increased drug resistance,[46] while inhibition of GST expression by antisense cDNA increased the sensitivity to several anticancer drugs.[47] Finally, exposure of cells to a specific inhibitor of γ-GCS, buthionine sulfoximine (BSO), decreases multidrug resistance to doxorubicin[48–50] and vincristine.[51,52]

One of the mechanisms behind GST mediated MDR is the capacity of GST to regulate the mitogen-active protein kinase (MAPK) pathway via protein–protein interactions. Elevated GSTπ sequesters and inhibits signalling kinases, like c-Jun N-terminal kinase 1 (JNK1), thereby inhibiting apoptosis and stimulating cell proliferation. This explains the fact that several anticancer drugs cause GST-mediated MDR, while they are not characterized as substrates of GST ([53–55] reviewed by[42,43]). A well known anti-cancer drug, which cytotoxic activity depends on the JNK1 signalling pathway, is cisplatin. Inhibition of the JNK1 pathway was shown to be associated with a decrease in cisplatin-induced apoptosis.[56]

Table 2 Localisation and substrates of multidrug resistance proteins

	Tissue	Localisation	Oncolytic substrates
MRP1/ABCC1	Ubiquitous Low in adult human liver, high in proliferating hepatocytes and liver cancer cell lines	Plasma membrane, basolateral	Anthracyclines, epipodophyllotoxins, *Vinca* alkaloids, arsenic and antimonial oxyanions, folic acid, methotrexate, HIV protease inhibitors, camptothecins, anthracenedione GSH-X (GSH conjugate doxorubicin), glucuronide-X (etoposide-glucuronide), sulfate-X
MRP2/ABCC2/cMOAT	Liver, small intestine, kidney, colon, gallbladder, placenta and lung	Plasma membrane, apical	Anthracyclines, epipodophyllotoxins, *Vinca* alkaloids, cisplatin, methotrexate, HIV protease inhibitors GSH-X, glucuronide-X (glucoronidated-SN38), sulfate-X
MRP3/ABCC3	Adrenal gland, pancreas, gut, gall bladder, placenta, liver, kidney and prostate	Plasma membrane, basolateral	Methotrexate, etoposide, taniposide, epipodophyllotoxins, glucuronide-X, sulfate-X
MRP4/ABCC4/MOATB	Ovary, testis, adrenals, lung, intestine, prostate	Plasma membrane, apical basolateral in some tissues	Nucleotide analogues, methotrexate GSH-X, sulfate-X
MRP5/ABCC5/MOATC	Ubiquitous	Plasma membrane, basolateral. Apical in microcapillary endothelial cells brain	Nucleotide/nucleoside analogues GSH-X
MRP6/ ABCC6	Kidney, liver, skin, tracheal and broncheal epithelium, intestinal mucosa, corneal epithelium, cardivascular	Basolateral	Anthracyclines, epipodophyllotoxins, cisplatin GSH-X
MRP7,8, 9 ABCC 10,11,12	Widely expressed	Basolateral	Cyclic nucleotides, GSH-X, glucuronide-X, sulfate-X

MRP (reviewed by[2,35–38]).

The other role of GSTs in the development of MDR is via the direct detoxification of chemotherapeutic drugs or their metabolites.

UDP-glucuronosyl transferase enzymes

UDP-glucuronosyl transferases (UGT) transfer glucuronic acid from UDP-glucuronic acid to drugs or their metabolites, resulting in more hydrophilic glucuronides. The human UGT superfamily consists of 2 families (UGT1 and UGT2) and 3 subfamilies (UGT1A, UGT2A and UGT2B), and the majority of the substrates of UGT are glucuronidated by multiple UGT enzymes, making it difficult to distinguish which UGT enzyme is predominantly responsible for the glucuronidation reaction. UGTs have a variety of endogenous substrates like bilirubine and steroid hormones, but also exogenous substrates, including some anticancer drugs (Table 6).

In contrast to GST and γ-GCS, until now only few data have been published about the role of UGTs and glucuronidation in MDR. Enhanced drug clearance and MDR to anticancer drugs due to glucuronidation was shown for 7-ethyl-1-hydroxycamptothecin (SN-38), the active metabolite of the chemotherapeutic drug irinotecan (CPT-11) and Nu/ICRF505, an antraquinone–tyrosine conjugate.[57,58] In addition, human colon cancer biopsies were shown to express levels of UGT sufficient to catalyze glucuronidation, although a marked interpatient variation was observed.[58] Despite the lack of in vitro and clinical data on UGT and MDR, glucuronidation by UGT enzymes is a unique mechanism that can therefore be expected to contribute significantly to MDR of several chemotherapeutic drugs.

Coordinate action between phase II enzymes and MRP

GST and γ-GCS and MRP transport

Although substantial evidence has been provided that GSTs play a role in MDR, the associations between MDR and the expression of GSTs are not always unequivocal.

Table 3 In vitro and in vivo studies of the involvement of multidrug resistant proteins (MRPs) in multidrug resistance to oncolytic drugs

MRP	Model	Involved in resistance to	Reference
MRP1	Fibroblast mouse cell lines (wildtype and (Mdr1a−/−1b−/−Mrp1−/−))	Anthracyclins, epipodophyllotoxins, camptothecins, vincristine, arsenite	138
MRP1	W9.5 embryonic stem cells (wildtype and Mrp+/− and Mrp−/−)	Etoposide, teniposide, vincristine, doxorubicin, daunorubicin, arsenite	139
MRP1	Human ovarian carcinoma 2008 cells	Methotrexate	140
MRP1	Wildtype and Mrp−/− mice	Etoposide, vincristine	141,142
MRP2	Human ovarian carcinoma 2008 cells	Methotrexate	140
MRP2	Melanoma MeWo Cis 1 cell line	Cisplatin	143
MRP2	Epithelial MDCKII cell line (wildtype, MRP2+/+)	Paclitaxel	144
MRP2	Wild type and tamoxifen resistant MCF7 cell line	Tamoxifen	145
MRP2	Wildtype and MRP+/+ Chinese hamster overy cell line and pig kidney epithelial LLC-PK1	Vincristine, cisplatin	146
MRP2	Subset resistant cell lines	Cisplatin	147
MRP2	Human embryonic kidney HEK-293 cell line	Etoposide, cisplatin, doxorubicin, epirubicin	87
MRP2	HepG2 transfected with MRP2	Cisplatin, doxorubicin, vincristine, camptothecins	148

Table 4 Oncolytic substrates of the phase II enzyme gluthatione *S*-transferase (GST) (reviewed by[3,42,44,134,149])

Substrate	GST (sub)-family	Reference
Melphalan	A1-1, P1-1	47,150,151
Chlorambucil	A1-1, P1-1	83,152–155
Mechlorethamine	—	156
Cyclophosphamide	—	157–159
Phosphoramine mustard	—	157–159
Ifosfamide	—	160
Cisplatin	P1-1	47,161
Nitrosocimetidine	—	162
1-Methyl-2-nitro-1-nitrosoguanidine (MNNG)	—	162
Nitrogen mustard	—	163
Hydroxyalkenals	—	164
Busulfan	A1-1	165,166
Thiotepa	A1-1, P1-1	167
Acrolein	A1-1, M	168
Carmustine (BCNU)	M3-3	169
Ethacrynic acid	P1-1	170
Adriamycin	P1-1	47
Etoposide	P1-1	47

BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea (Carmustine); MNNG, 1-methyl-2-nitro-1-nitrosoguanidine.

The discovery of the MRP transport family of proteins being responsible for the transport of GSH[30] and conjugative metabolites, like GSX,[40,59–62] led to the assumption that a coordinate interaction between MRP and GST co-expression might be necessary to achieve optimal MDR. The first evidence of this hypothesis was provided by the observations that MRP co-transports xenobiotics and GSH and that disruption of the MRP gene abrogates the co-transport of both exogenous compounds as well as GSH.[30] The fact that many cell lines that show a MRP drug resistance profile also have an increased GSTπ expression,[63,64] and that expression of the MRP1 gene is frequently co-induced together with γ-GCS,[65,66] also confirms the assumption that MRP forms an essential part of the thiol-based detoxification pathway.

In line with this hypothesis, an increase in cytosolic GST alone was shown not to be sufficient to confer resistance to several antineoplastic drugs, including drugs that are known to be substrates of GSTs.[67–70] This lack of potential of GSTs to induce optimal MDR is explained by the fact that hydrophilic cytotoxic GSX or their metabolites accumulate inside

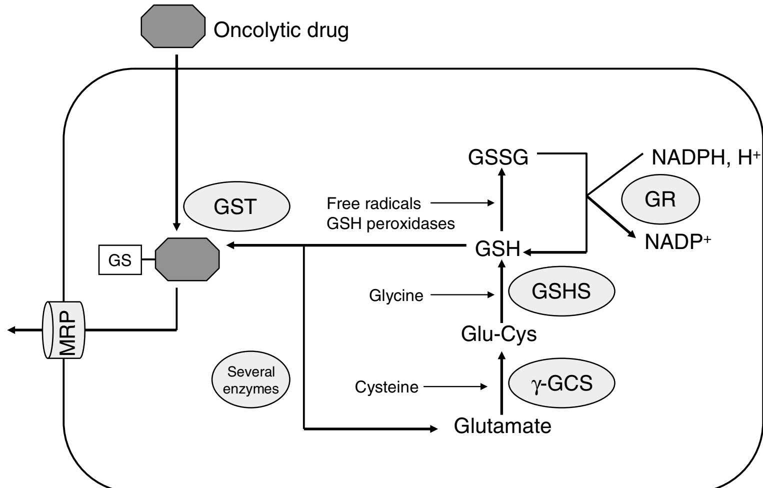


Figure 2 GSH metabolism and the conjugation of oncolytic drugs. Glutathione (GSH) is synthesized from the amino acids, glycine, cysteine and glutamate, for which the action of two enzymes is required: γ-glutamylcysteine synthase (γ-GCS) and GSH synthetase (GSHS). The reduction of GSH into glutathione disulfide (GSSG) is mediated by GSH peroxidases. This step is important for the protection of the cell against reactive oxygen species. The reduction of GSSG is catalyzed by GSH reductase (GR) in a process that requires NADPH. Glutathione *S*-transferases catalyse the conjugation of glutathione to electrophilic endogenous and exogenous compounds, among which are several oncolytic drugs. Multidrug resistance proteins (MRPs) are responsible for the transport of the conjugated metabolites out of the cell.[42,131,134]

Table 5 Experimental support of the involvement of glutathione metabolism and conjugation in multidrug resistance to oncolytic drugs

Mechanism	Model	Resistance to	Reference
Increased expression of			
GST	Lymphocytes of chronic lymphocytic leukaemia patients	Chlorambucil	154
GST	60 human cell lines of the NCI		64
γGCS	60 human cell lines of the NCI		64
γGCS	Cisplatin resistant human leukaemia HL-60 cells	Cisplatin	66
γGCS	13 human drug resistant cell lines		65
γGCS	Human colon tumour biopsy specimens		66
GST, γGCS	Several tumour types		3,171
Transfection with			
GST	COS-cells	Chlorambucil	46
GST-P1	Human colon cancer cell line M7609	Adriamycin, cisplatin, melphalan, etoposide	47
Inhibition of			
γGCS with BSO	Lung cancer cell lines	Doxorubicin	48,49
γGCS with BSO	Mice with human fibrosarcoma tumours	Doxorubicin	50
γGCS with BSO	KB cells	Vincristine	51
γGCS with BSO	Human embryonic kidney cells (HEK293)	Vincristine	52
Addition of			
N-acetylcysteine	Human embryonic kidney cells (HEK293)	Vincristine	52

the cell, and that other glutathione-dependent reactions can be directly or indirectly inhibited by increased intracellular GSX concentrations, thereby limiting the detoxification ability of the cell.[20,70] Furthermore, the conjugation reaction may be reversible and accumulated GSX in the cell can be regenerated into the cytotoxic drug[20] (Fig. 3). In addition, although most conjugation reactions lead to the detoxification of the parent drug or its metabolites, some glutathione conjugates have also been shown to be toxic.[71,72] It is therefore expected that transport of the conjugated metabolites out of the cell might contribute to multidrug resistance against GST substrates. Indeed, the results of several studies confirm that a combined expression of MRP and GST leads to a MDR that is greater than the MDR conferred by either protein alone. Priebe et al.[73] showed that the conjugation of the anthracyclines

Table 6 Oncolytic substrates of the phase II enzyme UDP-glucuronosyltransferase (UGT)

Sustrate	UGT (sub)-family	Reference
7-Ethyl-10-hydroxycamptothecin (SN38, metabolite of irinotecan)	1A1, 1A9, 1A10	57,172–174
Etoposide	1A1	175,176
Tamoxifen	1A4, 1A10, 2B7, 1A8	177,178
Thiocoraline	1A1, 1A9	179
NU/ICRF 505	1A9	57,172

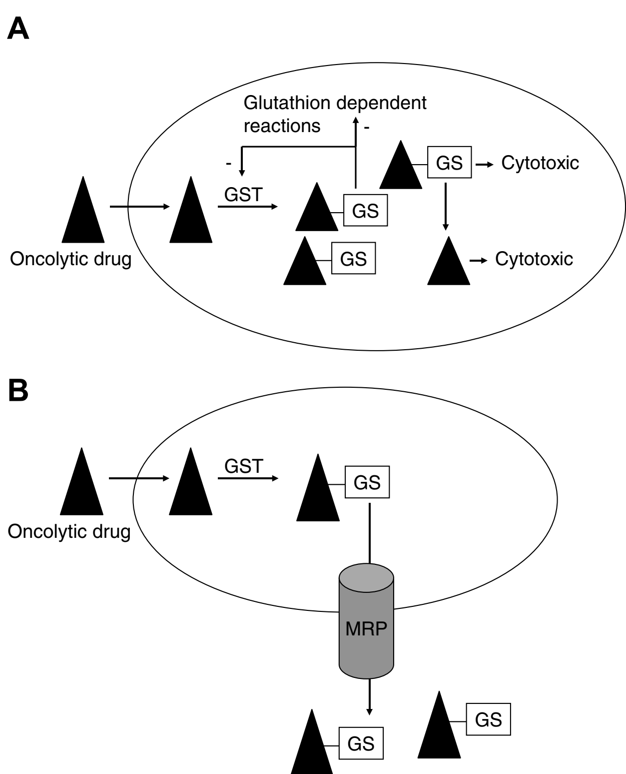


Figure 3 Hypothesis of the coordinate action between GST and MRP. Without the presence of MRP transporters, there is an accumulation of GSX or its metabolites in the cell. These might directly or indirectly inhibit other glutathione-dependent reactions thereby limiting the detoxification capacity of the cell. In addition, the conjugates might be regenerated into the cytotoxic drug, or the conjugates themselves might be cytotoxic (A).[20,70] Transport of conjugates out of the cell by MRP leads to multidrug resistance (B).

doxorubicin and daunomycin increased their elimination by MRP. A synergistic effect on MDR to the nitrogen mustard chlorambucil, was found in cells having a combined expression of MRP1 or MRP2 and GSTα,[70,74] while MRP1 or MRP2 activity was shown to be necessary for the full protection of cells against the toxic effects of the GSTπ substrate 4-nitroquinoline 1-oxide (NQO).[75–77] Coordinated expression of GSTπ and MRP1 also leads to higher levels of resistance to ethacrynic acid,[78,79] doxorubicin,[80] chlorambucil,[79] etoposide,[79] vincristine[79] and arsenic.[81] GSTμ was shown to act in synergy with MRP1 to provide MDR against vincristine,[82] while GSTα required MRP1 activity for optimal protection against chlorambucil.[83]

Evidence for a coordinate interaction between MRP and GSH comes from studies using BSO, which inhibits GSH synthesis, and *N*-acetylcysteine, a pro-gluthatione drug.

BSO decreases MRP-mediated multidrug resistance to several anticancer drugs, like vincristine, daunorubicin and doxorubicin,[50–52,84,85] while *N*-acetylcysteine enhances MDR.[52,86]

UGT and MRP transport

Anologous to GSX conjugates, the metabolites formed after glucuronidation (Glucuronide-X) are very hydrophilic and have to be excreted out of the cell by drug transporters, especially by MRP1–3[62,87] (Table 1). Both in vitro as well as in vivo transporters like MRP2 were shown to be involved in the excretion of the glucuronide of SN-38.[88–90] Using *Mrp2* and *Mrp3* knockout mice it was shown that the pharmacokinetics of morphine-3 glucuronide (M3G) is significantly changed; M3G is accumulated in the liver and bile, and reduced in the plasma.[91,92] These results clearly show an association between MRP and UGTs. There is however less evidence that there is indeed a synergistic effect of MRP and UGTs on MDR. Only Cummings et al.[93] showed that a coordinate expression of MRP and UGT is necessary to confer optimal MDR of colon cancer cells to NU/ICRF-505.

Co-regulation of expression of MRP, GST and UGT

Nrf2–ARE

As various studies have proven the concept that there is a coordinate action of phase II enzymes and MRP in MDR, the question is whether there is also a coordinate regulation of the expression of these proteins, as has been shown for CYP3A4 and Pgp.[23,27] The already mentioned connection between a MRP drug resistance profile and an increased GSTπ expression, shown in many cell lines, is indeed indicative for a shared regulatory mechanism of MRP and GST expression.[63,64] The frequently observed co-expression pattern between MRP1 and γ-GCS in MDR cell lines and in tumours also suggests a common underlying regulatory mechanism.[65,66] In addition, the expression of both MRP1 as well as γ-GCS has been shown to be co-induced by oxidative stress, some xenobiotics, but also anticancer agents like cisplatin and alkylating agents.[65,66,94] In rats exposed to 1,7-phenanthroline an induction of both UGT1A6 as well as MRP3 was observed.[95]

Thus far, several regulatory elements have already been identified that can control the expression and inducibility of GSTs, its related enzymes like γ-GCS, UGTs or MRPs (Fig. 4).

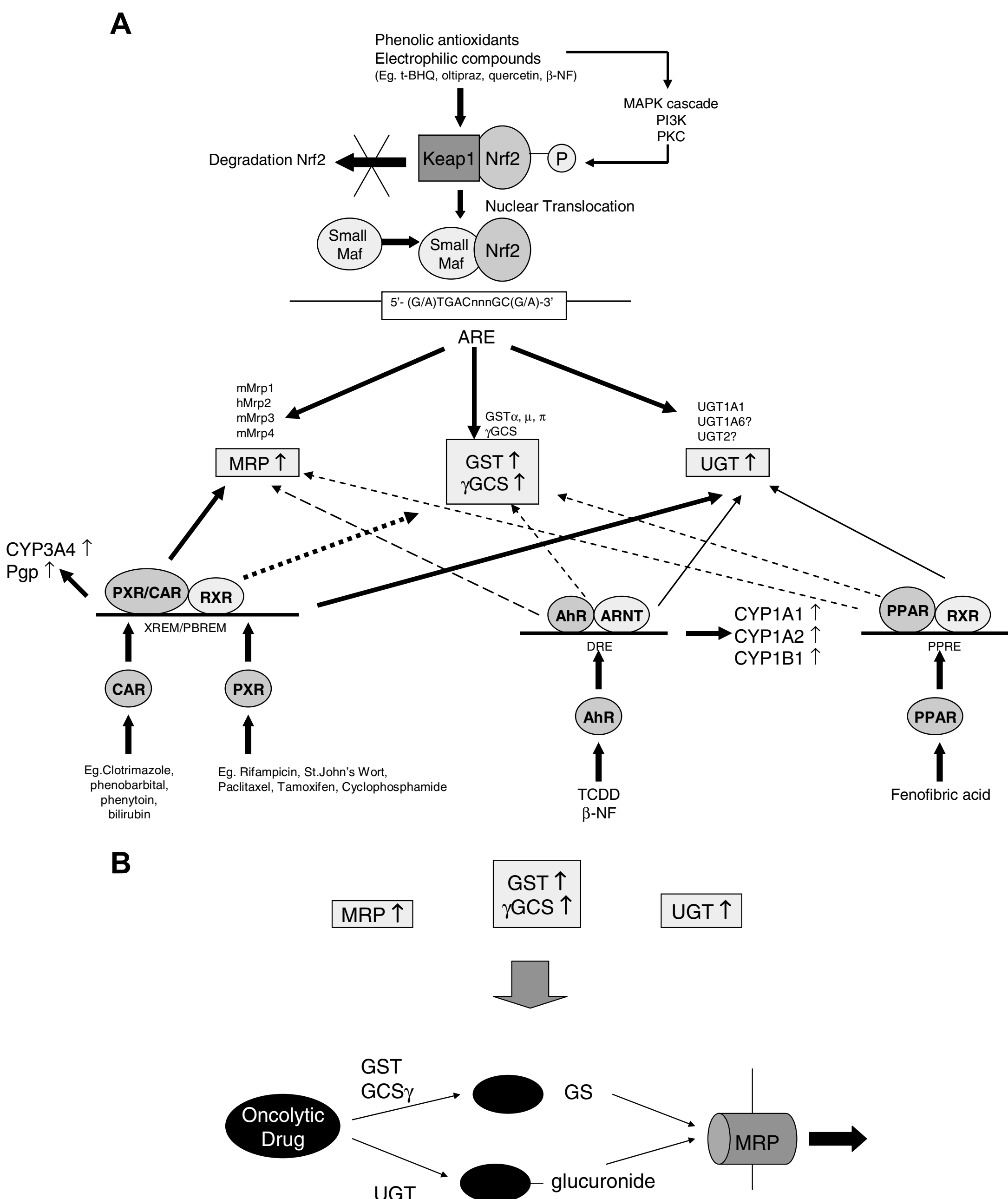


Figure 4 Co-regulation of expression of MRP, GST and UGT. (A) Normally Nrf2 is sequestered in the cytoplasm by Keap1, making Nrf2 sensitive for degradation. Upon exposure of cells to antioxidants or electrophilic compounds, binding between Nrf2 and Keap 1 is disrupted, Nrf2 is translocated to the nucleus, binds to the antioxidant response elements (ARE) as a heterodimer with small Maf proteins, and transcription of phase II enzymes and MRPs is activated. Protein kinase C (PKC) phosphorylates Nrf2 which can alter the binding of Nrf2 to Keap1. In addition, other transduction pathways such as the MAPK cascade and phosphatidylinositol 3-kinase (PI3K) affect the activation process of Nrf2.[45,107,135] Upon activation by several, structurally unrelated, ligands the nuclear receptors pregnane X receptor (PXR) and constitituve androstane receptor (CAR) bind to the xenobiotic response elements (XREM/PBREM) as a heterodimer with the retinoid X receptor (RXR). PXR was shown to be involved in the induction of MRPs and UGTs, but induction of GST is not proven yet (dotted arrows).[126–128,136] Ligand binding activates the nuclear translocation of the aryl hydrocarbon receptor (AhR), and heterodimerization with ARNT facilitates gene transcription after binding of the complex to dioxine response elements (DRE).[137] Just like PXR and CAR, the peroxisome proliferator-activated receptors (PPARs) form heterodimers with RXR. These complexes bind to the PPAR response elements (PPREs). Both AhR and PPARs have been shown to be involved in the induction of some UGT-enzymes. Evidence of their involvement in GST and MRP induction is limited.[126–128] (B). The induction of GSTs, UGTs and MRPs leads to the increased conjugation and efflux of oncolytic drugs, thereby making the cell multidrug resistant. (t-BHQ, — *tert*-butylhydroquinone; β-NF, β-naphtoflavone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin).

However, the most likely candidate for a concerted regulation of expression of GSTs, UGTs and MRPs are the antioxidant response elements (ARE; also referred to as electrophile response elements or EpREs), which have a common 5′-(G/A)TGACnnnGC(G/A)-3′ motif.[96] The ARE was first identified in the rat GST Ya gene,[97] and has now been detected in many genes of phase II enzymes, among which are genes coding for other GSTs,[98,99] γ-GCS[99,100] and UGT1A1.[101] Recently, ARE-like sequences were also identified in the mouse *Mrp2*,[102,103] *Mrp1*[103–105] and the *Mrp3* and *Mrp4* gene,[103] suggesting the possibility for a common regulatory mechanism for GSTs, γ-GCS and MRPs.

Itoh et al. (1997) identified the main transcription factor that mediates ARE-driven transcription to be nuclear factor-erythroid 2 p45-related factor 2 (Nrf2).[106]

Under basal conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1), thereby targeting Nrf2 for ubiquitination and proteasome degradation. Therefore, normally, Nrf2 has a short half-life. Upon exposure of cells to inducers like oxidants and electrophiles, Keap1 binding and repression of Nrf2 is disrupted, resulting in translocation of Nrf2 to the nucleus. In the nucleus, Nrf2 forms a heterodimer with small Maf proteins and, via interaction with the ARE, transcription of phase II enzymes is activated (reviewed by [45,107]) (Fig. 4). Various signal transduction pathways have been shown to be involved in the Nrf2 activation, like the protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K) and MAPK pathway.

The evidence for the role of Nrf2–ARE in the expression of phase II enzymes and MRPs was mainly provided in studies using *Nrf2* knockout mice. Disruption of the *Nrf2* gene decreased both the constitutive as well as the inducible expression of class Alpha, class Mu and class Pi glutathione transferases,[106,108–113] γ-GCS [111,114–116] and UGT.[113,115] Nrf2 was also shown to be necessary for the constitutive and inducible expression of MRP1 in mouse embryo fibroblast.[104]

The central role of Nrf2 is also confirmed by the fact that both phase II enzymes as well as MRPs can be induced by currently identified inducers of ARE-driven transcription by Nrf2, like: β-naphtoflavone (β-NF), menadione, butylated hydroxyanisole (BHA), methyl mercury hydroxide, *tert*-butylhydroquinone (t-BHQ), acetaminophen, oltipraz and pyrrolidine dithiocarbamate,[101,110,112] but also by several antioxidants from the diet like broccoli seeds and isothiocyanates such as sulforaphane,[117,118] organosulfur compounds,[119] phenolic antioxidants like green tea phenols,[120] and flavonoids like quercetin [121] and curcumin.[122] Although the presence and functionality of ARE regulatory elements in most UGT enzymes, with the exception of UGT1A1, have not been confirmed yet, some UGTs, like UGT1A6 and UGT2, are induced by these inducers, thereby suggesting a role for ARE–Nrf mediated transcriptional regulation of UGT.[118,123]

Only recently, Vollrath et al.[102] showed a Nrf2 mediated co-regulation of MRP2, GST and γ-GCS. After identifying the ARE-sequence in the *Mrp2* gene they showed that the *Mrp2* promotor activity was significantly enhanced by overexpression of Nrf2 and the activators of the ARE–Nrf pathway; BHA and β-NF. Furthermore, after exposure to several known Nrf2 activators, a co-induction pattern was observed for MRP2, GSTα and γ-GCS in mouse and human hepatoma cell lines, suggesting a common mechanism of transcriptional activation.[102] The same coordinate induction of MRP2 and γ-GCS was observed when the cells were exposed to the synthetic antioxidant bucillamine or its oxidized metabolite SA 981.[124] The coordinate induction of UGT1A6 and MRP2 in Caco-2 cells after exposure to t-BHQ and quercetin also suggests a common Nrf–ARE mediated mechanism.[125]

Thus, consistent with the observation that redox-active compounds can induce not only GSTs, UGTs and related enzymes, but also MRPs, the published data confirm a Nrf2–ARE mediated synergistic action of phase II enzymes and MRPs during oxidative stress caused by oncolytic drugs (Fig. 4).

Other nuclear receptors

Several other transcription factors and their accompanying response elements have been identified to play a role in the transcriptional regulation of phase II enzymes and MRPs.

Nuclear receptors that have shown to be involved in the induction of MRPs are CAR, PXR, the peroxisome proliferator-activated receptor (PPARα) and the aryl hydrocarbon receptor (Ahr) (reviewed by[126,127]). All of these receptors were also reported to be involved in the regulation of the transcription of UGTs (reviewed by[128]), however, as far as we know, there are currently no data on a coordinate induction of UGTs and MRPs involving these receptors. Transcriptional regulation of GSTs can also be mediated by Ahr and PXR, although in the case of GSTs Nrf2 seems to be the main receptor involved.[126] However, the strong PXR agonist St. John's Wort did induce both MRP2 and GSTπ in the liver of rats.[129]

A cross-talk between the different routes and nuclear receptors is also possible. Studies of the *Nrf2* promotor suggest that Nrf2 is a target gene of AhR, and Köhle and Bock[130] propose a model in which there is a tight coupling between Ahr and Nrf2 mediated induction of phase I and phase II enzymes.

Although there is substantial evidence of a coordinate regulation of the expression of phase II enzymes and MRPs, much remains to be clarified, like the exact molecular mechanism involved, the role of other nuclear receptors and possible cross-talk.

Discussion

Multidrug resistance to chemotherapeutic drugs is a main problem in oncology. In the past, research has mainly focused on the role of Pgp, however, it is increasingly recognized that the disposition of oncolytic drugs is also influenced by the more recently discovered MRPs. In addition, evidence is accumulating that phase II enzymes play an important role in MDR by mediating different cellular processes like conjugation of electrophilic drugs or their metabolites, activation of drug transport, modulating the redox status of cells, and via the regulation of cell signaling and repair mechanisms.[131] A synergistic interaction between these two routes of MDR, MRP and phase II enzymes is observed, which is most likely regulated by a common molecular mechanism: the Nrf2–ARE pathway. However, thus far most studies have been performed employing

animal models, and the role of the Nrf2—ARE pathway in the transcriptional regulation of human phase II enzymes and MRPs remains to be defined. Furthermore, there is a need for more studies investigating other possible regulatory mechanisms. In this review only the phase II enzymes GST and UGT were included, and not sulfotransferases (SULT), as limited information is available on the involvement of these enzymes in MDR and their transcriptional regulation. However, the substrate specificity of SULTs and UGTs largely overlap, thus also SULTs are expected to play a role in the MDR of selected chemotherapeutic drugs. In addition, other mechanisms of MDR might also be regulated by a common mechanism, as it was already shown that there is an induction of the DNA repair enzyme ERCC-1 in response to cisplatin. The transcriptional activator responsible for this elevated expression has thus far not been identified yet.[13] All together, obtaining more information about the combined action and regulation of phase II enzymes, MRPs and other mechanism of drug resistance is necessary and will be an aid in developing new methods to limit MDR and to improve the chemotherapeutic treatment of cancer patients.

At the moment, physicians should be aware that the Nrf2—ARE pathway is stimulated by anti-oxidants compounds found in food, herbs and nutritional supplements. As the use of complementary alternative medicines like herbs and vitamins among cancer patients is increasing,[132] this might lead to herb-drug interactions that stimulate MDR to chemotherapeutic drugs. In addition, in an ARE reporter gene assay, selected anticancer drugs themselves have been shown to be weak activators of Nrf2[133] which could induce MDR against other co-administered drugs or against the drug itself.

Further investigation into the mechanism of co-regulation of MRPs and drug conjugating enzymes and the potential clinical implications in oncology is therefore warranted.

Conflict of interest statement

All authors hereby declare that there are no financial and personal relationships with other people or organizations that could inappropriately influence their work.

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JOURNAL OF INTERFERON & CYTOKINE RESEARCH
Volume 00, Number 00, 2015
DOI: 10.1089/jir.2014.0210

FULL MANUSCRIPT

Liposomal Glutathione Supplementation Restores T_H1 Cytokine Response to Mycobacterium tuberculosis Infection in HIV-Infected Individuals

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Cytokines are signaling biomolecules that serve as key regulators of our immune system. $CD4^+$ T-cells can be grouped into 2 major categories based on their cytokine profile: T-helper 1 (T_H1) subset and T-helper 2 (T_H2) subset. Protective immunity against HIV infection requires T_H1-directed CD4 T-cell responses, mediated by cytokines, such as interleukin-1b (IL-1b), IL-12, interferon-g (IFN-g), and tumor necrosis factor-a (TNF-a). Cytokines released by the T_H1 subset of CD4 T-cells are considered important for mediating effective immune responses against intracellular pathogens such as Mycobacterium tuberculosis (M. tb). Oxidative stress and redox imbalance that occur during HIV infection often lead to inappropriate immune responses. Glutathione (GSH) is an antioxidant present in nearly all cells and is recognized for its function in maintaining redox homeostasis. Our laboratory previously reported that individuals with HIV infection have lower levels of GSH. In this study, we report a link between lower levels of GSH and dysregulation of T_H1- and T_H2-associated cytokines in the plasma samples of HIV-positive subjects. Furthermore, we demonstrate that supplementing individuals with HIV infection for 13 weeks with liposomal GSH (lGSH) resulted in a significant increase in the levels of T_H1 cytokines, IL-1b, IL-12, IFN-g, and TNF-a. lGSH supplementation in individuals with HIV infection also resulted in a substantial decrease in the levels of free radicals and immunosuppressive cytokines, IL-10 and TGF-b, relative to those in a placebo-controlled cohort. Finally, we determined the effects of lGSH supplementation in improving the functions of immune cells to control M. tb infection by conducting in vitro assays using peripheral blood mononuclear cells collected from HIV-positive individuals at post-GSH supplementation. Our studies establish a correlation between low levels of GSH and increased susceptibility to M. tb infection through T_H2-directed response, which may be relieved with lGSH supplementation enhancing the T_H1 response.

Introduction

According to the World Health Organization (WHO) more than 36 million people have died so far due to human immunodeficiency virus (HIV)-related illnesses and an estimated 35.3 million people are currently living with HIV infection (Piot and others 2001; WHO 2014). CD4 T helper (T_H1) lymphocytes are crucial regulators of both cell-mediated immune responses against microbial infection and their loss during advanced stages of HIV infection will lead to progression to AIDS (acquired immunodeficiency syndrome) (Ray and others 2006). CD4 T-cells direct the immune system in 2 major pathways: cytokines produced in T_H1 pathway favor a cascade of immune responses suited for controlling intracellular infections, or the opposing T_H2 pathway, which ensures a humoral-mediated defense against extracellular pathogens (Romagnani 2000).

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Our laboratory has previously reported that HIV-positive individuals have significantly lower levels of T_H1 cytokines, interleukin-1b (IL-1b), IL-12, interferon-g (IFN-g), and tumor necrosis factor-a (TNF-a), combined with elevated levels of immunosuppressive T_H2-associated cytokines, such as IL-10, in the plasma (Guerra and others 2011; Vera Tudela and others 2014). While the mechanisms of HIV-rooted immunosuppression are wide and complex, our laboratory has demonstrated an important relationship between HIV disease progression and a physiologically vital biomolecule, glutathione (GSH) (Morris and others 2014).

GSH, an antioxidant present in all eukaryotic cells, is a tripeptide composed of the amino acids glutamine, cysteine, and glycine. GSH plays a role in many aspects of cell physiology and is essential in maintaining redox homeostasis in the cells (Herzenberg and others 1997; Lushchak 2012). We previously reported that the levels of GSH were significantly diminished in individuals with HIV infection due to decreased levels of GSH de novo synthesis enzymes and due to excessive oxidative stress (Morris and others 2014). We also demonstrated that decreased levels of GSH in individuals with HIV infection was accompanied by impaired innate and adaptive immune responses against Mycobacterium tuberculosis (M. tb) infection (Guerra and others 2011, 2012; Morris and others 2012; Morris and others 2013a, 2013b, 2013c, 2013d, and 2014). Because oxidative stress has a profound effect on altering the viability of CD4 T-cells, we believe that depletion of GSH will increase the risk for opportunistic infections.

In this study we propose that the circulating plasma levels of cytokines produced by T_H1 subset of CD4 T-cells, such as IL-12, IL-2, and IFN-g, would be significantly lowered in individuals with HIV infection, and the plasma levels of cytokines known to induce oxidative stress (IL-6) and suppress the immune system (IL-10 and TGF-b), would be significantly elevated. We hypothesize that an increase in the intracellular GSH through oral liposomal GSH (lGSH) supplementation for 13 weeks will increase the production of cytokines, such as IL-12, IL-2, IL-1b, and IFN-g, allowing individuals with HIV infection to control opportunistic infections resulting from a weakened immune state. We also propose a decrease in the levels of IL-10, TGF-b, and IL-6 in individuals with HIV infection upon completion of a 13-week lGSH supplementation regimen. Reinforcing our hypothesis, we expect decreased oxidative stress and improved control of M. tb infection at the completion of the study.

Our results indicate that restoring the levels of GSH in individuals with HIV infection alleviated oxidative stress, increased the production of IL-1b, IL-12, and IFN-g, decreased the levels of IL-10, TGF-b, and IL-6, and favored successful control of M. tb infection.

Materials and Methods

Study participants

The Western University of Health Sciences Institutional Review Board approved the study. Statistically relevant sample size was determined by conducting a power analysis. The sample size consisted of individuals between the ages of 21 and 65 years. Inclusion criteria required that all participants were able to read and write English and were diagnosed with HIV before 2012. The exclusion criteria ruled out individuals with allergies, chronic disease, hepatitis, and those who were pregnant. It also excluded individuals that were classified as part of a vulnerable population. A total of 25 individuals were recruited to participate in this study without gender preference. A total of 10 out of 25 individuals were HIV negative and 15 were positive for HIV infection. E-mail and flyers were used to recruit the volunteers for this study from Pomona, California, and nearby areas. The purpose of the study and what was required from each participant was explained to each individual before obtaining their signed consent. Background information for the HIV-positive individuals, including duration of HIV infection and form of antiretroviral treatment, if any, was obtained. HIV-positive individuals were randomly divided into 2 groups, with 1 group receiving GSH supplements formulated in liposomes (lGSH) and the second group receiving empty liposomes as a placebo.

Study overview

Approximately 30 mL of blood was drawn from all of the participants using a Vacutainer Safety Lok Blood Collection Kit (364606; BD Biosciences). After the first blood draw, subjects were given lGSH or placebo supplements and instructed to take one and a half teaspoons of formula twice a day, once in the morning and once in the evening, for 7 weeks. Three teaspoons of lGSH & 15 mL (about 1,260 mg reduced GSH).

At the 7-week time point, participants were asked to come back for a second visit for more supplements. All participants were instructed to continue taking supplements to the completion of 13 weeks and asked to come back for a third and final visit for a final blood draw.

Separation of blood components from whole blood

Density gradient centrifugation using Ficoll-Paque PLUS (10040757; GE Healthcare) was used to separate plasma, red blood cells (RBCs), and peripheral blood mononuclear cells (PBMCs) from whole blood. Plasma and RBCs were aliquoted into separate vials and stored at - 80 for further analysis. PBMCs were washed 3· with 1· phosphate-buffered saline (PBS) and were resuspended in RPMI media composed of RPMI, l -glutamine, HEPES (1-041-CM; Corning Cellgro), and 5% human AB serum. PBMC counts were determined using a hemocytometer. Two hundred microliters of PBMCs corresponding to 10^5 cells were plated on a tissue culture plate precoated with 0.005% poly-l -lysine, and incubated overnight at 37 C to allow monocyte adherence.

Plasma cytokine measurement

To establish a baseline value for the levels of cytokines in healthy and HIV group, cytokine levels were measured in plasma samples from healthy subjects and individuals with HIV infection immediately after recruitment before any intervention. Cytokines were also measured in plasma samples collected from HIV-positive subjects at 13 weeks post-lGSH (or placebo) supplementation to determine whether lGSH supplementation result in any significant changes in the levels of cytokines. Cytokine levels were measured using enzyme-linked immunosorbent assay (ELISA). The cytokines that were measured in the plasma samples isolated from the participants belonging to the 2 study groups include: IFN-g, IL-1b, IL-2, IL-6, IL-10, IL-

12, TGF-b, and TNF-a (IFN-g, Cat. No. 88-7316; IL-1b, Cat. No. 88-7010; IL-2, Cat. No. 88-7025; IL-6, Cat. No. 88-7066; IL-10, Cat. No. 88-7106; IL-12, Cat. No. 88-7126; TGF-b, Cat. No. 88-8350; and TNF-a, Cat. No. 88-7346; eBioscience ELISA Ready-SET-Go!). The cytokine levels were measured following the manufacturer's protocol.

GSH measurements

Baseline levels of total GSH in healthy and HIV group were measured in PBMCs immediately after recruitment before any intervention. GSH levels were also measured in PBMCs isolated from individuals with HIV infection at baseline and 13 weeks post-lGSH supplementation. A GSH Detection Kit from Arbor Assays (K006-H1) was used to measure concentrations of total GSH. The GSH measurements were done following the manufacturer's protocol. Results were corrected for protein levels and were reported in mM GSH/mg protein.

Reactive oxygen species measurements

Quantifying reactive oxygen species (ROS) levels in plasma and cell lysates of RBCs and monocytes, derived from individuals with HIV infection at pre and post intervention:

ROS production was determined by 2 methods: (1) measurement of malondialdehyde (MDA) (end product of lipid peroxidation, an indirect measure of ROS production) in plasma samples and lysates of RBCs and monocytes (2) CellROX staining of monocytes, CD4, and CD8 T-cells, followed by quantification of fluorescence by flow cytometry (FACS).

MDA measurements

MDA is a byproduct of lipid peroxidation. Once MDA forms an adduct with thiobarbituric acid (TBARS) at 90 C–100 C, a color change occurs which can be measured colorimetrically at 530–540 nm. Baseline levels of MDA in healthy and HIV groups were measured in plasma samples and lysates of RBCs and monocytes, immediately after recruitment before any intervention. MDA levels were also measured in HIV-positive subjects at 13 weeks post-lGSH intervention in plasma samples and lysates of RBCs and monocytes. MDA levels were measured using a TBARS Assay Kit (10009055; Cayman Chemical). The assay procedure included with the kit was followed to obtain MDA sample concentrations. Results were corrected for protein levels and were reported in mM MDA/mg protein.

ROX staining and flow cytometry analysis

The cell-permeant CellROX green reagent (C10444; Life Technologies) is nonfluorescent in a reduced state and produces bright near-infrared fluorescence upon oxidation. The resulting fluorescence can be measured using flow cytometry. In addition to allowing ROS detection in live cells, the signal is retained after formaldehyde fixation. PBMCs isolated from the blood of healthy and HIV group after recruitment before any intervention, and from HIV-positive individuals after lGSH intervention, were treated with 5 mM CellROX green reagent and incubated at room temperature for 30 min in the dark. Stained PBMCs were centrifuged at 800g for 5 min and resuspended in 100 mL PBS. Antibodies conjugated to the fluorescent markers such as CD14-PE (12-0149; eBioscience), CD4-Cy5 (15-0049; eBioscience), and CD8-Cy5 (15-0088; eBioscience) were added to the appropriate tubes containing PBMCs and incubated in the dark at 4 C for 30 min. Cell suspension was centrifuged 3· at 800g for 5 min to remove excess staining and then suspended in 1 mL 1· cold PBS. The fluorescence was quantified by flow cytometry. Results were analyzed using FlowJo software version 7.6.5.

PBMC infection studies

PBMCs isolated from blood drawn from individuals with HIV infection at 13 weeks post supplementation were diluted as follows to yield 10^5 cells/mL: 200 mL of PBMCs was diluted to a total volume of 1 mL by adding 800 mL RPMI media. PBMCs were infected with H37Rv with multiplicity of infection of 10:1 and incubated for 1 h for the uptake of bacteria by the phagocytic cells. Extracellular bacteria were removed by washing the PBMCs 3· with RPMI. During each washing step, PBMCs were centrifuged at 1,800 rpm for 10 min, cell-free supernatants were discarded and the pellet was resuspended in RPMI. After the final wash, infected PBMCs were resuspended in fresh media and then distributed in tissue culture plates. The infected PBMCs were terminated at 1 and 72 h postinfection. Infected PBMCs were lysed by adding 150 mL of cold sterile water. Lysates were diluted and plated on 7H11 containing glycerol and albumin dextrose complex for mycobacterial colonies.

Statistical analysis

Statistical data analysis was performed using GraphPad Prism Software version 6. Levels of cytokines, MDA, GSH, and ROX were compared between 8 HIV-positive individuals in the lGSH supplementation group and 7 HIV-positive individuals in the placebo group after 13 weeks of supplementation using the unpaired t-test with Welch correction. Baseline levels of cytokines, MDA, GSH, and ROX were also compared between all 15 HIV-positive individuals and 10 healthy subjects using unpaired t-test with Welch correction. Reported values are in means. Statistical data analysis of CellROX staining and flow cytometry was completed using the non-parametric version of the unpaired t-test, Mann–Whitney test. Reported values are in medians.

Results

Baseline CD4 T-cell counts in healthy subjects and individuals with HIV infection

Seven out of the total 15 participants who were HIV-positive had CD4 T-cells that were below the minimum normal value of 500 cells/mm^3. Eight out of the total 15 participants who were HIV-positive had normal CD4 T-cell counts of 500 cells/mm^3 and above. Participants who were not positive for HIV and were classified as healthy had CD4 T-cell counts more than 500 cells/mm^3.

Baseline levels of TGF-b and GSH in healthy subjects and individuals with HIV infection

We observed a significant increase in the levels of TGF-b in individuals with HIV infection. TGF-b levels in HIV-positive individuals were 2· greater than their healthy counterpart (Table 3). The baseline levels of total GSH in PBMCs

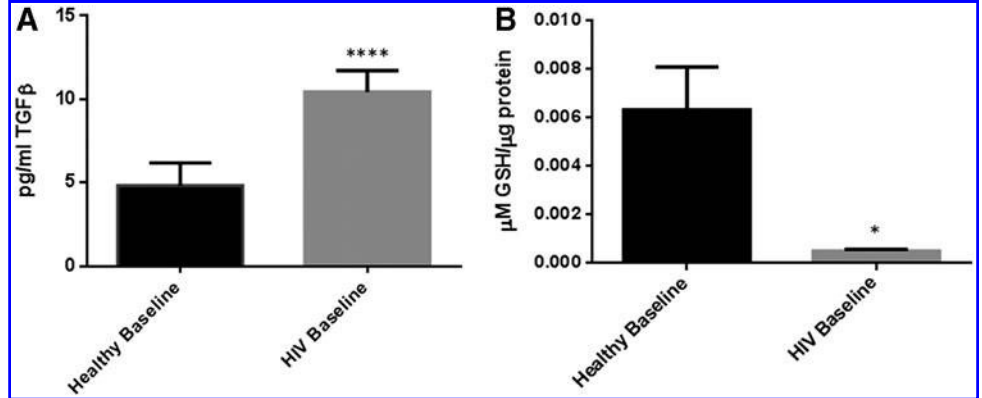

FIG. 1. Baseline comparison of TGF-b and total glutathione (GSH) levels between healthy subjects and individuals with HIV infection. There was a significant increase in the levels of TGF-b in plasma samples collected from individuals with HIV infection compared to healthy individuals (A). Data represent mean – SE from comparing baseline levels of 10 healthy volunteers and 15 HIV-positive individuals, $^{*}P < 0.05$. Assay of total GSH showed a significant decrease in the levels of total GSH in peripheral blood mononuclear cell (PBMCs) isolated from HIV-positive individuals compared to healthy subjects (B). Data represent mean – SE from comparing baseline levels of 10 healthy volunteers and 15 HIV-positive individuals, $^{*}P < 0.05$.

isolated from HIV-positive individuals were significantly lower compared to healthy volunteers (Fig. 1A). In comparison to healthy volunteers, there was a 12-fold decrease in the levels of total GSH in PBMCs isolated from HIV-positive individuals (Fig. 1B and Table 1).

Baseline levels of IL-6 and free radicals in healthy subjects and individuals with HIV infection

The proinflammatory cytokine IL-6 was significantly increased by 16-fold in plasma samples of individuals with HIV infection compared to healthy volunteers (Fig. 2A and Table 1). In line with the increased levels of IL-6, there was a significant increase in the levels of MDA in plasma, RBCs, and monocytes isolated from individuals with HIV infection compared to the healthy volunteers (Fig. 2B–D). We observed a 2-fold increase in the levels of MDA in plasma and monocytes of individuals with HIV infection compared to healthy volunteers (Fig. 2B–C). Furthermore, we observed a 9-fold increase in the levels of MDA in RBCs isolated from individuals with HIV infection compared to healthy volunteers (Fig. 2D). There was also a significant increase in ROS production in $CD14^+$ cells, $CD4^+$, and $CD8^+$ T-cells isolated from individuals with HIV infection as indicated by the intensity of ROX staining (Fig. 2E–G). There was a 5-fold increase in the ROX intensity in $CD14^+$ cells isolated from HIV-positive individuals compared to healthy volunteers (Fig. 2E and Table 1). We also observed a 2- and 7-fold increase in the ROX intensity in $CD4^+$ T-cells (Fig. 2F and Table 1) and $CD8^+$ T-cells (Fig. 2G and Table 1) respectively, that were isolated from individuals with HIV infection compared to healthy volunteers.

Baseline levels of IL-12, IL-2, and IFN-c in healthy subjects and individuals with HIV infection

The levels of T_H1 cytokines such as IL-12, IL-2, and IFN-g were significantly diminished in plasma samples isolated from individuals with HIV infection compared to healthy volunteers (Fig. 2A–C). A significant 8-fold decrease in the levels of IL-12 was observed in plasma samples isolated from individuals with HIV compared to healthy volunteers (Fig. 3A and Table 2). We also observed a 2-fold decrease in the levels of IL-2 in plasma samples isolated from HIV-positive individuals compared to healthy volunteers (Fig. 3B). Finally, we observed a 3-fold decrease in the levels of IFN-g in plasma samples isolated from HIV-positive individuals compared to healthy volunteers (Fig. 3C and Table 2).

Table 1. Changes in the Oxidative Stress Markers Before and After lGSH Supplementation

Redox changes in the immune cells	Function	Changes in the levels of oxidative stress markers in individuals with HIV infection compared to healthy subjects	Changes in the levels of oxidative stress markers in individuals with HIV infection at 3 months post-supplementation with lGSH
IL-6	Induces oxidative stress and inflammation	16· increase	2· decrease
Oxidative stress	Inflammation and cell death	5· increase in monocytes 2· increase in CD4 T cells 7· increase in CD8 T cells	6· decrease in monocytes 4· decrease in CD4 T cells 8· decrease in CD8 T cells
GSH	Antioxidant; has antimycobacterial and immune enhancing effects	12· decrease in PBMC	2· increase in PBMC

GSH, glutathione; IL-6, interleukin-6; lGSH, liposomal GSH; PBMC, peripheral blood mononuclear cell.

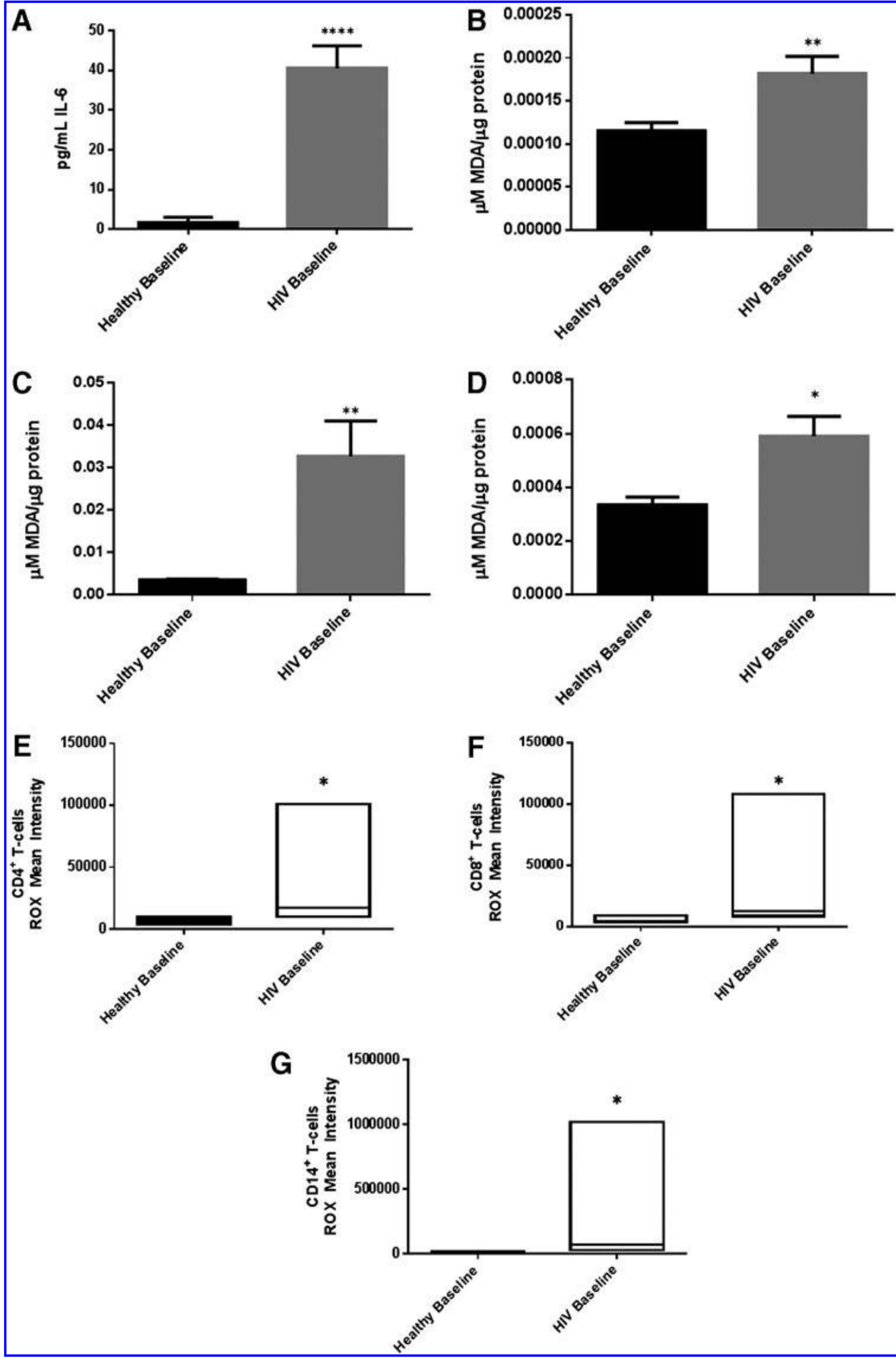

FIG. 2. Baseline comparison of the interleukin-6 (IL-6) and reactive oxygen species (ROS) markers between healthy volunteers and HIV-positive individuals. We observed a significant increase in the levels of the proinflammatory cytokine, IL-6 in plasma samples collected from individuals with HIV infection compared to healthy individuals (A). Data represent mean – SE from comparing baseline levels of 10 healthy volunteers and 15 HIV-positive individuals, $****P < 0.00005$. Malondialdehyde (MDA), a marker of lipid peroxidation and oxidative stress was significantly increased in plasma (B), red blood cells (RBCs), $**P < 0.005$ (C), and monocytes $**P < 0.005$ (D) from HIV-positive individuals compared to healthy subjects. Data represent mean – SE from comparing baseline levels of 10 healthy volunteers and 15 HIV-positive individuals, $*P < 0.05$. ROS production was also quantified by CellROX staining. CellROX green dye is nonfluorescent in its reduced state and produces bright near-infrared fluorescence upon oxidation. The resulting fluorescence can be measured using flow cytometry. Flow cytometry analysis indicates a significant increase in ROX mean intensity in $CD4^+$ T-cells (E), $CD8^+$ T-cells (F), and $CD14^+$ cells/monocytes (G) from HIV-positive individuals compared to healthy subjects. Data represent medians with range, $*P < 0.05$ when comparing baseline levels of healthy volunteers with HIV-positive individuals using the nonparametric version of the t-test, Mann–Whitney test.

Baseline levels of IL-1, TNF-a, IL-17, and IL-10 in healthy subjects and individuals with HIV infection

The levels of IL-1b, TNF-a, and IL-17 were also significantly diminished in plasma samples isolated from individuals with HIV infection compared to healthy volunteers (Fig. 4A–D). We detected a 12-fold decrease in IL-1b levels and 3-fold decrease in the levels of IL-17 in plasma samples isolated from individuals with HIV compared to healthy volunteers (Fig. 4A–B). We also saw an 11-fold decrease in the levels of TNF-a in plasma samples isolated from individuals with HIV infection compared to healthy volunteers (Fig. 4C and Table 2). IL-10 levels were 6· higher in plasma samples of individuals with HIV compared to the healthy volunteers (Fig. 4D and Table 2).

Supplementation of HIV-positive individuals with either lGSH or empty liposomes (placebo)

A total of 15 individuals with HIV infection were recruited for this study without gender preference. The HIV group was further categorized into the placebo group and the

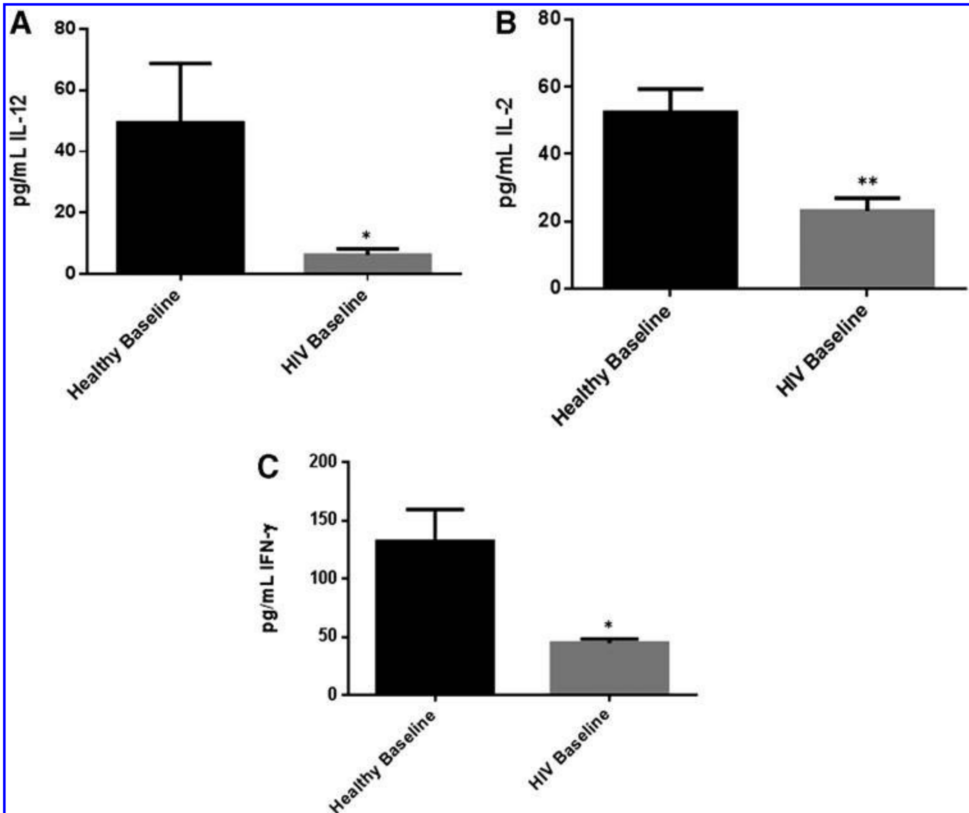

FIG. 3. Baseline comparison of T_H1 cytokine (IL-12, IL-2, and interferon-g [IFN-g]) levels between healthy volunteers and HIV-positive individuals. Assay of T_H1 cytokines showed significant decrease in the levels of IL-12 (A), IL-2 (B), and IFN-g (C) in plasma samples collected from individuals with HIV infection compared to healthy individuals. Data represent mean – SE from comparing baseline levels of 10 healthy volunteers and 15 HIV-positive individuals, $^*P < 0.05$, $^{**}P < 0.005$.

lGSH treatment group. A total of 7 individuals with HIV infection were included in the placebo group and a total of 8 individuals with HIV infection were recruited to the lGSH treatment group. The supplementation regimen lasted for 13 weeks. Volunteers were asked to visit the clinic 3· during the study. During the first visit (V1), blood was drawn from the participants for clinical and research laboratory tests (to measure the baseline levels of cytokines, free radicals, and GSH), and to receive supplements sufficient to last for 7 weeks. The second visit (V2) was on the seventh week of the study and during this visit, participants had clinical encounters and received additional supplements to last for another 6 weeks (till the end of the study). During the third visit (V3 on week 13 of the study), blood was drawn from the participants for both research and clinical laboratory tests. Before conducting the trial, baseline levels of cytokines, free radicals, and GSH were first compared between the placebo group and lGSH treatment group using an unpaired t-test with Welch correction to demonstrate that there was no significant difference between the 2 groups during visit 1. None of the participants developed any adverse reactions to either placebo or lGSH.

Changes in the levels of TGF-b and GSH at 13 weeks post supplementation with lGSH

lGSH supplementation in HIV-positive individuals for 13 weeks resulted in a 3-fold decrease in the levels of TGF-b (Fig. 5A and Table 3) along with a 2-fold increase in the levels of total GSH (Fig. 5B and Table 1). Supplementation

Table 2. Changes in the Levels of Immune-Stimulating Cytokines Before and After lGSH Supplementation

Cytokine	Function	Changes in the levels of cytokines in individuals with HIV infection compared to healthy subjects	Changes in the levels of cytokines in individuals with HIV infection at 3 months post-supplementation with lGSH
IL-12	Polarizes CD4 T-cells toward T_H1 response	8· decrease	3· increase
IFN-g	Enhances macrophage control of mycobacterial infection	3· decrease	2· increase
IL-1b	Facilitates lymphocyte-directed immunity	12· decrease	10· increase
TNF-a	Induces granuloma formation and enhances the intracellular effector mechanisms	11· decrease	2.5· increase

IFN-g, interferon-g; TNF-a, tumor necrosis factor-a.

Table 3. Changes in the Levels of Immunosuppressive Cytokines Before and After lGSH Supplementation

Cytokine	Function	Fold increase in the levels of cytokines in individuals with HIV infection compared to healthy subjects	Changes in the levels of cytokines in individuals with HIV infection at 3 months post-supplementation with lGSH
TGF-b	Limits T-cell clonal expansion; downregulates glutamine cysteine ligase catalytic subunit	2· increase	3· decrease
IL-10	Lowers T_{H1} responses; suppresses effector mechanisms in macrophages	6· increase	6· decrease

with empty liposomes for 13 weeks showed no significant difference in the levels of TGF-b and total GSH between visit 1 and visit 3 in the placebo group (Fig. 5).

Changes in the levels of IL-6 and free radicals at 13 weeks post supplementation with lGSH

There was no significant difference in the levels of IL-6, MDA, and ROX between visit 1 and visit 3 of the placebo group (Fig. 6B, D, F, H, J, L). Interestingly, treatment with lGSH for 13 weeks significantly decreased the levels of IL-6 as observed by a 2-fold decrease in the levels of this cytokine from visit 1 to visit 3 (Fig. 6A and Table 1). The decreased levels of IL-6 in the lGSH treatment group was accompanied by a significant decrease in the levels of MDA in the plasma (Fig. 6C), RBCs (Fig. 6E), and monocytes (Fig. 6G) as evident by a 2-fold decrease in the levels of MDA in plasma, RBC, and monocyte samples between visit 1 and visit 3 (Fig. 6C, E, G). Furthermore, there was a 4-fold decrease in the intensity of ROX staining in $CD4^+$ T-cells (Fig. 6I and Table 1), 8-fold decrease in the $CD8^+$ T-cells (Fig. 6K and Table 1), and 6-fold decrease in the ROX staining in $CD14^+$ cells from the lGSH treatment group at 13 weeks post-lGSH supplementation (Fig. 6M and Table 1).

Changes in the levels of IL-12, IL-2, and IFN-c at 13 weeks post supplementation with lGSH

There was a significant increase in the levels IL-12 and IFN-g in HIV-positive individuals at 13 weeks post supplementation with lGSH (Fig. 7A–C and Table 2). We observed a 3-fold increase in the levels of IL-12 (Fig. 7A and Table 2) and approximately a 2-fold increase in the levels of IFN-g (Fig. 7B and Table 2). We also observed a tremendous 10-fold increase in the levels of IL-1b (Fig. 8A and Table 2) and a 2.5 increase in the levels of TNF-a (Fig. 8B and Table 2) in the lGSH treatment group. Importantly, supplementation with lGSH for 13 weeks resulted in a 6-fold

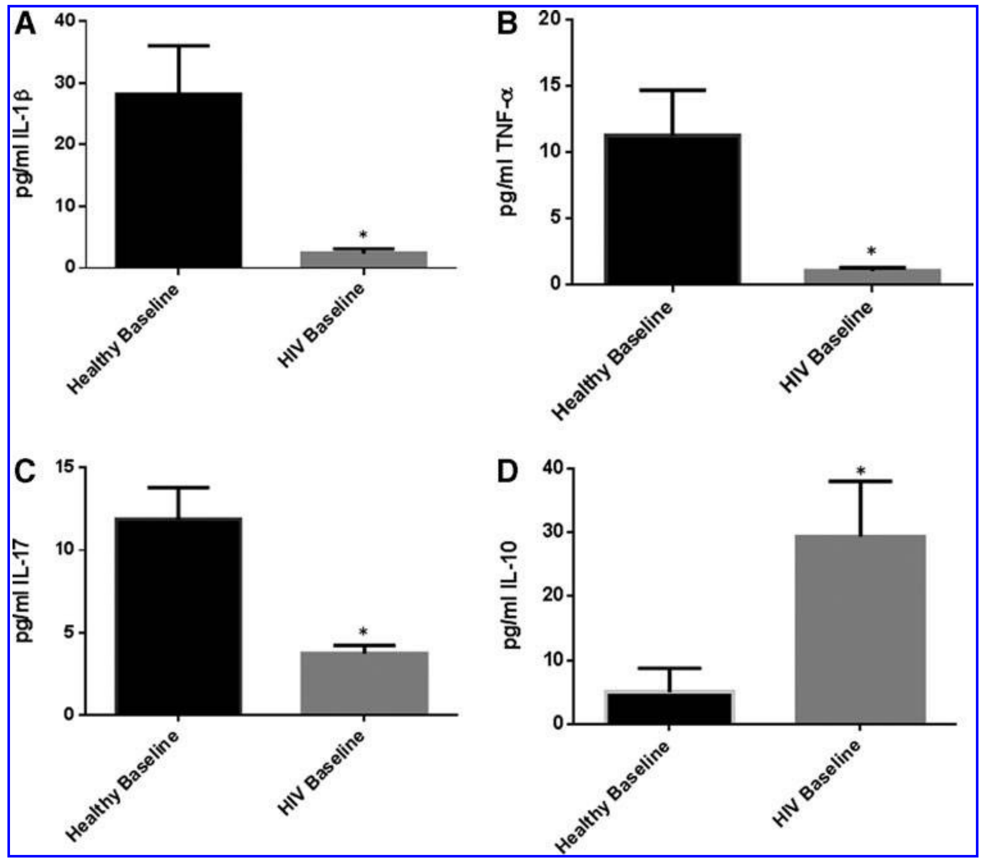

FIG. 4. Baseline comparison of IL-1b, tumor necrosis factor-a (TNF-a), IL-17, and IL-10 levels between healthy volunteers and HIV-positive individuals. We observed a significant decrease in the levels of IL-1b (A), TNF-a (B), and IL-17 (C) in plasma samples collected from individuals with HIV infection compared to healthy individuals. There was also a significant increase in the levels of IL-10 (D) in plasma samples collected from individuals with HIV infection compared to healthy individuals. Data represent mean – SE from comparing baseline levels of 10 healthy volunteers and 15 HIV-positive individuals, *P < 0.05.

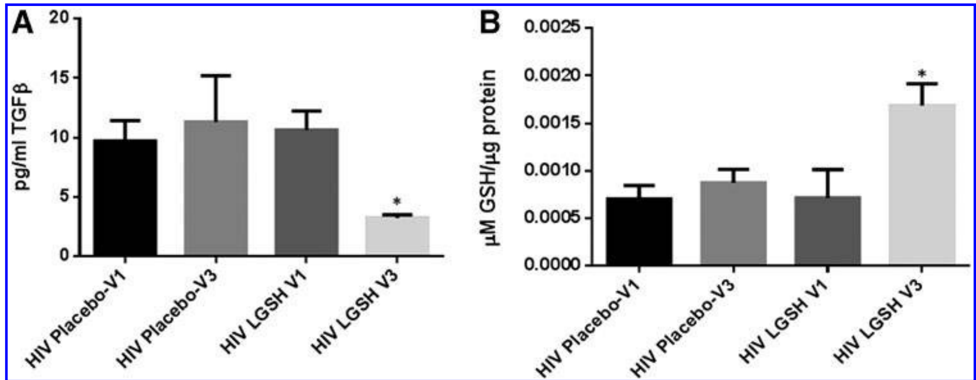

FIG. 5. Changes in the plasma TGF-b levels pre- and post-GSH supplementation. Sandwich enzyme-linked immunosorbent assay (ELISA) was performed to compare the cytokine levels between pre-supplementation (V1) and post-supplementation (V3). Assay of cytokines showed a significant decrease in the levels of TGF-b in plasma samples collected from the liposomal GSH (lGSH)-treatment group after 13 weeks of supplementation. There was no significant difference between the levels of TGF-b from the placebo group when comparing visit 1 and visit 3 (A). Data represent mean – SE, *P < 0.05 when comparing pre- and post-supplementation levels within placebo or lGSH group. GSH assay was performed to compare the levels of total GSH between pre-supplementation (V1) and post-supplementation (V3). Assay of GSH showed a significant increase in the levels of total GSH in PBMCs of the lGSH-treatment group after 13 weeks of supplementation. There was no significant difference between the levels of total GSH from the placebo group when comparing visit 1 and visit 3 (B). Data represent mean – SE, *P < 0.05 when comparing pre- and post-supplementation levels within placebo or lGSH group.

decrease in the levels of IL-10 (Fig. 8C and Table 3). We did not observe any noticeable changes in the levels of IL-2 and IL-17 in the lGSH group between visit 1 to visit 3 (data not shown). There was no significant difference in the levels of IL-1b, TNF-a, IL-17, and IL-10 in the placebo group.

Decreased survival of M. tb in PBMCs isolated from individuals with HIV infection at 13 weeks post supplementation with lGSH

We observed a significant decrease in the intracellular survival of H37Rv in PBMCs isolated from HIV-positive individuals at 13 weeks post supplementation with lGSH (Fig. 9). In contrast to the lGSH group, there was a 2-fold increase in the intracellular survival of H37Rv in PBMCs isolated from the placebo group (Fig. 9).

Discussion

We previously demonstrated a pattern of HIV-induced immunosuppression, wherein those cytokines belonging to T_H1 subsets were significantly decreased in individuals who were positive for HIV infection (Guerra and others 2011; Morris and others 2012). GSH, a tripeptide with key immunological functions, has been shown to be depleted in individuals with HIV infection (Ray and others 2006; Morris and others 2012). We further demonstrated that deficient GSH levels in individuals with HIV infection trigger impaired cytokine production leading to enhanced susceptibility of HIV-positive individuals to M. tb infection (Venketaraman 2011; Morris and others 2014; Vera Tudela and others 2014). These findings established the foundation for this study.

TGF-b, a cytokine produced by macrophages and regulatory T-Cells (T-Regs), is known for its role in regulating the immune responses by specifically limiting T-cell clonal expansion and proliferation (Kehrl and others 1986; Lotz and Seth 1993; Letterio and Roberts 1998; Garba and others 2002). Studies have shown that TGF-b can diminish the levels of GSH by downregulating the expression of glutamine cysteine ligase catalytic subunit), the rate limiting step enzyme involved in the synthesis of GSH (Chung and others 2003). In this study, we observed that the TGF-b levels were significantly higher in individuals with HIV infection compared to the healthy subjects at the baseline time point (Fig. 1A and Table 3). Increased levels of TGF-b in individuals with HIV infection correlated with significant decrease in the levels of GSH in the PBMCs (Fig. 1B and Table 1). We expected a decrease in TGF-b at 13 weeks, accounting for the effects of GSH supplementation in reducing immunosuppressive cytokines (Fig. 5A). Our data indicated a strong link between GSH supplementation in HIV-positive subjects for 13 weeks and significant reduction in the levels of TGF-b (Fig. 5A and Table 3). Reduced levels of TGF-b that occurred in individuals with HIV infection after 13 weeks supplementation with lGSH correlated with significant increase in the levels of GSH in the PBMCs (Fig. 5B and Table 1). Our findings establish an inverse correlation between TGF-b levels and GSH synthesis and support the previous findings that TGF-b has the ability to downregulate the de novo synthesis of GSH.

IL-6, a proinflammatory cytokine has been shown to induce oxidative stress and systemic inflammation (Wassmann and others 2004; Maeda and others 2010). We observed a significant increase in the levels of IL-6 in the plasma samples of HIV-positive individuals compared to the healthy group (Fig. 2A). Interestingly, lGSH supplementation for 13 weeks resulted in a significant decrease in the levels of IL-6 in HIV-positive individuals (Fig. 6A and Table 1).

Aligned with the lower levels of GSH in PBMCs, we observed that the levels of free radicals were significantly increased in individuals with HIV infection. The MDA assay results showed elevated levels of free radicals in the

plasma, monocytes, and RBCs of individuals with HIV infection compared to the healthy cohort (Fig. 2B–D). GSH supplementation for 13 weeks resulted in a marked decrease in the levels of MDA in the same blood components (Fig. 6C, E, G).

The baseline levels of total GSH in PBMCs were significantly decreased in HIV-positive individuals (Fig. 1B) and this decrease was accompanied by a notable elevation in the intensity of ROX staining in monocytes, $CD4^+$ T-cells, and $CD8^+$ T-cells in HIV-positive individuals in

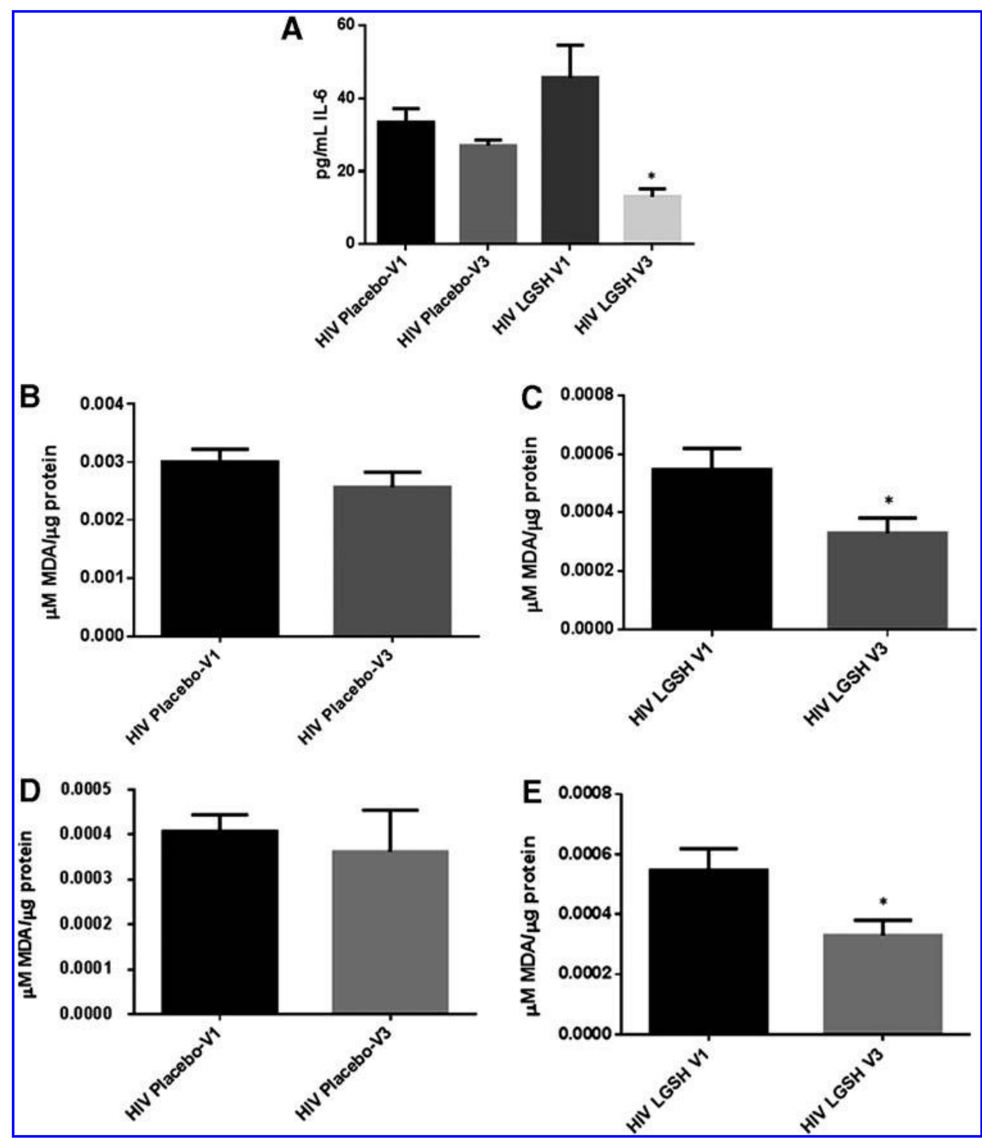

FIG. 6. Difference in plasma IL-6 levels and ROS markers pre- and post-GSH supplementation. Sandwich ELISA was performed to compare the cytokine levels between pre-supplementation (V1) and post-supplementation (V3). Assay of cytokines showed a significant decrease in the levels of IL-6 in plasma samples collected from the lGSH-treatment group. There was no significant difference between the levels of IL-6 from the placebo group when comparing visit 1 and visit 3 (A). Data represent mean – SE, $*P < 0.05$ when comparing pre- and post-supplementation levels within placebo or lGSH group. MDA assay was performed to compare the levels of MDA, a byproduct of lipid peroxidation, between pre-supplementation (V1) and post-supplementation (V3). Assay of MDA showed that there was no significant difference between the levels of MDA in plasma (B), RBC (D), and monocytes (F) from the placebo group when comparing visit 1 and visit 3. The levels of MDA in plasma (C), RBC (E), and monocytes (G) of the lGSH-treatment group significantly decreased after 13 weeks of supplementation. Data represent mean – SE, $**P < 0.005$ when comparing pre- and post-supplementation levels within placebo or lGSH group. Flow cytometry analysis of CellROX mean intensity, an indicator of ROS production, was completed to compare the levels of ROS production in different cell populations between pre-supplementation (V1) and post-supplementation (V3). Flow cytometry analysis of ROX showed that there was no significant difference between the median of ROX mean intensity $CD4^+$ T-cells (H), $CD8^+$ T-cells (J), and $CD14^+$cells/monocytes (L) from the placebo group when comparing visit 1 and visit 3. Median of ROX mean intensity in $CD4^+$ T-cells (I), $CD8^+$ T-cells (K), and $CD14^+$cells/monocytes (M) from the lGSH-treatment group significantly decreased after 13 weeks of supplementation. Data represent medians with range, $*P < 0.05$when comparing pre- and post-supplementation levels within placebo or lGSH group using the non-parametric version of the t-test, Mann–Whitney test.

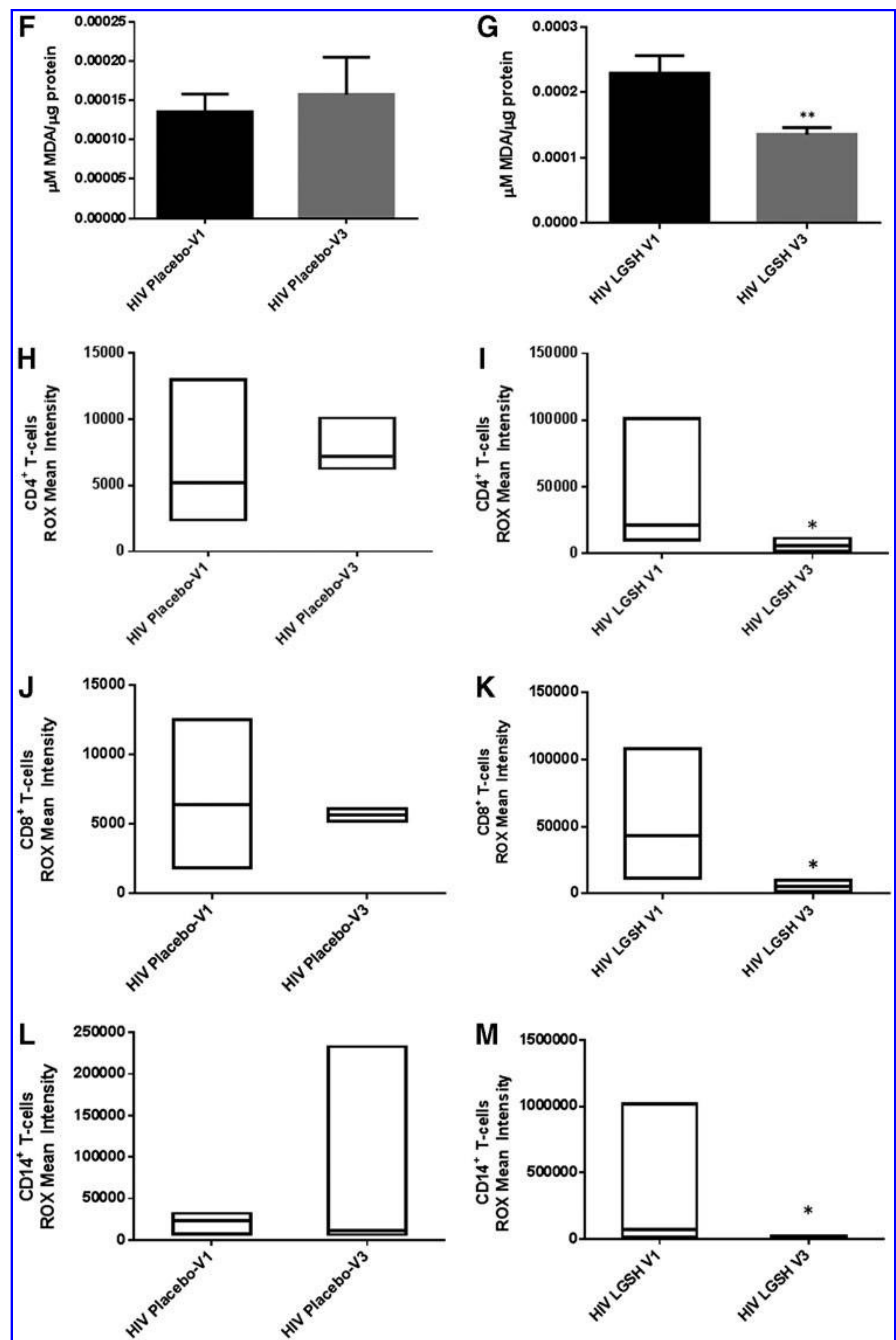

FIG. 6. (Continued).

comparison to healthy individuals (Fig. 2E–G and Table 1). lGSH supplementation for 13 weeks resulted in a significant decrease in the intensity of ROX staining in monocytes, $CD4^+$ T-cells, and $CD8^+$ T-cells from HIV-positive individuals (Fig. 6I, K, M, and Table 1). We offer this finding as a plausible explanation for the positive correlation between GSH restoration and normalized levels of IL-6 and free radicals.

In line with the diminished levels of GSH and increased levels of TGF-b, IL-6, and free radicals, we found decreased levels of T_H1-specific cytokines such as IL-12, IL-2, and IFN-g in HIV-positive individuals (Fig. 3 and Table 2). We also found increased levels of the T_H2-directing cytokine IL-10 in the plasma samples of HIV-positive participants (Fig. 4B and Table 3). Expanding on our hypothesis, we proposed that GSH supplementation in individuals with HIV infection should increase T_H1 cytokines while decreasing T_H2 cytokines, resulting in a more effective immune response against M. tb infection. Our study measured cytokine levels in the plasma samples derived from HIV-positive individuals

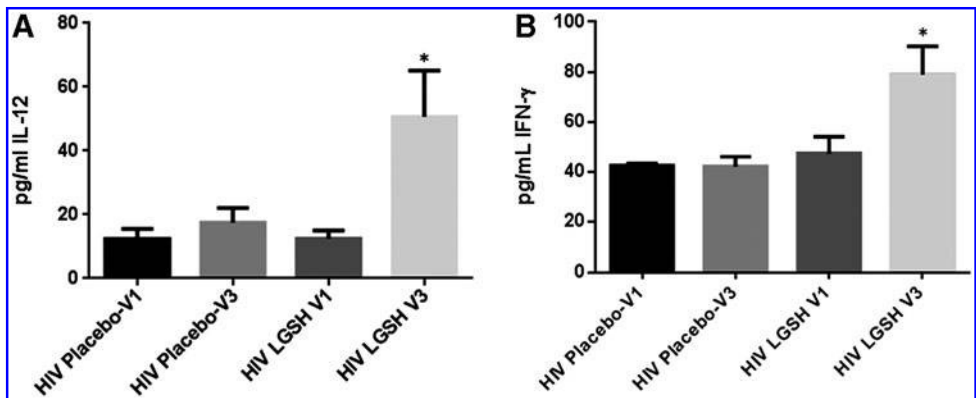

FIG. 7. Changes in the plasma T_H1 cytokine (IL-12 and IFN-g) levels pre- and post-GSH supplementation. Sandwich ELISA was performed to compare the cytokine levels between pre-supplementation (V1) and post-supplementation (V3). Assay of cytokines showed a significant increase in the levels of IL-12 (A) and IFN-g (B) in the plasma samples collected from the lGSH-treatment group after 13 weeks of supplementation. There was no significant difference between the levels of IL-12 (A) and IFN-g (B) from the placebo group when comparing V1 and V3. Data represent mean – SE, *P < 0.05 when comparing pre- and post-supplementation levels within the placebo or lGSH group.

before and after supplementation with oral lGSH. HIV-positive individuals receiving a placebo treatment (empty liposomes) were carefully analyzed alongside this group.

In line with our hypothesis that decreased GSH in HIV infection reduces the levels of IL-12 in favor of a T_H2-directed effect, we expected levels of IL-12 to be increased after 13 weeks of lGSH supplementation. Our data indicates that relative to the placebo-controlled cohorts, IL-12 levels increased in the lGSH supplement group after 13 weeks of supplementation (Fig. 7A and Table 2).

Likewise, we expected an increase in the levels of IFN-g after lGSH supplementation, but not after placebo

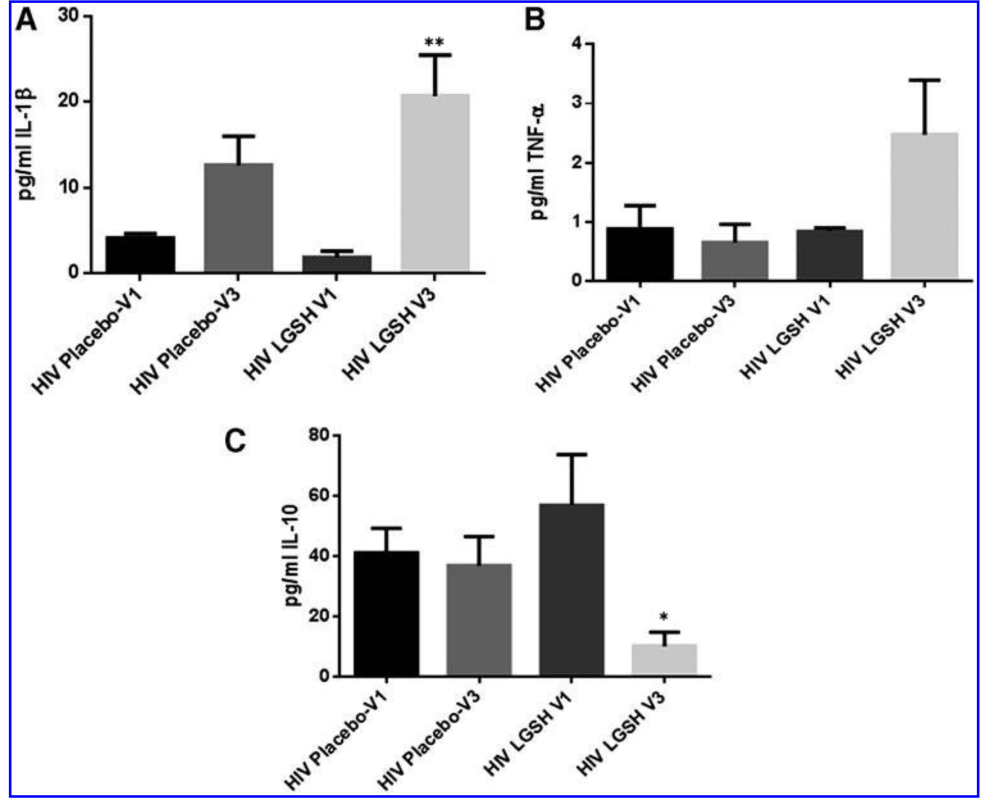

FIG. 8. Changes in the plasma IL-1b, TNF-a, and IL-10 levels pre- and post-GSH supplementation. Sandwich ELISA was performed to compare the cytokine levels between pre-supplementation (V1) and post-supplementation (V3). Assay of cytokines showed a significant increase in the levels of IL-1b (A) and TNF-a (B) in plasma samples collected from the lGSH-treatment group after 13 weeks of supplementation. On the other hand, a decrease in the levels of IL-10 was observed in the plasma samples collected from the lGSH-treatment group after 13 weeks of supplementation (C). There was no significant difference between the levels of IL-1b (A), TNF-a (B), and IL-10 (C) from the placebo group when comparing V1 and V3. Data represent mean – SE, *P < 0.05 and **P < 0.005 when comparing pre- and post-supplementation levels within placebo or lGSH group.

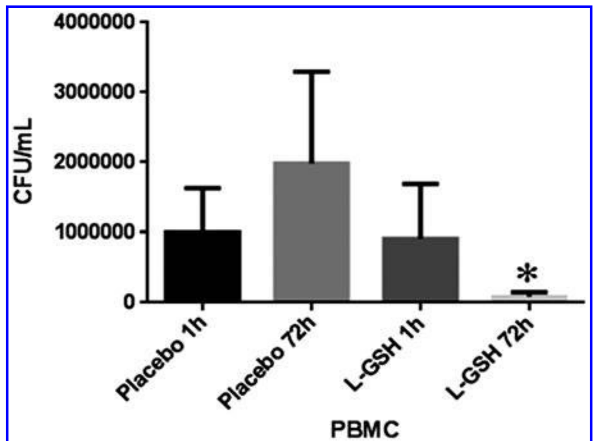

FIG. 9. Decreased survival of Mycobacterium tuberculosis (M. tb) in PBMCs isolated from individuals with HIV infection at 13 weeks post supplementation with lGSH. The figure illustrates significant decrease in the intracellular survival of H37Rv in PBMCs isolated from individuals with HIV infection at 13 weeks post-supplementation with lGSH. Data represent mean – SE, *P < 0.05when comparing colony-forming unit counts between 1 and 72 h time points of the lGSH group.

supplementation. Our results indicate a significant increase in the levels of IFN-g in individuals with HIV infection at 13 weeks post supplementation with lGSH (Fig. 7B and Table 2).

While the aforementioned cytokines are important regulators of adaptive immunity, our hypothesis also anticipated an increase in the levels of IL-1b and TNF-a in the lGSH treatment group.

Baseline levels of IL-1b and TNF-a were markedly decreased in HIV-positive individuals compared to healthy individuals at the initiation of our study (Fig. 4A, B and Table 2). Our results showed a significant increase in the levels of IL-1b and TNF-a in individuals with HIV infection at 13 weeks post lGSH supplementation (Fig. 8A, B, and Table 2).

IL-2 was hypothesized to increase in individuals with HIV infection after the participants had completed the 13-week long lGSH supplementation regimen. However, our data showed no significant difference in the levels of IL-2 after lGSH treatment (data not shown).

We found that baseline levels of IL-17 were significantly lower in individuals with HIV infection compared to healthy volunteers (Fig. 7C). However, our data showed no significant difference in the levels of IL-17 after lGSH treatment (data not shown).

We observed that the baseline levels of IL-10 were significantly higher in individuals with HIV compared to healthy volunteers (Fig. 4D and Table 3). Elevated levels of IL-10 as observed in the plasma samples from individuals with HIV infection will increase the risks for opportunistic infections. lGSH supplementation was effective in reducing the levels of IL-10 at the 13-week mark (Fig. 8C and Table 3). Finally, we observed a significant decrease in the intracellular survival of H37Rv in PBMCs isolated from individuals with HIV infection at 13 weeks post lGSH supplementation (Fig. 9).

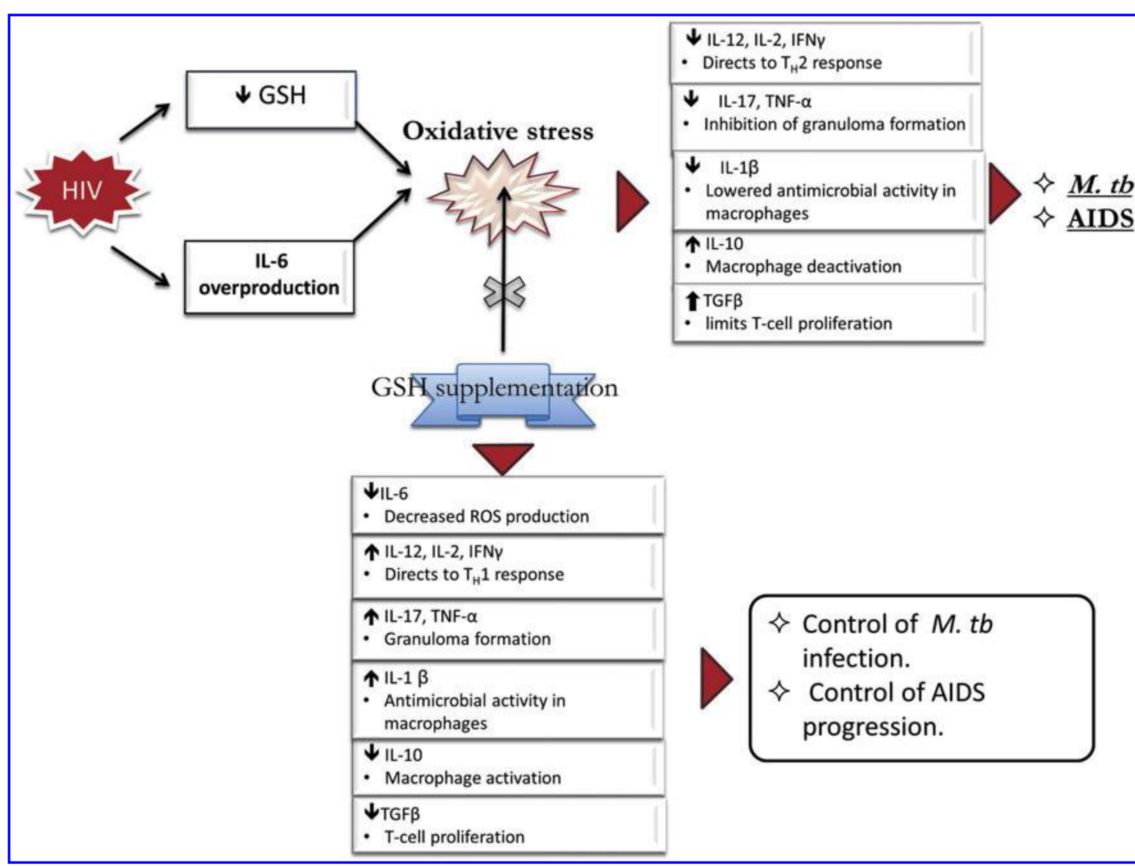

FIG. 10. Effects of GSH supplementation in restoring the immune cell functions against M. tb infection.

Our findings indicate that there is an imbalance created in the cytokine profiles due to the HIV disrupting the body's normal physiological processes and supplementation with lGSH can restore immune responses that could be of great advantage to HIV patients in managing opportunistic infections (Fig. 10).

Acknowledgments

The authors appreciate the funding support from Western University of Health Sciences to conduct this study. The authors thank Your Energy Systems for providing lGSH for this study and for the funding support. Their sincere thanks to all their study participants for taking time out of daily lives to make their study possible.

Author Disclosure Statement

No competing financial interests exist.

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Received 17 November 2014/Accepted 27 May 2015

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CASE REPORT

The Use of Intravenous Glutathione for Symptom Management of Parkinson's Disease: A Case Report

Madalyn Otto, ND; Tracy Magerus, ND; Jeffrey Langland, PhD

ABSTRACT

Intravenous glutathione has been suggested empirically to improve Parkinson's disease (PD) symptoms of tremor and rigidity, but there is limited supporting research. This case report demonstrates both subjective and objective symptom improvement of a conventionally-treated patient suffering from PD when adjunctive intravenous glutathione was administered. In addition to suggesting clinical benefit, this case also suggests an effective therapeutic frequency of therapy and a minimal therapeutic dose. The consistent pattern of improvement following glutathione injections asserts that this therapy may improve symptoms common to PD patients and can offer additional quality of life that would be otherwise unattainable to these patients. (*Altern Ther Health Med.* [E-pub ahead of print.])

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Parkinson's disease (PD) is a clinical diagnosis based on the presentation of motor and nonmotor features including resting tremor, bradykinesia, and rigidity. Other motor symptoms include hypomimia, dysphagia, micrographia, dysarthria, autonomic dysfunction, cognitive changes and sleep disorder. The primary symptom of bradykinesia in PD is best correlated to the loss of dopaminergic neurons in the substantia nigra of the brain.[1] Deterioration of the substantia nigra and loss of dopamine is the hallmark biochemical feature of this disease and contributes to the impaired motor function seen in disease progression. There are no biomarkers or imaging for diagnosis outside of clinical presentation. Left untreated, PD causes severe disability and even death within 5 to 10 years in more than half of patients. Increased function and longevity in recent decades are attributed to the administration of levodopa and the medications with which it is combined.[2,3] There is currently no medication regimen that can arrest or reverse the disease process.

Although the etiology of dopaminergic neuronal destruction in PD remains unknown, there are contributing factors and possible mechanisms underlying its development. One of the most common of these hypotheses is excessive oxidative processes acting on the central nervous system (CNS) producing large amounts of free radicals, thereby depleting its antioxidant capacity, particularly of glutathione.[4,5] The oxidative stress hypothesis is evidenced by a decrease in glutathione without a corresponding deficiency in glutathione transferase, which might otherwise suggest a disorder of glutathione synthesis rather than depletion.[6] Studies have revealed a 40% reduction in CNS glutathione in patients with early PD supporting its role in the pathogenesis of this disease. Glutathione deficiency in PD patients seems to occur significantly in the substantia nigra and not in other brain regions.[7] As a result of these findings, small clinical trials have been conducted to evaluate the use and efficacy of glutathione in patients with Parkinson's disease. The results of these studies suggest a positive effect of this therapy.[8-10]

Due to the fact that the hallmark biochemical feature of PD is a loss of dopaminergic neurons, the mainstay conventional treatment for PD continues to be carbidopa/levodopa. Levodopa is a dopamine precursor that crosses the blood-brain barrier to be converted to dopamine, thereby replacing brain

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Table 1. Treatment Regimen in the Course of Adjunctive Alternative Treatment

Drug	Stalevo (L/C/E)	Azilect	NAC	Silybum	GSH IV	GF diet
Dose	100/25/200 mg, 5×/d	1 mg QD	100 mg BID	3 caps	1400 mg in NS IV 2-3 ×/wk	85% to 95% of the time

Note: Conventional treatment included Stalevo (levadopa/carbidopa/entacapone) 200 mg/50 mg/200 mg taken 5 times daily and Azilect (rasagiline) (1 mg QHS). Natural treatments included oral *N*-acetyl-cysteine (100 mg BID), oral *Silybum marianum*, and IV glutathione injections performed 2-3 times weekly and dosed at 1400 mg GSH diluted in normal saline. Gluten-free diet was also implemented by the patient with adherence of 85% to 95%.

Abbreviations: NAC, *N*-acetyl-cysteine; GSH, glutathione; IV, intravenous; GF, gluten free.

levels of this neurotransmitter. Carbidopa is a decarboxylase inhibitor that maximizes levodopa conversion to dopamine in the CNS rather than in peripheral tissues. Both drugs are used only to treat the symptoms of the disease and do not address the etiology of dopaminergic neuronal death. Initial PD treatment generally includes levodopa, often combined with carbidopa. Levodopa has a particularly short half-life (90 to 20 minutes), which seems to increase the risk of side effects, namely motor fluctuations.[11] As the disease advances, the duration of improvement decreases substantially. At this time, a monoamine oxidase (MAO-B) inhibitor or a catechol-*O*-methyltransferase (COMT) inhibitor is commonly added to the regimen. MAO inhibitors do not slow the progression of the disease better than levodopa does, but rather they slow the enzymes that inactivate dopamine, maintaining the carbidopa/levodopa effect for longer when used adjunctively.[11] There is no evidence that progression to motor fluctuation is decreased.[2] The patient in this case report had been prescribed Azilect (rasagiline), an MAO-B inhibitor, during his conventional course of treatment. COMT inhibitors inhibit peripheral levodopa metabolism, thereby enhancing its action in the CNS. The drug Stalevo, which the patient in this case report had been previously prescribed, is a combination of carbidopa/levodopa and entacapone (a COMT inhibitor) and will be referred to by its brand name (Stalevo) from this point forward.

The following case demonstrates not only efficacy of intravenous (IV) glutathione as an adjunctive treatment for the management of PD symptoms, but also the pattern of symptom improvement following treatment.

PRESENTING CONCERNS

The patient was a 61-year-old white male, nonsmoker, working in commercial real estate management. He was diagnosed with PD 5 years prior at age 56 years. The patient began carbidopa/levodopa (Sinemet) treatment at the time of diagnosis. Azilect was added 2 months later. He had no other significant health concerns but had a first-degree relative also diagnosed with PD. At the time of diagnosis, he was experiencing a stiffened gait, dysarthria, micrographia, and hypomimia. As the disease progressed, he also suffered from cognitive symptoms of mental fatigue, lack of motivation, and poor memory.

At the time that alternative treatment was sought 4 years after diagnosis, the patient had been taking a consistent dose of Stalevo (200 mg/50 mg/200 mg 5 times per day) and Azilect (1 mg QD) for 1 year prior, which had been helping to manage his symptoms.

INTERVENTION

The patient sought alternative therapies to be added to his conventional medication regimen to help better control his mental and motor PD symptoms. Immediately prior to seeking alternative medical care, the patient self-prescribed a gluten-free diet after reading that it may be helpful for Parkinson's symptoms. He continued this diet with estimated 85% to 95% adherence throughout treatment. Later that same month, the patient was assessed at our office and additional adjunctive treatments were initiated (Table 1). Glutathione injections were administered twice weekly at a 1400-mg dose diluted in normal saline for the first few months of treatment, administered at 8:00 AM every 3 to 4 days. The patient eventually received IV therapy 3 times per week, as this was found to be the optimal frequency of dosing to achieve the greatest consistent effect. The patient was also prescribed oral *N*-acetyl-cysteine (NAC), a precursor to glutathione as well as *Silybum*, an herbal hepatoprotective agent used in multidrug patients.[12] He continued his 5-times-per-day oral dosing of Stalevo along with Azilect throughout the course of treatment.

OUTCOMES

The patient's primary symptoms during treatment included dysarthria, micrographia, and hypomimia along with cognitive symptoms of mental fatigue, lack of motivation, and poor memory. Because the patient describes the latter (cognitive) symptoms interchangeably, they will be discussed as a combined symptom of mental function from this point forward. Outcome assessment of alternative therapies was discerned based on patient's subjective symptom changes and objective findings from the patient's friends and family. Symptoms were subjectively rated by the patient on a scale of 1 to 10 to describe lowest to highest level of function (1, unable to function; 10, the highest level of function before having PD) in the course of conventional treatment and throughout the course of alternative treatments.

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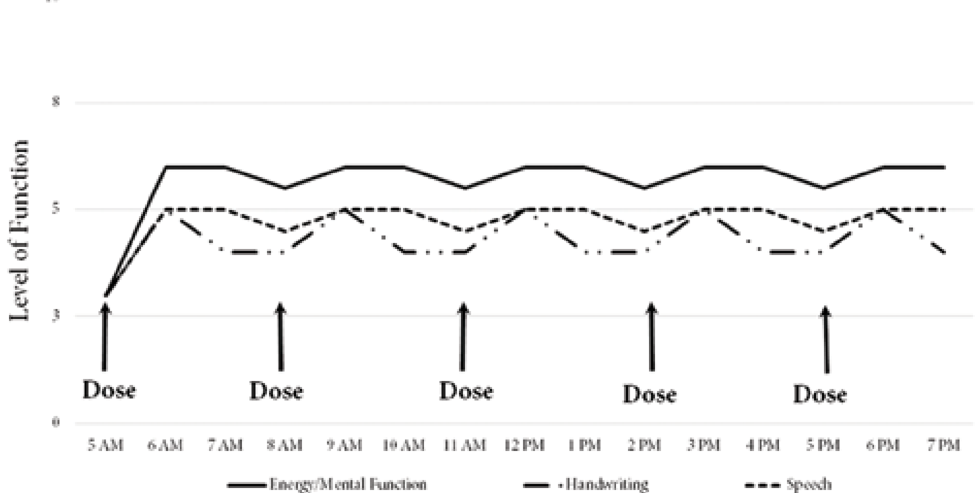
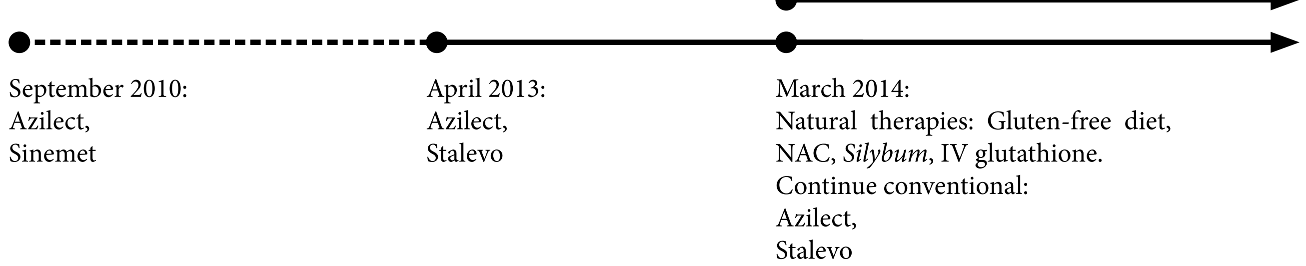
Figure 1. Level of Motor and Mental Function Throughout the Day When Taking Stalevo 5× per Day



Note: Figure 1 shows symptom management on Stalevo and Azilect alone. Graph illustrates a daily snapshot of mental functioning, speech, and handwriting when administered conventional therapies alone. The patient's dosing schedule of Stalevo (levodopa/carbidopa/entacapone) was 5 times daily and Azilect was 1 time daily. Within 1 hour of a dose of Stalevo, patient experienced maximum benefit in symptoms followed by a plateau and regression until the following dose. Maximum improvement occurred 1 hour after a dose of Stalevo. Maximum regression occurred upon waking or after the longest period of time between doses. Mental function was rated by the patient at 3/10 upon waking, improved to a maximum of 6/10 after dosing Stalevo, and regressed to 5/10 by the time the next dose was taken. The patient defines 1/10 as an inability to function and 10/10 is defined as his level of function before he had Parkinson's disease.

Table 2. Timeline of Therapy Intervention



Note: Patient began carbidopa/levodopa (Sinemet) treatment upon diagnosis in 2010. He was additionally prescribed Azilect 2 months later. In 2013, he was switched to Stalevo in addition to Azilect and maintained this regimen until the time of this publication. In 2014, natural therapies were added including oral NAC and *Silybum*, a gluten-free diet, and biweekly IV glutathione injections.

Abbreviations: NAC, *N*-acetyl-cysteine; IV, intravenous.

While taking Stalevo and Azilect alone, the patient rated his mental and motor function to be a maximum of 4-6/10 (Figure 1) as long as he maintained a 5-times-per-day dosing schedule of Stalevo. If he missed a single dose, he noted that his motor and mental function decreased. Figure 1 represents the level of function the patient experienced on a typical day when taking Stalevo and Azilect alone. Despite this moderate improvement, he continued to have significant difficulty with his occupational and home tasks. It was because of this that the patient sought additional treatments.

All alternative treatments were started during the same month (Table 2). The patient reported that he did not perceive a notable change with a gluten-free diet or with NAC and *Silybum* supplementation. He was not completely compliant with the diet and did not notice fluctuations in symptoms with dietary indiscretions.

During the first 3 weeks of alternative treatment including IV glutathione therapy, the patient experienced no change in symptoms. After 3 weeks of alternative treatments, he began to experience a significant improvement in his

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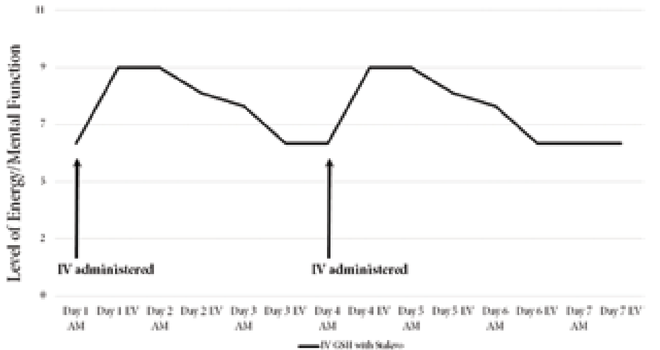
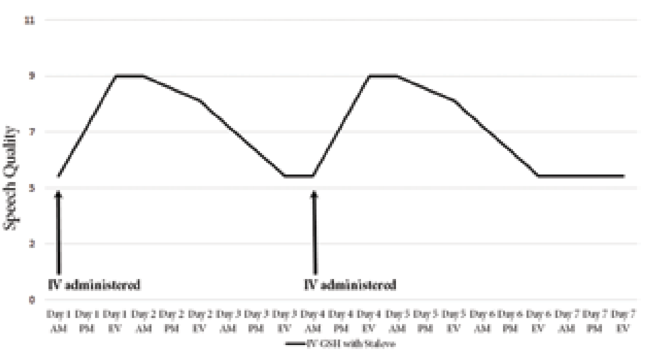
Figure 2. Level of Mental Function During the Week When Receiving Glutathione Injections



Note: Figure 2 shows mental function following IV glutathione injections. Graph illustrates symptom improvement in the course of a week in relation to IV glutathione injections. Glutathione injections were administered at 8:00 AM on days 1 and 4. Approximately 8 hours after an injection, patient felt that his mental functioning improved to 9/10 that would maintain fully for approximately 24 hours before regression would occur. Rating scale is defined as follows: 1/10, inability to function; 10/10, level of function before diagnosis of Parkinson's disease.

Abbreviations: IV, intravenous; GSH, glutathione.

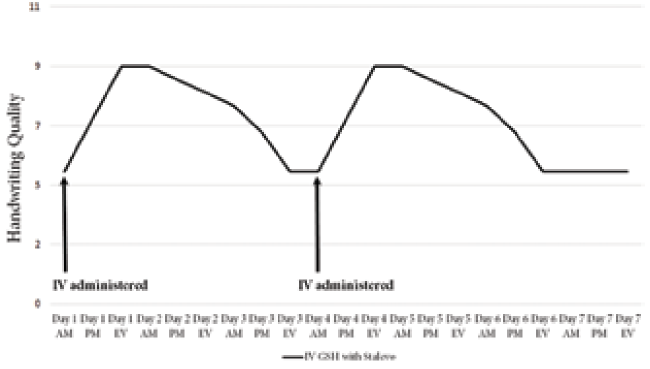
Figure 3. Level of Speech Quality During the Week When Receiving Glutathione Injections



Note: Figure 3 shows speech function following IV glutathione injections. Graph illustrates symptom improvement in the course of a week in relation to IV glutathione injections. Glutathione injections were administered at 8:00 AM on days 1 and 4. Approximately 8 hours after an injection, patient felt that his speech improved to 9/10 that would maintain fully for approximately 24 hours before regression would occur. Rating scale is defined as follows: 1/10, inability to articulate speech; 10/10, quality of speech before diagnosis of Parkinson's disease.

Abbreviations: IV, intravenous; GSH, glutathione.

Figure 4. Level of Mental Function During the Week When Receiving Glutathione Injections



Note: Figure 4 shows handwriting ability following IV glutathione injections. Graph illustrates symptom improvement in the course of a week in relation to IV glutathione injections. Glutathione injections were administered at 8:00 AM on days 1 and 4. Approximately 8 hours after an injection, patient felt that his speech improved to 9/10 that would maintain fully for approximately 24 hours before regression would occur. Rating scale is defined as follows: 1/10, inability to write legibly; 10/10, quality of handwriting before diagnosis of Parkinson's disease.

Abbreviations: IV, intravenous; GSH, glutathione.

symptoms following IV glutathione treatment that surpassed the benefit of Stalevo and Azilect alone. Figures 2, 3, and 4 represent a typical week of mental and motor function observed following twice-weekly IV glutathione along with other medications. Glutathione injections were administered at 8:00 AM on days 1 and 4. Approximately 8 hours after an injection, the patient would feel a surge of improvement in all measured symptoms that would maintain fully for approximately 24 hours before any decline was seen. He rated his level of function at 9/10 for all primary symptoms immediately following IV treatment. Symptoms would return to the baseline function of Stalevo/Azilect-only treatment (4-6/10) within 36 hours or until the patient received another injection.

This pattern was consistent for all his primary symptoms and was reproduced with each subsequent injection in the period of 1 year. If an injection or series of injections was missed due to schedule conflict or vacation, the patient reported that his level of function would regress to the baseline Stalevo/Azilect level seen in Figure 1. As mentioned in the Intervention section, the patient was initially administered glutathione injections twice weekly but eventually received injections 3 times per week as this was found to be the optimal frequency of dosing to achieve the greatest consistent effect. The patient reported that within a few months of consistent IV glutathione treatments, his coworkers and friends began to remark that his facial expression improved dramatically. This symptom had

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previously caused social and occupational difficulties and remained improved in the course of treatment. Since the improvement of masked facies occurred after several months' duration of the alternative treatment regimen, the patient reports that this is when he experienced maximal benefit.

DISCUSSION

This case study not only suggests that the efficacy of IV glutathione as an adjunctive treatment in a condition that is very difficult to treat, but it also demonstrates the pattern and timing of symptom improvement following treatment. This offers insight to a treatment protocol that could produce the greatest improvement for patients with PD. All-natural interventions including diet changes (self-prescribed), oral nutrient therapy, and IV therapy were initiated at the same time. Because all treatments were maintained, it is unclear the extent of the role each played or whether they were necessary to see clinical benefit. Although several treatments were initiated together, maximum improvement consistently correlated with IV glutathione treatments. In addition, delaying injections consistently correlated with regression in function. This suggests that glutathione was the primary cause of symptom improvement. The patient in this case had a more advanced case of PD and was still significantly affected by the introduction of IV glutathione. Noticeably, after receiving IV glutathione treatments for 1 year with sustained improvement, no adverse effects were reported. This protocol represents a treatment for patients with PD patients who have progressed past the early stages of the disease.

Two previous trials that have been conducted to evaluate the use of IV glutathione clinically in patients with PD have shown promising results. One study was conducted to evaluate the effect of IV glutathione in 9 early stage, untreated patients with PD. All patients experienced significant reduction in symptoms for 2 to 4 months posttreatment with no adverse effects.[8] A later randomized, placebo-controlled, double-blind trial investigated the effect of IV glutathione in medicated patients with PD in the course of 4 weeks and found only mild symptom improvement in patients with virtually no adverse effects.[9] These trials both illustrate the possible subjective improvement of PD symptoms, but neither treated patients for more than 30 days.

The patient in this case did not experience any subjective benefits until the third week of treatment and experienced benefit in facial expression after several months of treatment. This finding suggests that there may be a loading dose required before seeing a therapeutic effect and suggests that there is a repletion period before maximal effect is observed. Neither previous clinical study remarked on a pattern in the symptom improvement, which is the highlighted clinical feature in this case report. The establishment of this pattern can help guide a clinician in prescribing an optimal therapeutic regimen using this therapy. An important finding in both trials as well as this case report is that IV glutathione therapy appears extremely safe. No serious side effects or adverse events occurred.

As shown in this case, administration of IV glutathione in addition to daily conventional pharmacotherapy yielded symptom improvement of PD superior to that from pharmacotherapy alone. This case report suggests that IV glutathione should be investigated on a larger scale as a treatment option for PD both in early and later stages of the disease. Trials should consider a loading-dose time period of at least 2 weeks before symptoms should be expected to improve as well as a repletion period where maximal improvement may be expected after 2 months of consistent treatment at a frequency of 2 to 3 injections per week. This treatment represents a very low risk approach with superior beneficial outcomes.

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Title: Glutathione versus N-Acetyl Cysteine for the Brain

"N-Acetyl Cysteine May Support Dopamine Neurons in Parkinson's Disease: Preliminary Clinical and Cell Line Data" 2016 [1]

Blog and Literature Review

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This study, and the related papers, show a complexity in trying to treat nervous system issues via manipulation of glutathione. As I look at posts people make and opinions posed it is clear that this evolving area of neurobiology needs more "fleshing out" for clinicians. This is not a bad thing – every time there is this type of confusion it indicates that new information needs to be incorporated into our existing knowledge base (and that treating human beings isn't as easy as it looks). So:

Sometimes… being old has advantages. In the case of this study [1] it is seeing a protocol I used in the 1990's replicated (as if some new epiphany) and assessed in a real trial setting. Nothing wrong with that at all because they now have many methods of assessment we did not have all those years ago when what we had to go on was our wits and some sparse data. The fact that our patients improved is likely a shock to modern researchers such as Monti et.al.

Modern era physicians may say "why were you using NAC in Parkinson's (and other neurodegenerative disease) when you could use glutathione?" Excellent question, and the answer is "we did not have it". I suppose it is like my early memories of television, all black and white with two or three channels and trying to describe that to a kid with an IPhone. So how did those of us in that generation come on to the idea of glutathione "support" for neurological disease?

The few of us doing it understood biochemistry and neurobiology enough to realize that thiols like NAC (and ALA which we were able to get for parenteral use later as well) may have a beneficial effect in the brain as well as the liver and other organs, and could support the formation of glutathione (which at the time was not available in any form as a therapy). So protocols very much like this one (IV NAC plus oral NAC on non IV days) were developed and used. If I were asking someone from an older time who had already used this protocol my questions would be:

- Why don't you do it any more, or do you?

- Why, if glutathione purportedly does not cross the blood brain barrier, do we not use more NAC for CNS issues?

- Do the "newer" protocols using intranasal, oral liposomal or IV glutathione really work better?

- Can we make glutathione therapy (from any substrate) more efficient and effective?

- How about all this new "SNP" knowledge. Does that have an effect?

All are excellent questions and in light of my historical background with this topic and protocol I will discuss all of them in relationship to this study. The current paper states the following conclusions: "The results of this preliminary study demonstrate for the first time a potential direct effect of NAC on the dopamine system in PD patients, and this observation may be associated with positive clinical effects. A large-scale clinical trial to test the therapeutic efficacy of NAC in this population and to better elucidate

the mechanism of action is warranted." If one reads the entire paper it is clear that this was a reasonably designed in vitro and vivo study. As mentioned earlier we in the past surmised these effects and used NAC (oral and IV) in this same setting. In light of that I will now answer the questions posed above.

Why don't you do it any more, or do you?

As soon as IV glutathione (GSH) (and later oral liposomal or other absorbable forms) became available I and others saw better clinical results using glutathione over NAC exclusively. That said however, in those who are responsive (see SNP discussion below), I do use oral NAC and or ALA as a glutathione support in all such cases. Conversely since IV glutathione became available I have almost never ordered or used IV NAC simply because the (in my opinion anecdotally) outcomes are superior with IV glutathione.

- Why, if glutathione purportedly does not cross the blood brain barrier (BBB), do we not use more NAC (especially IV) for CNS issues?

I would like to (hopefully) stop the misinformation on the BBB-GSH connection. I have read most of the data on this and am very convinced that (based on scientific investigation) GSH is transported as GSH across the BBB. This is not even taking into account the leaky BBB in neuro-inflammatory conditions which (even if GSH couldn't be transported across the BBB the inflammation would allow it). But under physiologic conditions the BBB does (yes DOES) transport GSH into the brain circulation intact. Tsuji [2] demonstrated this in a seminal neuroscience paper and cites multiple supporting references. So yes, go ahead and use GSH.

Any GSH that makes it into the plasma can get to the brain without "being dismantled to cysteine etc. before transport" as is often unfortunately stated emphatically.

If you have lost track, the point of NAC originally as used was as a substrate for GSH (it is the rate limiting amino acid for GSH) hence the study using NAC we are reviewing. So if we can supply GSH to the brain by intranasal, IV, IM or oral methods isn't that better?

As a quick note: Why use NAC in this study instead of GSH? NAC and its relative l-cysteine are approved medication by the USP and FDA. GSH is not.

- Do the "newer" protocols using intranasal, oral liposomal or IV glutathione really work better?

I would argue, based on having done this with NAC alone or with NAC orally with IV GSH as augment, that the modern advances in GSH availability and delivery ARE an improvement on the NAC alone. No, the paper we are discussing does not discuss this. They cannot as they needed a single intervention medication. That does not make the science (or our experience with) of GSH change however.

- Can we make glutathione therapy (from any substrate) more efficient and effective?

The tenets of improving GSH therapy are as follows: Give GSH and if applicable GSH precursors (as in NAC and ALA). Assure redox balance long term (as described in earlier posts) [3]. Assure GSH is supported by its many cofactors. [4] In general (unless a genomic condition precluded use) I always give NAC or ALA orally as substrate between IV or Intranasal GSH use. And in all cases I recommend cofactor use.

- How about all this new “SNP” knowledge. Does that have an effect?

Glutathione Synthetase (GSS) and SNPs in this region are connected with poor utilization of precursors for GSH formation (such as ALA and NAC). A look to the National Library of Medicine database can provide more specifics of this SNP area as well as related pathogenic studies. [http://www.ncbi.nlm.nih.gov/gene/2937]. The bottom line here is that the more GSS SNPs one has the more they need GSH and the less that precursors such as ALA and NAC will help.

Clinical Summary:

This well designed trial does show benefit directly to neurons via supplementation with NAC. Our understanding of the benefit of GSH for conditions such as Parkinson’s disease may be partly related to the GSH effect and potentially to the NAC effect. At this point we cannot know to any certainty which has what effect. What many years of clinical use of all these agents has taught me is that combination and well-rounded therapy always surpasses a single agent approach.

Supplement with parenteral, intranasal or oral GSH (my current choices are Acetyl Glutathione or Liposomal Glutathione for oral use). Supply GSH cofactors as well as ReDox triplet cofactors (see references below). And if using a non-oral form of GSH provide oral NAC or ALA between. If the patient has GSS SNP’s consider that NAC and or ALA will have much less efficacy.

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The Use of Nebulized Glutathione in the Treatment of Emphysema: a Case Report

Davis W. Lamson, ND, Matthew S. Brignall, ND

Abstract

We present the case of a 95-year-old man with an acute respiratory crisis secondary to emphysema and apparent bronchial infection. Treatment with nebulized glutathione led to a rapid resolution of the crisis, as well as a marked improvement in the chronic course of the disease. This treatment has been used since for a number of patients with emphysema. The safety and bioavailability of this method of delivery have been established in human studies. Preliminary results suggest efficacy for nebulized administration of glutathione in this patient population. We suggest this treatment can be considered an option for acute respiratory crises due to COPD.
(*Altern Med Rev* 2000;5(5):429-431)

Introduction

Chronic obstructive pulmonary disease (COPD), a designation which includes emphysema, is a leading cause of death in America. This case study reports on the successful treatment of both acute and chronic emphysema with a novel agent.

Much of the tissue damage in emphysema is thought to be mediated by an oxidative down-regulation of the activity of α-1-proteinase inhibitor.[1] This down-regulation has been shown *in vitro* to be slowed by glutathione, a sulfhydryl-containing tripeptide known to be a major antioxidant in the lung.[2]

Glutathione concentrations in bronchoalveolar fluid have been found to be inversely correlated with the degree of inflammatory activity in the lungs of smokers.[3] Thiol compounds (i.e., compounds containing an –SH group) like glutathione have a history of use as mucolytics as well.[4] Previous clinical trials of nebulized reduced glutathione have demonstrated the bioavailability and safety of up to 600 mg twice daily.[5,6] The absorption of oral glutathione remains controversial, with animal studies suggesting significant absorption and some human studies showing little to none.[7,8] Based on these findings, it appears inhalation might be the preferred route of administration for respiratory and perhaps systemic effect. We report the case of a man with an acute respiratory crisis due to emphysema and apparent bronchial infection that responded favorably to treatment with nebulized glutathione.

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Case Report

In 1997, a 95-year-old male with emphysema presented in a wheelchair and using an oxygen tank and mask necessitated by his acute illness. He was alert, responsive, and reported a productive cough with colorless sputum. His breathing was obviously labored. He refused hospitalization and antibiotic treatment. We chose to try a single trial dose of 2 ml of a 60 mg/ml glutathione solution (prepared by Apothecure Pharmacy, Dallas, TX) nebulized and inhaled over a 5-10 minute period. Due to the obvious immediate benefit, it was decided to continue this treatment with twice-daily administration and close monitoring by his family of his overall condition. He returned to the office in three days without wheelchair or oxygen tank. He showed no signs of respiratory distress, and no adventitious lung sounds were noted on auscultation. The patient reported his breathing was better than it had been in years. He continued daily treatment with glutathione until his death from congestive heart failure over two years later.

Conclusion

While resolution of the acute episode due to a mucolytic effect was the desired outcome of the glutathione treatment, the lasting improvement in breathing was unexpected. Since we have no serial spirometry data available on this patient, placebo effect cannot be ruled out as an explanation for his marked response. However, given the progressive nature of his disease, the dramatic and rapid change in physical findings, and the emphatic insistence of the patient for continued treatment, we believe placebo response to be an unlikely explanation.

We have subsequently prescribed this preparation for six patients with emphysema, five of whom reported improved breathing after a single in-office application and who later requested to continue treatment. We also have found nebulized glutathione is best administered daily from 4 ml vials. We have also seen improved respiratory function associated with nebulized glutathione treatment in cases of chronic bronchitis and asthma.[9] In the case of asthma patients we feel it is advisable to check urinary sulfite excretion to verify proper metabolism of sulfur compounds, as certain individuals appear to experience exacerbation of respiratory symptoms from exogenous sulfur compounds.[10] In three cases of non-small cell lung cancer with effusion, the effusion resolved completely. Given the safety and promise of this treatment, combined with the paucity of other effective treatments for emphysema, we suggest this treatment be considered for widespread use.

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Is your Alternative Medicine Therapy Helping Cancer Patients?

The National Cancer Institute, the U.S. Federal government's agency responsible for cancer research, invites you to submit data from your best cases for review by conventional research and alternative medicine experts. Successful approaches can receive financial support and recommendations for further research, visibility among the clinical research community, and feedback on the strengths and limitations of your approach. For details or a submission package, contact NCI's office of Cancer Complementary and Alternative Medicine, National Cancer Institute, EPN/Suite 102, Bethesda, MD 20892; (301) 435-7980 (phone); (301) 480-7980 (fax); ncioccam-r@mail.nih.gov (email); or http://occam.nci.nih.gov and click on Best Case Series Program.

Please contact me with any questions or comments you may have about this announcement. Thank you for your consideration.

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Eur J Nutr
DOI 10.1007/s00394-014-0706-z

ORIGINAL CONTRIBUTION

Randomized controlled trial of oral glutathione supplementation on body stores of glutathione

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Received: 24 January 2014 / Accepted: 16 April 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract
Purpose Glutathione (GSH), the most abundant endogenous antioxidant, is a critical regulator of oxidative stress and immune function. While oral GSH has been shown to be bioavailable in laboratory animal models, its efficacy in humans has not been established. Our objective was to determine the long-term effectiveness of oral GSH supplementation on body stores of GSH in healthy adults.
Methods A 6-month randomized, double-blinded, placebo-controlled trial of oral GSH (250 or 1,000 mg/day) on GSH levels in blood, erythrocytes, plasma, lymphocytes and exfoliated buccal mucosal cells was conducted in 54 non-smoking adults. Secondary outcomes on a subset of subjects included a battery of immune markers.
Results GSH levels in blood increased after 1, 3 and 6 months versus baseline at both doses. At 6 months, mean GSH levels increased 30–35 % in erythrocytes, plasma and lymphocytes and 260 % in buccal cells in the high-dose group ($P < 0.05$). GSH levels increased 17 and 29 % in blood and erythrocytes, respectively, in the low-dose group ($P < 0.05$). In most cases, the increases were dose and time dependent, and levels returned to baseline after a 1-month washout period. A reduction in oxidative stress in both GSH dose groups was indicated by decreases in the oxidized to reduced glutathione ratio in whole blood after 6 months. Natural killer cytotoxicity increased >twofold in the high-dose group versus placebo ($P < 0.05$) at 3 months.
Conclusions These findings show, for the first time, that daily consumption of GSH supplements was effective at increasing body compartment stores of GSH.

Keywords Glutathione · Supplementation · Antioxidant · Immune function

Electronic supplementary material The online version of this article (doi:10.1007/s00394-014-0706-z) contains supplementary material, which is available to authorized users.

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Introduction

Glutathione (GSH) is the major endogenous intracellular antioxidant. It has numerous functions including protecting cells against oxidative stress, detoxification of toxins and carcinogens, posttranslational regulation of protein function and maintenance of immune function [1–5]. Nearly, all tissues in the body synthesize GSH by sequential addition of the precursor amino acids, cysteine (Cys), glutamic acid (Glu) and glycine (Gly) through enzymatic catalysis by two ATP-dependent enzymes, glutamine cysteine ligase (GCL) and GSH synthetase (GS) [6, 7]. The maintenance of tissue levels of GSH is critical for maintaining health,

Published online: 05 May 2014

preventing diseases and age-related biological insults. Even partial GSH depletion impairs immune function [8] and increases susceptibility to a wide range of xenobiotics [9] and oxidative damage [10]. Low GSH levels are associated with increased risks of numerous diseases including cancer [11], cardiovascular diseases, arthritis and diabetes [12, 13].

There is a wide range of inter-individual variability in blood and tissue GSH levels, and low levels can be associated with exposure to oxidants/drugs/toxins, poor nutrition and other factors. GSH levels are also dependent on the availability of its precursor amino acids, Cys, Glu and Gly, with Cys most often considered as rate limiting. Consequently, intracellular GSH levels can be depleted in certain tissues including liver by short periods of fasting such as that which occurs overnight [14, 15].

Increasing GSH represents a potentially important approach to counteract disorders associated with GSH depletion, enhance detoxification capacity and protect against disease. Oral GSH supplementation represents one such strategy for enhancing tissue GSH levels. The use of oral GSH is supported by studies linking high dietary GSH intake with high blood levels and reduced risk of cancer [16, 17]. Studies in animal models have shown that oral GSH, administered either in the diet or by gavage, increases plasma and tissue GSH levels [18–23] and protects against aging-related impairments in immune function [24, 25], influenza infections and cancer [26–29]. These effects of oral GSH have been accounted for, in part, by the direct absorption and transport of intact GSH in the small intestine [19, 21].

There is less data on the bioavailability of oral GSH in humans. While GSH was found to be absorbed and transported in human intestinal epithelial cells in vitro [30] and in buccal mucosal cells in vivo [23], results from a clinical study of oral GSH, administered as a single dose (150 μmol/kg) to 7 healthy adults, showed no significant effect on plasma GSH levels during a 4.5 h period [31]. However, the rapid turnover of GSH in human plasma would likely make it difficult to detect an increase in plasma after a single oral dose. Thus, our current objectives were to determine the long-term effects of daily oral GSH supplementation on GSH levels in different body stores. We conducted a randomized, double-blinded, placebo-controlled trial of oral GSH at two doses, 250 and 1,000 mg/day, administered for 6 months in healthy adults on the levels of GSH in different blood compartments and exfoliated buccal mucosal cells. GSH oxidation products, GSH disulfide (GSSG) and GSH protein mixed disulfides (GSSP), are commonly used biomarkers of oxidative stress [32]; thus, we also examined the effects of oral GSH on GSSG/GSH and GSSP/GSH ratios in blood. Since intracellular GSH plays a key role in the maintenance and regulation of certain immunological functions [33, 34] including the activation of lymphocytes and functional activity of NK cells [35–37], secondary endpoint analysis included the assessment of hematologic measurements of immune function including neutrophil phagocytosis, neutrophil respiratory burst, lymphocyte proliferation and natural killer (NK) cell cytotoxicity in a subset of subjects.

Materials and methods

Study protocol

The study (ClinicalTrials.gov identifier: NCT01044277) was approved by the Institutional Review Board of the Penn State College of Medicine in accordance with the Helsinki Declaration of 1975 as revised in 1983. The study design is summarized in the CONSORT form (Fig. 1). All clinical activities were conducted at the Penn State Hershey Cancer Institute, Hershey, PA. Healthy subjects were recruited by the study coordinator from the local Hershey/Harrisburg, PA area using fliers, newspaper and radio advertisements, online announcements and word of mouth. Interested individuals were prescreened by telephone to assess potential eligibility. Individuals who passed the initial screening were asked to visit the clinic where, after signing the informed consent, they were screened for eligibility based upon the following criteria: Healthy male and female non-smokers, 30–79 years of age, not taking antioxidant supplements for at least 1 month. Eligible subjects were randomly assigned to one of three treatment groups with equal probability: 250 mg GSH per day orally (provided as two 125 mg capsules); 1,000 mg GSH per day orally (provided as two 500 mg capsules); and placebo (provided as two capsules). Glutathione (Setria®) was provided by Kyowa Hakko USA, Inc., and capsules were formulated as follows: GSH 125 mg (125 mg GSH, 360 mg cellulose); GSH 500 mg (500 mg GSH, 15 mg cellulose); and placebo (470 mg cellulose). Both subjects and investigators were blinded to the group assignment. At baseline, trained nurse interviewers administered a structured questionnaire to each subject to collect information on demographics, occupation, lifestyle habits, medical history, usage of medication and dietary supplements, alcohol consumption and past cigarette smoking history.

University pharmacists dispensed either active supplements or placebo at the baseline visit. Subjects were provided instructions for supplement usage and instructed to continue taking the capsules for 6 months, maintain a daily pill diary and return all unused capsules. There was a 1-month washout period between 6 and 7 months where subjects discontinued their supplementation to assess the reversibility of any GSH-related changes. After 1, 3 and 6 months, subjects returned to the clinic to return their

Enrollment

Assessed for eligibility (n=98)

Excluded (n=37)
- Not meeting inclusion criteria (n=12)
- Declined to participate (n=12)
- Other reasons (n=13)

Randomized (n=61)

Allocation

Allocated to Placebo (n=21)
- Received intervention (n=20)
- Did not receive intervention (n=1)

Allocated to 250 mg/d GSH (n=20)
- Received intervention (n=20)
- Did not receive intervention (n=0)

Allocated to 1000 mg/d GSH (n=20)
- Received intervention (n=20)
- Did not receive intervention (n=0)

Follow-Up

Lost to follow-up (n=0)
Discontinued intervention (n=4)
- dropped out (n=3)
- enrolled in competing trial (n=1)

Lost to follow-up (n=0)
Discontinued intervention (n=2)
- moved (n=1)
- enrolled in competing trial (n=1)

Lost to follow-up (n=0)
Discontinued intervention (n=0)

Analysis

Analysed (n=16)
- Excluded from analysis (n=0)

Analysed (n=18)
- Excluded from analysis (n=0)

Analysed (n=20)
- Excluded from analysis (n=0)

Fig. 1 Subject flowchart summarized according to consolidated standards of reporting trials

unused capsules and, at the 1- and 3-month time points, to receive new capsules. At baseline and after 1, 3, 6 and 7 months, biological samples including blood, urine and exfoliated buccal mucosal cells were collected and processed as described below. Outcome measures included GSH levels in whole blood, erythrocytes, plasma and lymphocytes at baseline and 1, 3, 6 and 7 months and in exfoliated buccal mucosal cells at baseline and 3, 6 and 7 months. Secondary outcomes included immune function activities including NK cell cytotoxicity and lymphocyte proliferation at baseline and 3 months and respiratory burst and neutrophil phagocytosis at baseline and 3 and 6 months.

Subjects

The clinical phase of the study was conducted from February, 2010, to September, 2011, over which time, 61 subjects were enrolled (Fig. 1). A total of 60 received intervention, of which 6 dropped out for reasons including relocation ($n = 1$), enrollment in competing trials ($n = 2$) and reports of adverse effects ($n = 2$, both in the placebo group) and one was dropped for lack of compliance. A total of 54 completed at least 6 months of the study protocol and were included in the analyses. The characteristics of these 54 participants are summarized in Table 1 including, age, sex, race and BMI. All subjects were non-smokers (having not smoked for >1 year) and had no history of chronic disease or antioxidant or GSH supplement usage within the past month. Most subjects were female (76 %), white (92 %) and ranged in age from 28 to 72 years (mean = 46.6 years). Fifteen of subjects had a BMI > 30 kg/m^2. There were no significant differences in subject characteristics by treatment group. Compliance was assessed by pill count and daily pill diary entries.

Collection and processing of biological samples

Exfoliated buccal mucosal cells were obtained after a mouth rinse with distilled water and brushing of the cheeks and gums with a soft tooth brush. Subjects then rinsed with 20 ml of saline, which was collected and stored at 4 °C until centrifugation (6,000×g for 10 min) on-site within 1 h after collection. Cells were washed three times with

Table 1 Study subject characteristics

N	Placebo 16	GSH (250 mg/day) 18	GSH (1,000 mg/day) 20	All 54
Age (year)				
Mean	48.2	44.3	47.6	46.6
SD	12.1	8.01	10.7	10.3
Range	31–68	30–59	28–72	28–72
Sex *n* (%)				
Female	12 (75 %)	15 (83 %)	14 (70 %)	41 (76 %)
Male	4 (25 %)	3 (17 %)	6 (30 %)	13 (24 %)
Race/ethnicity *n* (%)				
White	14 (88 %)	16 (88 %)	19 (95 %)	49 (92 %)
Black	2 (12 %)	1 (6 %)	1 (5 %)	4 (7 %)
Asian	0 (0 %)	1 (6 %)	0 (0 %)	1 (2 %)
Education (year)				
Mean	11.2	12.0	11.0	11.4
SD	2.4	3.6	2.0	2.3
BMI (kg/m^2)				
Female				
Mean	24.6	25.9	26.4	25.8
SD	4.26	3.90	5.80	4.72
Male				
Mean	27.6	22.2	30.1	27.5
SD	5.45	4.61	3.99	5.26
All				
Mean	25.3	25.2	27.5	26.2
SD	4.50	4.14	5.49	4.86

saline, and packed cells were stored at −80° C until analysis.

Blood samples were collected between 9:00 am and 1:00 pm from an antecubital vein into three tubes containing sodium heparin as an anticoagulant and immediately placed on ice. Tubes were mixed by gentle shaking, and a 2.5-ml aliquot of whole blood was removed for analysis of neutrophil phagocytosis and respiratory burst (see below). Two 0.5-ml aliquots of whole blood were removed and stored at −80 °C for future analyses. The remaining blood was centrifuged for 10 min at 1,300×*g* to obtain plasma, buffy coat and red cell fractions. Multiple 0.5-ml aliquots of plasma were placed into 1.5-ml cryovials and immediately frozen at −80 °C. Packed red cells were washed three times in saline, aliquoted (0.5 ml each) into multiple cryovials and frozen at −80 °C. Buffy coat fractions were combined, and lymphocytes were isolated by Ficoll–Hypaque density gradient centrifugation. In brief, after addition of 3 ml of Ficoll, buffy coats were centrifuged at 400*g* for 30 min at 19 °C. Lymphocyte layers were removed and washed two times in PBS, followed by centrifugation at 250*g* for 10 min. After the final wash, lymphocytes were re-suspended in 5 ml PBS. Cell number was assessed after addition of 40 µl trypan blue to 10 µl of cell suspension using a hemocytometer. Cells were re-suspended in 95 % FBS, 5 % DMSO at concentrations of 2.5×10^6 cells/ml, frozen at −80 °C and stored in liquid nitrogen until analysis of GSH, lymphocyte proliferation or NK cell cytotoxicity (see below). Buccal cells were mixed with an equal volume of PBS and centrifuged at 5,000*g* for 2 min. Cells were kept on ice until acid extraction as described below.

Analytical procedures

Glutathione

The synthesis and regulation of GSH can vary in different cells and tissues. Consequently, we measured GSH in a variety of compartments including whole blood, erythrocytes, plasma, lymphocytes and exfoliated buccal mucosal cells. For whole blood or red cells, 0.8 ml of 5 % (w/v) metaphosphoric acid (MPA) was added to 0.2 ml of blood or packed cells. Samples were centrifuged for 2 min at 14,000*g*, and supernatants were stored at −80° until analysis of free GSH. Levels of GSH and GSSG were

determined in MPA extracts as described previously [38, 39]. GSSP was measured in acid-insoluble pellets after reduction with KBH_4 and re-acidification with MPA as described previously [32]. While GSH in whole blood is concentrated in erythrocytes, lower but measurable levels can also be detected in plasma. GSH in plasma is present mostly in its oxidized forms, GSSG and GSSP [40]. Therefore, plasma samples were first reduced with sodium borohydride prior to analysis to allow for the measurement of total GSH (free + bound) [40].

To prevent the oxidation of lymphocyte and buccal cell GSH that can occur during sample processing, these samples were also first reduced with sodium borohydride. In brief, 400 µl of 5 % MPA was added to aliquots of packed cells containing $\sim 5 \times 10^6$ cells. After vigorous mixing and incubation at room temperature for 15 min, samples were centrifuged at 14,000*g* for 2 min. Supernatants were stored at −80 °C until analysis for GSH.

Glutathione levels were expressed on a per milliliter basis for plasma and whole blood, per gram hemoglobin for erythrocytes, per 10^6 cells for lymphocytes and per gram protein for buccal cells. Protein concentrations were measured by the bicinchoninic acid procedure (Pierce, Rockford, IL). Hemoglobin was determined spectrophotometrically using Drabkin's reagent [41].

Glutamate-cysteine ligase *activity* was determined in red cells by measuring the product, γ-glutamylcysteine, formed after incubating cell lysates with cysteine and glutamic acid, as described previously [42].

Cyst(e)ine (cystine and cysteine) was analyzed in plasma according to the spectrophotometric method of Gaitonde [43].

Immune function

Neutrophil phagocytosis and respiratory burst assays were performed on fresh whole blood samples, which were available from 16 subjects from the placebo group, 18 subjects from the 250 mg/day GSH group and 20 subjects from the 1,000 mg/day GSH group at baseline and 3 and 6 months. Lymphocyte and NK cell assays were performed on frozen purified lymphocyte cell fractions. Fewer samples were available for these analyses; samples with a sufficient number of viable cells were available from 8 to 9 subjects per group at baseline and 3 months for the lymphocyte proliferation assay and 5 to 6 subjects per group at baseline and 3 months for the NK cell cytotoxicity assay.

Neutrophil phagocytosis was determined in fresh whole blood samples (0.1 ml aliquots) incubated with *E. coli* labeled with a pH-sensitive dye using the pHrodo™ *E. coli* BioParticles® Phagocytosis kit (Invitrogen). Dye-containing cells were analyzed with a FACScan flow cytometer (Becton–Dickinson) using the 488-nm laser for excitation and emission within the FL2 channel. Results were expressed as a phagocytosis index calculated as the FL2 geometric mean of granulocytes incubated at 37 °C/FL2 geometric mean of granulocytes incubated at 4 °C. Thus, the higher the index value, the greater the extent of phagocytosis.

Neutrophil respiratory burst in fresh whole blood samples was determined by measuring the oxidation of dihydrorhodamine 123 by hydrogen peroxide produced by activated neutrophils as described previously [44]. In brief, 0.1-ml aliquots of whole blood in triplicate were exposed to 1 µM phorbol 12-myristate 13-acetate (PMA) for 15 min to activate granulocytes and then exposed to the fluorescence probe dihydrorhodamine 123. Fluorescent cells were detected using a FACSCanto flow cytometer, and the portion of granulocytes that showed increased fluorescence was determined along with the geometric mean of FL1 fluorescence intensity per cell. Results are expressed as the respiratory burst index calculated as the FL1 geometric mean of PMA-stimulated cells/non-PMA-stimulated cells. Thus, the higher the index, the greater the respiratory burst activation.

Lymphocyte proliferation was measured at 2 time points. Lymphocytes were thawed, washed three times and counted to determine cell viability. Cells were plated in triplicate at both 1×10^5 and 5×10^4 cells per well of a 96-well flat-bottom plate in RPMI-1640 plus GlutaMAX supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 25 ng/ml sodium pyruvate, 10 mM HEPES and 50 µM 2-mercaptoethanol. Cells were rested for 48 h at 37 °C and 5 % CO_2 followed by addition of media alone or 2 µg/ml of the T-cell mitogen phytohemagglutinin (PHA). Cultures were incubated for further 72 h, and 1 µCi of ^{3}H-thymidine was added for the last 6 h of the assay. Cells were harvested using a PHD™ cell harvester (Brandel, model 290) per the manufacturer's instructions. Intracellular radioactivity was measured by liquid scintillation counting, and results were expressed as CPM.

Natural killer cell cytotoxicity was assessed using a standard ^{51}Chromium-release assay. Briefly, lymphocyte samples from each of the five time points were thawed, washed three times, counted to determine cell viability, added in triplicate to 96-well V-bottom plates in complete RPMI-1640 supplemented with 10 % FBS and rested for 48 h at 37 °C and 5 % CO_2. Human K562 cells [45] grown in complete RPMI-1640 medium were labeled overnight with 200 µCi sodium ^{51}Chromate in saline, washed three times and then added to lymphocytes (1×10^4 cells per well) at an effector/target cell ratio of 10:1. After incubation for 4 h at 37 °C and 5 % CO_2, cells were pelleted by centrifugation and supernatants were analyzed for radioactivity by gamma counting. Results are expressed as

percent of target cells lysed (% lysis) calculated as (cpm experimental − cpm spontaneous release)/(cpm maximum − spontaneous) × 100 as described previously [46].

Statistics

Sample size and power estimates were based on a two-tailed type I error of 0.05, 15 % standard deviation, $n = 16$ per group and >80 % power. Minimal detectable percent differences in GSH between treatment groups and placebo were ~11 %. Descriptive statistics were provided as means and standard deviations. The normality of data distribution was assessed using the Kolmogorov–Smirnov goodness-of-fit test. Group differences at baseline were assessed by ANOVA followed by Tukey's post hoc test or χ^2 where appropriate. General linear mixed models with repeated measurement (Proc mixed) were used to test for the effects of intervention, period and their interactions with changes in all outcome variables. Potential confounding by age and gender was assessed, and none were observed. Subgroup analyses of age groups (<40 years of age, >40–50 years of age and >50 years of age) and gender revealed no interactions. Correlations of changes in outcomes with levels at baseline or between measures were evaluated using Pearson (r) correlations. All statistical tests were two-sided, and significance was set at $P < 0.05$. SAS 9.3 software (SAS Institute, Inc., Cary, NC, USA) was used for all statistical analyses.

Results

Compliance and adverse effects

Overall, compliance was ≥94 % based on both pill counts and pill diary entries. By pill count, compliance was 95.5 ± 6.25 % (mean ± SD) in all subjects and did not differ between treatment arms (placebo, 97.6 ± 3.01; 250 mg GSH, 93.7 ± 8.38; and 1,000 mg GSH, 95.5 ± 5.54). Four subjects had an overall compliance rate of <90 % including three in the low-dose GSH group and one in the high-dose GSH group.

No serious adverse effects were reported by the study participants regardless of arm. All potential adverse events reported were minor including colds, stomach virus, lightheadedness, back pain, hot flashes, soft stools, eye twitching, headaches, ear infection, urinary tract infection and constipation. None were attributed to any specific treatment arm. The number of these events reported was similar in each arm of the study (placebo, 20; 250 mg GSH, 18; and 1,000 mg GSH, 19). Two individuals dropped out because of allergy-like symptoms, both of which were in the placebo group.

Effects of oral GSH supplementation on body GSH stores

In whole blood, mean GSH levels significantly increased in both low- and high-dose GSH groups after 1, 3 and 6 months of administration versus baseline (Fig. 2). No increase was observed in the placebo group. At 6 months, the increased levels were higher in the 1,000 mg dose group (31 %) than in the 250 mg group (20 %) ($P < 0.05$). In the 1,000 mg group, GSH levels increased in a time-dependent manner from 1 to 6 months of supplement administration. In the 250 mg group, equivalent increases were observed after 1, 5 and 6 months. In both dose groups, GSH levels decreased toward baseline levels following the 1-month washout period. However, the levels in the 1,000 mg group remained significantly greater than baseline after washout ($P < 0.05$).

In erythrocytes, mean GSH levels significantly increased after 1, 3 and 6 months in the 1,000 mg group and after 6 months in the 250 mg group (Fig. 2). Similar to whole blood, maximum increases in GSH levels in erythrocytes of about 35 % were observed after 6 months. In both dose groups, GSH levels decreased toward baseline levels after the 1-month washout period.

The effects of oral GSH on plasma total GSH levels are summarized in Fig. 3. Concentrations ranged from ~0.2 to 8 nmol/ml, which are <1 % of levels in whole blood. Increases in plasma GSH levels were observed in the 1,000 mg group after 3 and 6 months of administration. While the mean level at 6 months was higher than baseline in the low-dose group, this increase was not statistically significant. In the high-dose group, the increase appeared to be time dependent with levels progressively increasing from 3 to 6 months of administration, after which time GSH levels decreased toward baseline levels after the 1-month washout period. Non-significant time-dependent increases were found in the 250 mg group. There were no changes in the placebo group.

The effects of oral GSH administration on lymphocyte total GSH levels are summarized in Fig. 3. Values are expressed on a per million cell basis and ranged from ~0.7 to 6.7 μmol/10^6 cells. Increases in lymphocyte GSH levels were observed in the high-dose GSH group after 1, 3 and 6 months of administration. While the mean level at 3 and 6 months was higher than baseline in the low-dose group, these increases were not statistically significant. No changes were observed in the placebo group. A maximum increase of about 30 % was observed after 6 months in the high-dose GSH group. In the high-dose group, the increase appeared to be time dependent with levels progressively increasing

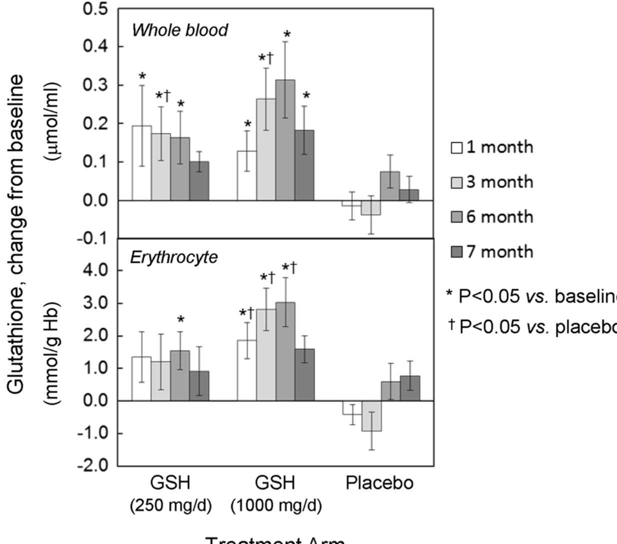


Fig. 2 Effect of oral GSH supplementation on GSH levels in whole blood and erythrocytes. Subjects were randomized to placebo ($n = 16$), 250 mg/day GSH ($n = 18$) and 1,000 mg/day GSH ($n = 20$). GSH or placebo supplementation continued for 6 months followed by a 1-month washout. Blood was collected at baseline and after 1, 3, 6 and 7 months. Free and protein-bound GSH was determined in whole blood (*upper panel*) and in packed erythrocytes (*lower panel*) as described in text. Erythrocyte GSH levels, expressed on a gram hemoglobin basis, are presented as changes from baseline. Bars are mean ± SE. General linear models with repeated measurement were used to test for effects of intervention, period and their interaction with study outcomes. *Change from baseline within group is statistically significant, $P < 0.05$. †Change from baseline is significantly different from placebo group, $P < 0.05$

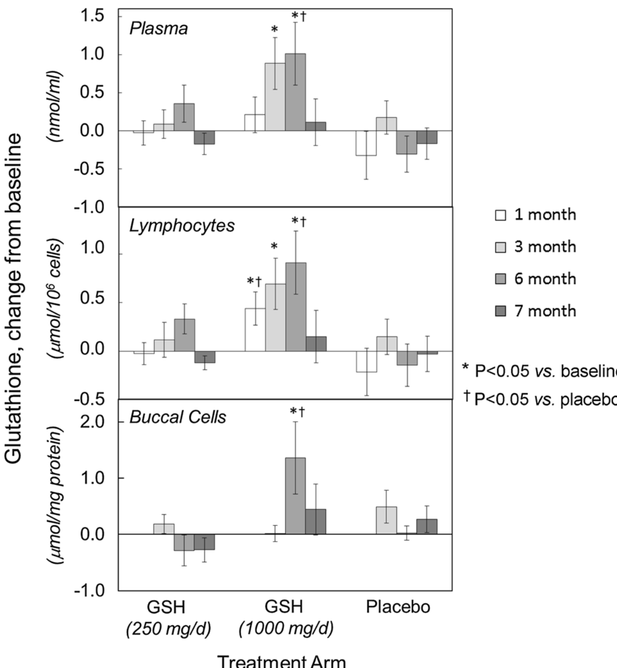


Fig. 3 Effect of oral GSH supplementation on total GSH in plasma, lymphocytes and buccal mucosa. Subjects were randomized to placebo ($n = 16$), 250 mg/day GSH ($n = 18$) and 1,000 mg/day GSH ($n = 20$). GSH or placebo supplementation continued for 6 months followed by a 1-month washout. Blood and buccal cells were collected at baseline and after 1, 3, 6 and 7 months. Total GSH was determined in plasma, lymphocytes and buccal cells as described in text. Lymphocyte and buccal cell GSH levels were expressed on a 10^6 cells and milligram protein basis, respectively, and are presented as changes from baseline. *Bars* are mean ± SE. General linear models with repeated measurement were used to test for effects of intervention, period and their interaction with study outcomes. *Change from baseline within group is statistically significant, $P < 0.05$. †Change from baseline is significantly different from change from baseline in the placebo group, $P < 0.05$

from 1–3 to 6 months of administration after which time GSH levels decreased toward baseline levels during the 1-month washout period.

The effects of oral GSH administration of exfoliated buccal cell levels are summarized in Fig. 3. Values are expressed on a per milligram protein basis and ranged from ~0.6 to 11 μmol/mg protein. While variation in GSH levels was high, a significant increase was observed in the high-dose GSH group after 6 months of administration. No changes were observed at other time points or in either the low-dose GSH or placebo groups.

GSH levels in whole blood, erythrocytes, plasma, lymphocytes and buccal cells at baseline and after 6 months are summarized for each treatment group in Supplemental Table 1. There were no effects of either sex or age observed for GSH levels in any blood compartment or buccal cells. Likewise, sex and age had no impact on changes in GSH levels due to treatment.

The effects of GSH supplementation on the levels of the GSH precursor cysteine in plasma and the activity of the rate-limiting GSH biosynthetic enzyme GCL in erythrocytes were also examined after 6 months (Supplemental Table 2). No changes were observed in cyst(e)ine concentrations or GCL activity in any of the groups.

Effects of oral GSH supplementation on GSH redox status in blood

The major oxidized forms of GSH, GSSG and GSSP were analyzed in whole blood, and the impact of oral GSH supplementation on the ratio of oxidized to reduced forms is summarized at baseline and after 6 months in Fig. 4. No differences were observed in the placebo group for any of the following ratios: GSSG/GSH, GSSP/GSH or (GSSG + GSSP)/GSH. However, GSSG/GSH and (GSSG + GSH)/GSSG ratios were both decreased significantly in the high-dose GSH supplementation group and GSSG/GSH was decreased significantly in the low-dose GSH supplementation group after 6 months.

Effect of oral GSH supplementation by baseline GSH levels

In order to determine whether effects of oral GSH differed by baseline GSH levels, changes by treatment arm in each of the measured outcomes were compared between individuals with levels below the median (<0.89 μmol/ml) and above the median (>0.89 μmol/ml). No significant differences were observed between the two groups at 6 months for GSH in blood, erythrocytes, plasma, lymphocytes and buccal cells (data not shown). Likewise, changes in GSH levels after 6 months in blood, erythrocytes, lymphocytes, plasma and buccal cells were not significantly correlated with GSH levels at baseline for subjects in the GSH-treated groups ($n = 38$) (blood, $r = 0.35$; erythrocytes, $r = 0.043$; plasma, $r = -0.11$; lymphocytes, $r = 0.01$; and buccal cells, $r = 0.018$).

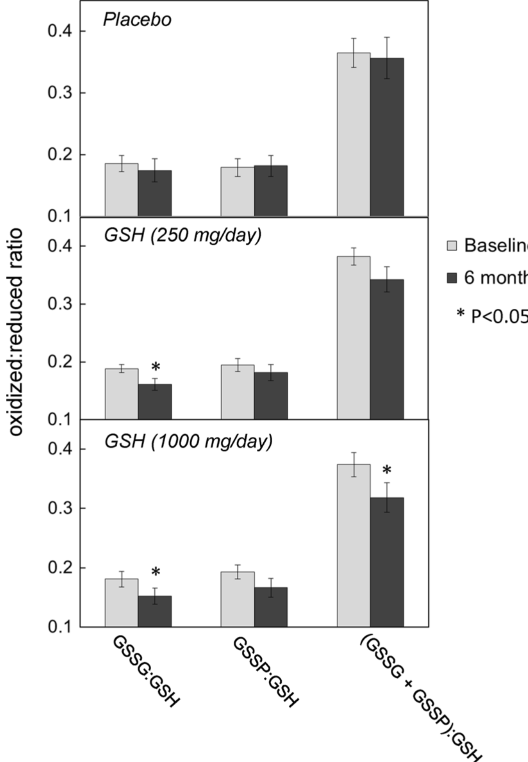


Fig. 4 Effect of oral GSH supplementation on oxidized to reduced glutathione ratios in whole blood. Subjects were randomized to placebo ($n = 16$), 250 mg/d GSH ($n = 18$) and 1,000 mg/day GSH ($n = 20$). GSH or placebo supplementation continued for 6 months followed by a 1-month washout. Blood was collected at baseline and after 1, 3, 6 and 7 months. GSH and its major oxidized forms, GSSG and GSSP were determined in whole blood as described in text. *Symbols* and *bars* are mean ± SE

Correlation of GSH changes in different blood compartments and buccal cells

To examine whether changes in GSH levels within individuals were consistent among the different blood compartments and buccal cells, correlational analyses were conducted for individuals in the GSH-treated groups (Table 2). Changes in blood GSH were highly correlated with those in erythrocytes at 6 months ($r = 0.83$). In addition, changes in lymphocyte GSH were significantly correlated with those in both blood ($r = 0.55$) and erythrocytes ($r = 0.45$). Changes in plasma GSH were weakly correlated with those in erythrocytes ($r = 0.33$). No associations were observed for GSH changes in buccal cells.

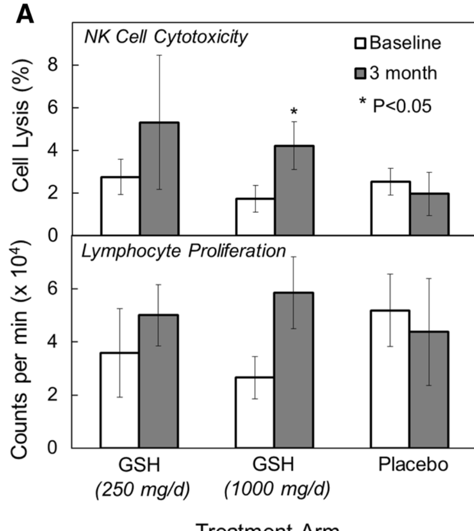
Effects of oral GSH on immune function markers in blood

The effects of GSH administration on lymphocyte proliferation are summarized in Fig. 5a. Increases in mean proliferative capacity were observed after 3 months in both GSH groups, but were not significant. No changes were evident in the placebo group. The effects of GSH administration on NK cell cytotoxicity are summarized in Fig. 5a. Increases in mean % lysis values were observed after 3 months in both GSH groups, but were only significant in the high GSH dose arm ($P_{\text{paired}} = 0.01$). No changes were evident in the placebo group. The effects of GSH administration on neutrophil phagocytosis and respiratory burst are summarized in Fig. 5b. No consistent or significant change in either index was observed by study arm after either 3 or 6 months.

Table 2 Correlation of GSH changes from baseline at 6 month in different blood compartments and buccal cells in GSH supplemented individuals

	Pearson's correlation coefficients*			
	Erythrocytes	Plasma	Lymphocytes	Buccal Cells
Blood	0.83 (<0.0001)	0.23 (0.18)	0.55 (0.0004)	0.04 (0.82)
Erythrocytes		0.33 (0.04)	0.45 (0.005)	−0.12 (0.53)
Plasma			0.30 (0.07)	0.02 (0.92)
Lymphocytes				0.05 (0.80)

* r (P value), $n = 38$



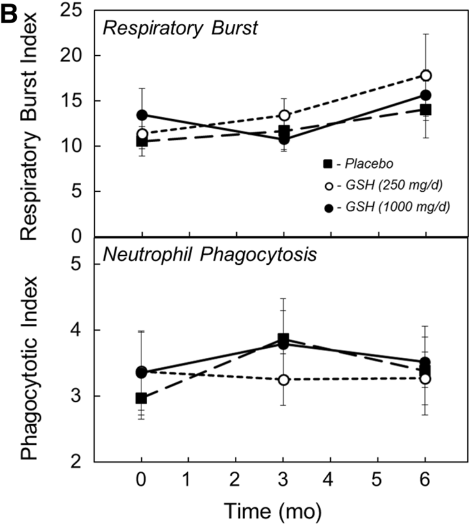


Fig. 5 Effect of oral GSH supplementation on immune function. Subjects were randomized to placebo, 250 mg/day GSH and 1,000 mg/day GSH. GSH or placebo supplementation continued for 6 months followed by a 1-month washout period. Blood and buccal cells were collected at baseline and after 1, 3, 6 and 7 months. Lymphocytes collected at baseline and at 3 months were isolated by density gradient centrifugation of whole blood on Ficoll–Hypaque and utilized for analysis of NK cell cytotoxicity and lymphocyte proliferation (**a**). NK cytotoxicity was assessed using ^{51}Cr-labeled human K562 cells as the target and measuring the percent of target cells lysed after incubation with lymphocytes for 4 h at 37 °C. Lymphocyte proliferation was assessed by measuring ^{3}H-thymidine incorporation after incubation with phytohemagglutinin (PHA) as described in the text. Results are expressed as CPM. *Bars* are mean ± SE. For NK cytotoxicity $n = 6$ for placebo and 1,000 mg/day GSH groups and $n = 5$ for 250 mg/day GSH group. For lymphocyte proliferation, $n = 9$ for placebo and 1,000 mg/day GSH groups and $n = 8$ for 250 mg/day GSH group. Fresh whole blood collected at baseline and at 3 and 6 months was used for assessment of neutrophil phagocytosis and respiratory burst (**b**). Phagocytosis was measured by incubation of whole blood with *E. coli* labeled with a pH-sensitive dye using the pHrodo^{TM} BioParticles kit (Invitrogen). Dye-containing cells were analyzed by flow cytometry, and results were expressed as a phagocytosis index calculated as the geometric mean of gated granulocytes incubated at 37 °C/geometric mean of granulocytes incubated at 4 °C. Respiratory burst was measured after incubation of whole blood with PMA followed by addition of the fluorescence probe dihydrorhodamine 123 and detection of fluorescent neutrophils by flow cytometry. Results are expressed as a respiratory burst index calculated as the geometric mean of PMA-stimulated cells/non-PMA-stimulated cells. *Symbols* and *bars* are mean ± SE. For phagocytosis and respiratory burst assays, $n = 16$, 18 and 20 for the placebo, 250 mg/day GSH and 1,000 mg/day GSH groups, respectively. *Significantly different from baseline by Student's t test, $P < 0.05$

Discussion

The results of this randomized, double-blinded, placebo-controlled study of long-term (6 months) supplementation with GSH at two doses (250 mg/day and 1,000 mg/day) demonstrate for the first time that orally administered GSH in supplement form increased body GSH stores in humans. These findings support the use of oral GSH supplementation as a strategy for increasing tissue GSH levels. Overall, these results are consistent with previous in vivo studies in laboratory animals and findings of specific intestinal transport systems for GSH in laboratory animals in humans [18–23, 30]. In two previous clinical investigations, oral GSH supplementation was not associated with increased tissue GSH levels. In one study, a single oral dose of GSH (0.15 mmol/kg) had no impact on plasma GSH levels after 4.5 h [31]. However, due to the very short half-life (1–2 min) of GSH in human plasma resulting from its rapid removal by tissues such as kidney that are rich in γ-glutamyl transpeptidase [47, 48], the accurate measurement of plasma GSH status in vivo is difficult and the ability to detect an increase in plasma GSH levels after a single oral dose is unlikely [40]. Our results were also not consistent with those from a recent study of oral GSH supplementation (1,000 mg/day) that showed no effects on erythrocyte GSH concentration and biomarkers of oxidative stress after 1 month [49]. However, in this study, GSH and GSSG measurements did not account for possible differences in erythrocyte volume and number which can significantly impact GSH levels. In addition, erythrocytes were not directly acidified immediately after collection, but rather after an initial hemolysis step which can greatly decrease the stability of both GSSG and GSH and lead to inaccurate measurement [50].

The increases in free GSH levels in whole blood and erythrocytes in the current study were dose dependent with the greatest increases occurring in the high-dose (1,000 mg/day) group. Increases in GSH were also time dependent, particularly in the high-dose group, where levels increased from 1–3 to 6 months and decreased

toward baseline after the 1-month washout period. Using this experimental design, the mechanism of GSH induction could not be ascertained. In animal models, direct intestinal absorption of GSH has been observed with transport being facilitated by specific proteins including the cystic fibrosis transmembrane conductance regulator (CFTR) [18, 19, 23, 30]. Facilitated transport of GSH has also been observed and characterized in human intestinal epithelial cells in vitro [30] and in buccal mucosal cells in vivo [23]. The extent to which direct absorption may be responsible for the present findings is not known. The progressive nature of the increase in GSH levels may suggest that changes in GSH metabolism may be occurring as a result of long-term GSH supplementation leading to greater steady-state levels. While it is possible that changes in dietary intake of precursor amino acids resulting in increased GSH biosynthesis could account for some of the observed changes, it is unlikely that group-specific changes in intake would occur with the randomized clinical trial design used. This is supported by our finding that plasma cyst(e)ine levels of erythrocyte GCL activity were unchanged.

In order to gain a more comprehensive assessment of supplementation on body GSH stores, we measured GSH levels in plasma, lymphocytes and exfoliated buccal mucosal cells in addition to whole blood and erythrocytes. In lymphocytes and buccal cells, only total GSH levels (free + bound) were measured since the in vivo levels of protein-bound GSH are very low in these cells and the extent of artifactual GSH oxidation during processing can be quite high. Total GSH was also measured in plasma since overall GSH levels in plasma are low and the majority of GSH is present in the oxidized state [40]. For most measures, GSH increases were both time- and dose responsive and were not impacted by baseline levels of blood GSH. Increases in erythrocyte GSH were highly correlated with those in lymphocytes and plasma supporting a generalized treatment effect of oral GSH on different blood compartments. While increases in buccal cells were not correlated with those in blood, it should be noted that buccal cell GSH levels are highly variable due to heterogeneity of cells obtained in the sampling procedure and the small number cells that are used for measurement which severely limits the power to detect changes and correlations. Overall, GSH was highly tolerated and its administration was not associated with any signs of adverse effects. The trial had high levels of compliance (95.5 % based on pill count), a low dropout rate and no reported significant adverse events in all treatment arms.

The impact of oral GSH on buccal cells is of interest given the previous epidemiologic findings where increased dietary intake of GSH from fruits and vegetables, and higher blood GSH levels were associated with decreased risk of oral cancer [17, 51]. Also, we recently identified a trinucleotide repeat polymorphism in the gene encoding the rate-limiting enzyme in GSH biosynthesis (GCL) which is linked with decreased GCL activity and GSH levels in vivo and, in case control studies, observed an association between this polymorphism and risk of oral and lung cancers [52, 53]. Our present findings suggest that the mechanism of GSH protection against oral cancer development may involve increased GSH levels in oral tissues. It is of interest to note that, in the previous study by Flagg et al. [17], the differences in dietary GSH intake between low and high oral cancer risk groups were 32–358 mg/day in men and 34–126 mg/day in women, well within the dose ranges used in the present intervention trial.

While the majority of GSH in cells is in the reduced form, GSH oxidation can occur resulting in the formation of GSSG or GSSP. The levels of both GSSG and GSSP are increased during periods of oxidative stress. The formation of GSSP, a process known as protein glutathionylation, is thought to play an important redox-sensitive regulatory role in the cell [4, 54]. The ratio of GSSG or GSSP to GSH has often been used as indicators of redox status or biomarkers of oxidative stress [32]. In the present study, we observed a significant decrease in the GSSG/GSH ratio as a result of both low- and high-dose GSH supplementation. This decrease may be indicative of a reduction in oxidative stress resulting from long-term GSH supplementation. Slight reductions in the ratio of GSSP/GSH were also observed, but did not reach the level of significance. Since the subjects were healthy non-smokers, the basal levels of oxidative stress would be expected to be low, thus limiting the ability to detect reductions by GSH. Larger differences in these ratios would likely be expected in individuals exposed to higher levels of free radicals and reactive oxygen species such as tobacco smokers.

Regarding immune parameters, in the high-dose GSH group after 3 months, a significant increase in NK cell cytotoxicity was observed. An effect of GSH supplementation on lymphocyte proliferation was suggested, but the sample sizes for these assays were small and results were not statistically significant. While the mechanisms for these effects are not known, they are consistent with previous in vitro studies demonstrating the importance of intracellular GSH levels in NK cytotoxic activity [55–58]. These studies show that even partial depletion of intracellular GSH can inhibit the effector phase of cytotoxic cell response as well as IL-2-dependent functions. Our results are also consistent with a previous clinical study in which low intracellular GSH levels were correlated with NK cell activity [59]. Overall, the present findings provide a basis for conducting larger studies focusing on immune function.

A limitation of the trial was that there were few male participants. We did not find significant differences in GSH

levels by sex at any time point in the study, but caution should be used when generalizing these findings to men. Gender-related differences in blood GSH levels have been noted in some studies [60, 61], but not in most others including those conducted by our group [32, 38, 62, 63]. Also, there were limited samples available for analysis of NK cell cytotoxicity and lymphocyte proliferation. While significant increases in NK cell cytotoxicity were observed, future studies with a larger sample size and additional time points are required to provide more detailed information on the impact of GSH on this important NK cell activity.

These findings may have implications regarding the treatment of diseases associated with reduced GSH levels [11]. HIV infection represents one such disease where GSH depletion is thought to be an important factor leading to impairments in immune function and disease progression [33, 37, 64]. GSH precursors including cysteine and N-acetylcysteine have been tested for their potential use in the control of symptoms of HIV infection with improvements being noted for some endpoints [65–67]. The use of GSH itself in HIV infection may have advantages over its precursors since it would not require GSH re-synthesis within cells via GCL, the activity of which is reduced in HIV+ macrophages [68]. Indeed, in vitro studies indicate that GSH is more effective in restoring immune function in macrophages from HIV-infected individuals that is *N*-acetylcysteine [68].

Overall, results from this study demonstrate for the first time the effectiveness of long-term GSH supplementation at increasing body stores of GSH in humans. GSH was well tolerated and was not associated with any negative side effects. Beneficial effects on immune function were observed, but additional studies are required to further elucidate the nature of these effects. Importantly, no negative impacts on immune function were observed. These new findings are consistent with studies in laboratory animals [18–23, 30] and suggest that oral GSH supplementation is an effective strategy for increasing body stores of GSH.

Acknowledgments We thank Ashley Knipe for her technical assistance with sample processing and Nate Sheaffer in the Penn State Hershey Flow Cytometry Core Facility. We thank Mr. Yoichiro Sugimura (deceased) and Ms. Erika Hashizume for their support in the design and implementation of this trial. This work was supported by Kyowa Hakko Bio Co., Ltd (JR). Glutathione (Setria®) supplements and placebo were provided by Kyowa Hakko Bio Co., Ltd. Kyowa Hakko Bio Co., Ltd had no role in the conduct, collection, management, analysis and interpretation of the data, or writing of the manuscript. Immunological analyses were supported in part by the Penn State Hershey Cancer Institute through the Clinical Correlative Immunology Laboratory (TS and JH).

Conflict of interest JPR received research support for this study and travel funds to present previous research findings from Kyowa Hakko Bio Co., Ltd. Kyowa Hakko Bio Co., Ltd is a biotechnology and fermentation company that provides glutathione (Setria®). None of the other authors have any conflicts of interest to disclose.

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eCAM Advance Access published May 17, 2007

doi:10.1093/ecam/nem040

Review

The Treatment of Pulmonary Diseases and Respiratory-Related Conditions with Inhaled (Nebulized or Aerosolized) Glutathione

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Reduced glutathione or simply glutathione (γ-glutamylcysteinylglycine; GSH) is found in the cytosol of most cells of the body. GSH in the epithelial lining fluid (ELF) of the lower respiratory tract is thought to be the first line of defense against oxidative stress. Inhalation (nebulized or aerosolized) is the only known method that increases GSH's levels in the ELF. A review of the literature was conducted to examine the clinical effectiveness of inhaled GSH as a treatment for various pulmonary diseases and respiratory-related conditions. This report also discusses clinical and theoretical indications for GSH inhalation, potential concerns with this treatment, its presumed mechanisms of action, optimal doses to be administered and other important details. Reasons for inhaled GSH's effectiveness include its role as a potent antioxidant, and possibly improved oxygenation and host defenses. Theoretical uses of this treatment include Farmer's lung, pre- and postexercise, multiple chemical sensitivity disorder and cigarette smoking. GSH inhalation should not be used as a treatment for primary lung cancer. Testing for sulfites in the urine is recommended prior to GSH inhalation. Minor side effects such as transient coughing and an unpleasant odor are common with this treatment. Major side effects such as bronchoconstriction have only occurred among asthma patients presumed to be sulfite-sensitive. The potential applications of inhaled GSH are numerous when one considers just how many pulmonary diseases and respiratory-related conditions are affected by deficient antioxidant status or an over production of oxidants, poor oxygenation and/or impaired host defenses. More studies are clearly warranted.

Keywords: aerosolized glutathione (GSH) – antioxidant – inhaled GSH – nebulized GSH – reduced GSH

Introduction

Reduced glutathione or simply glutathione (γ-glutamylcysteinylglycine; GSH) is found in the cytosol of most cells of the body (1). It is a tripeptide consisting of glycine, cysteine and glutamate. GSH functions in several enzyme systems within the body that assist with the quenching of free radicals and the detoxification of fat-soluble compounds (Table 1) (2–5). It also plays a significant metabolic role in supporting many different biochemical processes (e.g. amino acid transport, deoxyribonucleic acid synthesis and immune system augmentation) considered to be important mediators of health status (6).

Glutathione in the epithelial lining fluid (ELF) of the lower respiratory tract is thought to be the first line of defense against oxidative stress (6). The ELF concentration of GSH is 140 times that of serum concentrations with a redox ratio of $>9:1$ (7). In fact, alternations in alveolar and lung GSH metabolism are widely recognized as a central feature among many inflammatory lung diseases (8–14). In healthy lungs, the oxidant burden is balanced by local antioxidant defenses. However, in lung

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Table 1. Enzyme systems involving glutathione

Enzyme system	Function
Glutathione synthetase	Gamma-glutamyl cycle.
Riboflavin-containing glutathione reductase	Catalyzes the conversion of oxidized glutathione (glutathione disulfide; GSSG) to its reduced form.
GSH transferase isoenzymes	Conjugation of GSH with fat-soluble substances for liver detoxification and the detoxification of environmental carcinogens, such as those found in tobacco smoke.
Selenium-containing glutathione peroxidase (GPX)	Protects cells from hydrogen peroxides and lipid hydroperoxides. If not neutralized, these peroxides will damage cellular membranes and other vital cellular components.
Leukotriene C_4 synthase	Conjugation of leukotriene A_4 with GSH, resulting in the generation of leukotrienes C_4. Gamma-glutamyl transpeptidase then metabolizes leukotrienes C_4 to leukotrienes D_4.

diseases cellular damage and injury is mediated by an increased oxidant burden and/or decreased antioxidant defenses.

In inflammatory lung diseases, supplementation with exogenous sources of GSH would be necessary to reduce the oxidant load and/or correct for antioxidant deficiencies within the lungs. A few published clinical studies have shown the oral administration of GSH to be ineffective at increasing plasma levels when given to healthy subjects (15), or when used for the treatment of hepatic cirrhosis (16). If the oral administration of GSH cannot raise plasma levels in healthy and diseased patients, it is doubtful that this method of delivery would have any appreciable effects at increasing GSH concentrations within the lungs.

Intravenous administration might be effective since it bypasses the gastrointestinal tract, immediately enters the blood stream, and presumably would saturate body tissues such as the lungs. Unfortunately, the results of a study did not show intravenous administration to be effective at increasing GSH levels within the ELF (17). When 600 mg of GSH was delivered intravenously to sheep, the levels in the venous plasma, lung lymph and ELF increased only for a very brief period of time. However, when the same amount of GSH was delivered through inhalation (nebulized or aerosolized), the baseline GSH level in the ELF ($45.7 \pm 10\,\mu M$) increased 7-fold at 30-min ($337 \pm 64\,\mu M$), remained above the baseline level 1 h later ($P < 0.001$) and returned toward baseline levels by 2 h. Despite this short-term increase in GSH concentrations within the ELF, the inhalation method did not significantly increase the amount of GSH in the lung lymph, venous plasma and urine during the 2 h study period. The authors of this report concluded that inhalation specifically increased GSH levels at the lung epithelial surface.

Given that inhalation is the only known method that increases GSH levels in the ELF for a significant duration, a review of the literature was conducted to examine the clinical effectiveness of inhaled GSH as a treatment for various pulmonary diseases and respiratory-related conditions. Only reports involving human subjects were included in the analysis. The clinical and theoretical indications for GSH inhalation were summarized and potential concerns with this treatment reported. Other pertinent details such as its presumed mechanisms of action and optimal doses to be administered were compiled and evaluated.

Methods

Literature Search

Computer searches were conducted of English and non-English language articles in the Biomedical Reference Collection (1984 to August 2006), CINAHL (1982 to August 2006), MEDLINE (1965 to August 2006) and Nursing and Allied Health Collection (1985 to August 2006) databases. Articles were searched with the key search terms 'Nebulized Glutathione,' and 'Glutathione' in combination with 'Aerosol' OR 'Inhalation.' These keywords were also searched with words related to pulmonary and/or respiratory disease. To supplement the search, references of the articles found from the initial search were reviewed. Hand searching of relevant journals was also completed as part of the search.

Selection of Articles

To be included in the final review, articles had to report on the use and administration of inhaled GSH for pulmonary diseases and respiratory-related conditions in human subjects. Only peer-reviewed articles were reviewed.

Quality Assessment

An evidence grade was determined for each article. These evidence grades were adapted from the hierarchy of evidence developed by the Oxford Centre of Evidence Based Medicine (Table 2) (18).

Table 2. Grades of evidence

A	Systematic reviews of randomized controlled trials and/or randomized controlled trials with or without double-blind placebo control.
B	Systematic reviews of observational studies and/or high-quality observational studies including cohort and case-control studies and/or cohort 'outcomes' research and/or nonrandomized controlled trials.
C	Case-series, case-reports, and/or poor-quality cohort and case-control studies.
D	Expert opinion without explicit critical appraisal or based on physiology, bench research or 'first principles.'

Results

A total of 12 reports were screened (9,10,17,19–27). Only one report was excluded because it involved the use of inhaled GSH in sheep (17). In total, 11 articles were found to meet the inclusion criteria and were included in this review (9,10,19–27). Table 3 displays the characteristics of the studies included in this review.

Discussion

Based exclusively on the published evidence included in this review, inhaled GSH is potentially indicated for the following clinical conditions: cystic fibrosis (CF), chronic otitis media with effusion (OME), HIV seropositive individuals, idiopathic pulmonary fibrosis (IPF) and chronic rhinitis. These conditions were chosen since the published studies were of good quality, received A and B evidence grades, and their respective results demonstrated benefits from the use of GSH inhalation.

Inhaled GSH cannot be recommended as a potential treatment for emphysema since the quality of evidence is lacking at the present time. The emphysema case report had notable limitations since serial spirometry was not documented, and the placebo effect could not be ruled-out (22). However, this does not necessarily indicate that GSH inhalation would be of no benefit for emphysema patients. There is experimental and human data demonstrating a link between GSH, oxidant-derived damage and possible protection against the development of emphysema. An *in vitro* study demonstrated that GSH could retard the oxidant-mediated down-regulation of α-1-proteinase inhibitor activity in smokers' emphysema (28). This finding is important since one of the principal pathophysiological mechanisms of emphysema is the down-regulation of this enzyme by means of oxidative damage (29). Moreover, in a recent review of lung GSH and cigarette smoke-induced airway disease, increased GSH in the ELF of chronic smokers was presumed to be a protective adaptive mechanism against the development of chronic obstructive pulmonary disease (COPD) (30). Considering that not all chronic smokers go on to develop COPD, the authors in that review pointed out that genetic variations in the molecular mechanisms that regulate GSH metabolism might explain why some individuals are better protected against the development of COPD. It thus appears that emphysema patients are subjected to progressive tissue damage due, in part, to the consequences of GSH deficiency and/or genetic variations in GSH metabolism. Since GSH inhalation would presumably offer both antioxidant protection and GSH replenishment, this method of treatment would potentially benefit emphysema patients.

Asthma is another condition where inhaled GSH cannot be recommended since this treatment caused notable side effects (e.g. breathlessness, bronchoconstriction and cough) in the cited study (21). These side effects were linked primarily to the production of sulfites that occurred when GSH was in solution. GSH inhalation should continue to be explored as a potential treatment for asthma. None of the asthma patients in the study had their urine tested for sulfites. A positive test for sulfites would have eliminated these patients from entering the study. Accordingly, the results might have been much more favorable if patients without sulfite sensitivities were included.

This issue of asthma and sulfite sensitivities is an important one for clinicians to be mindful of. Sulfites are found in beer, wine, restaurant salad bars, seafood, potatoes, processed foods and many pharmaceuticals (31). Many asthma patients report being sensitive to sulfites. In an Australian study, ~30% of asthmatic patients reported being sensitive to sulfites in wine (32). A more recent and rigorous scientific study, however, demonstrated that asthma patients can tolerate varying amounts of sulfites in wines ranging from 20, 75 or 150 parts per million (ppm) (33). Only a small minority of patients in this study (4 of 24 self-reported wine-sensitive asthmatics) exhibited reactions when challenged with 300 ppm of sulfites. One report indicated that 4–8% of asthmatics are sensitive to sulfites (34). Other reports have estimated the incidence of sulfite sensitivity to be around 5–11% (35,36). Even though the exact percentage of sulfite-sensitive asthmatics is difficult to ascertain, sulfite sensitivity is an important factor to assess when using or evaluating research done on inhaled GSH.

Future Research Directions

There are additional clinical conditions that might benefit from this type of treatment, but further studies are necessary. One such condition is Farmer's lung (FL), which is a hypersensitivity pneumonitis caused by the inhalation of thermophilic actinomycetes and spores of *Aspergillus* specie (11). A study was undertaken to investigate the effect of pulmonary GSH levels after hay exposure in patients with FL and in asymptomatic farmers (AF) (11). Fifteen symptomatic patients with FL

Table 3. Summary of articles demonstrating the effectiveness of inhaled glutathione for the treatment of pulmonary diseases and respiratory-related conditions

Reference	Condition	*N*	Dosages of inhaled GSH	Outcome	Evidence grade
(21)	Asthma	Eight asthma patients [mean age, 29 ± 7 (standard deviation; SD) years]	600 mg once weekly for 3 months	A subset of patients with clinically stable mild asthma experienced a bronchoconstrictor effect when treated with inhaled GSH.	A: Randomized placebo-controlled trial
(23)	Chronic otitis media with effusion (chronic OME)	30 patients (3–12 years of age; mean age, 5.8 years) and 30 controls (3–12 years of age; mean age, 6.1 years)	600mg of GSH in 4 ml of saline subdivided into five 2-min sessions by nasal aerosol every 3–4 waking h for 2 weeks	GSH should be considered for the nonsurgical management of chronic OME.	A: Randomized placebo-controlled trial
(24)	Cystic fibrosis (CF)	Nine patients [mean age, 16.1 ± 1.44 (SD) years] received the S-nitrosoglutathione (GSNO) and 11 patients [mean age, 19.9 ± 3.45 (SD) years] received the phosphate-buffered saline (PBS) solution	0.05 ml/kg of 10 mM GSNO	The treatment group showed a modest improvement in oxygenation that was thought to be independent of the physiological effects of nitric oxide.	A: Randomized placebo-controlled trial
(27)	CF	19 patients (6–19 years of age) were randomized to treatment [mean age, 13.3 ± 4.1 (SD) years] or placebo groups [mean age, 12.9 ± 4.9 (SD) years]	Total daily dose administered to the patients in the treatment group was 66 mg/kg of body weight	GSH can improve clinical parameters in CF patients, and that effective treatment should include the correction of GSH deficiency.	A: Randomized placebo-controlled trial
(9)	Idiopathic pulmonary fibrosis (IPF)	10 patients with IPF [mean age, 46 ± 3 (SD) years] and 19 normal nonsmokers [mean age, 36 ± 3 (SD) years]	600 mg twice daily for 3 days	Inhaled GSH might be beneficial among IPF patients by reversing the oxidant–antioxidant imbalance.	B: Nonrandomized controlled trial
(19)	Human immunodeficiency virus (HIV) seropositive individuals	14 HIV seropositive individuals [mean age, 32 ± 2 (SD) years]	600 mg twice daily for 3 days	It is a reasonable therapeutic strategy to augment the deficient GSH levels of the lower respiratory tracts of HIV seropositive individuals.	B: Cohort 'outcomes' research
(20)	Chronic rhinitis	13 patients with chronic rhinitis and 13 healthy subjects (4–15 years of age for all subjects; mean age, 8.2 years)	600 mg daily for 14 days	Statistically significant improvement in nasal obstruction, rhinorrhea and ear fullness.	B: Nonrandomized controlled trial
(10)	CF	Seven CF patients [mean age, 25 ± 1 (SD) years]	600 mg of GSH for 3 days	Inhalation therapy with GSH does normalize the respiratory epithelial surface oxidant–antioxidant balance in CF patients.	B: Cohort 'outcomes' research
(25)	CF	21 patients with CF (16–37 years of age for all subjects)	300 or 450 mg three times daily for 14 days	Inhaled GSH can permeate the lower airways of the lungs and improve important parameters of lung function in CF patients despite not having any effect upon markers of oxidative injury.	B: Cohort 'outcomes' research

(continued)

Table 3. Continued

Reference	Condition	*N*	Dosages of inhaled GSH	Outcome	Evidence grade
(26)	CF	17 patients with CF (18–29 years of age for all subjects; mean age, 24 years)	450 mg three times daily for 14 days	Inhaled GSH did not affect the oxidative status of the patients who were tested, but it did favorably modulate their immune responses.	B: Cohort 'outcomes' research
(22)	Emphysema	One (95 year-old male)	120 mg of GSH in office, then 120 mg twice daily for 3 days, and continuation of treatment (dose unknown) for 2 years	When the patient returned for a follow-up visit, he no longer required the use of his wheelchair and oxygen. The striking results were unexpected and unlikely to be due to placebo alone.	C: Case report

[mean age, 42 ± 1 (SD) year] were compared with 10 AF [mean age, 43 ± 1 (SD) year] serving as the control group. All patients had baseline lung function testing and testing at various time intervals following hay exposures. The authors of this study concluded that FL and AF patients have characteristically different intrapulmonary levels of GSH, and that the pathogenesis of FL is likely related to GSH regulatory mechanisms. They also speculated that AF patients have a better ability to upregulate their pulmonary GSH levels, which would protect them against active disease. Clinical testing of inhaled GSH in patients with FL is warranted.

The administration of GSH inhalation before and/or immediately following exercise is another potential application of this novel treatment. Exercise is a known inducer of oxidative stress leading to free radical production, which can encourage lipid peroxidation and tissue damage among individuals with deficient and/or impaired antioxidant systems. As stated in the beginning of this report, selenium is a cofactor in the GPX enzyme that protects cells from hydrogen peroxides and lipid hydroperoxides. When under situations of oxidative stress, the GPX enzyme will markedly increase in the lungs as an antioxidant adaptive response (37). By supplying more GSH to the lung tissues, more of this enzyme might be available to help reduce the production of free radicals associated with exercise. Although these assumptions are very speculative, it does seem possible and even logical that GSH inhalation would benefit those who regularly exercise by increasing exercise tolerance, and by maintaining and/or replenishing the antioxidant systems within the lungs.

Multiple chemical sensitivity disorder (MCSD) is another condition that might be clinically responsive to this treatment. Patients with this disorder are known to have bronchial hyperreactivity and even exhibit asthma-like symptoms (38). Unlike asthma, MCSD is not associated with atopy and immunoglobulin E (IgE)-mediated allergic mechanisms (39). The prevailing theory explaining the cause of MCSD is a fusion between two separate theories—the neural sensitization and nitric oxide/peroxynitrite theories (40). This fusion theory, proposed by Pall, links long term potentiation of *N*-methyl-d-aspartate (NMDA) receptors at the synapses of nerve cells by glutamate and aspartate to an increased production of nitric oxide and its oxidant product, peroxynitrite (40,41). Treatment with antioxidants may improve symptoms of MCSD by reducing the peroxynitrite elevations and other biochemical dysfunctions that are associated with such elevations (40,41). Glutathione inhalation may be ideal since the primary route by which patients with MCSD get triggered is through smelling and breathing. Sulfite sensitivity would have to be considered since inhaled GSH could provoke adverse events. This treatment might be capable of providing antioxidant protection to both the upper and lower respiratory airways, which would theoretically help to reduce the symptoms of MCSD and the production of peroxynitrite. More research studies are necessary.

Two final conditions, cigarette smoking and lung cancer, are worth mentioning since they are intimately related to each other and are affected by GSH and its related enzymes. These conditions are influenced by the glutathione S-transferase (GST) group of enzymes that are found in significant quantities in the bronchioles and alveoli of the lungs (42), and in very high concentrations in the bronchial epithelium (43). Among smokers, a lack of the GST mu enzyme was thought to be associated with a greater risk of lung cancer, especially if there was a cancer and/or lung cancer history among the relatives of the patients in this study (44). Since the GST mu enzyme detoxify carcinogens in tobacco, any deficiency of this enzyme was presumed to be associated with an increased risk of lung cancer. However, a more recent study pertaining to the GST group of enzymes found no such association (45). In this meta-analysis, polymorphisms in the GST genes had no associations or weakly positive associations with risk factors for lung cancer. Despite the

need for more research, GSH inhalation might be beneficial for smokers to augment their GST enzymes, which would help facilitate the detoxification of carcinogens. Even though the best intervention for these patients would be smoking cessation, many patients lack the necessary willpower to quit. For these patients, regular GSH inhalation might reduce oxidants generated from cigarette smoke ($\sim 10^{14}$ free radicals/puff) (46), and the epithelial lung injury associated with smoking (47).

For lung cancer patients, the use of GSH inhalation is not recommended. Cancer cells use multiple mechanisms (e.g. altered transport of a drug, inhibition of drug-induced apoptosis and elevation of cellular GSH) to circumvent the cytotoxic effects of chemotherapeutic agents (48). Early research studies showed that GSH was able to reduce cytotoxicity to chemotherapeutic compounds by boosting the metabolism of drugs to less active compounds, or by the detoxification of free radicals (49,50). More recently, research has revealed that the levels of a specific GST enzyme increases among cancer cells with higher differentiation grades, and that these drug-resistant gene products are found in lung carcinomas at the time of surgical resection (51). There is also speculation that GSH might be capable of repairing drug-induced injury at the DNA level (48). A recent review article has described the involvement of glutathione in the detoxification or inactivation of platinum drugs—the most commonly employed drugs for the treatment of advanced stage lung cancer patients (52). Based on this information, it would be unwise and illogical to use GSH inhalation while lung cancer patients are undergoing active chemotherapy treatment.

Mechanism of Action

Inhalation of GSH results in a mechanism of action confined to the upper airways and lungs (Fig. 1), and will not influence plasma levels to a significant degree. In the studies that measured both lung and plasma levels of GSH, the plasma levels remained essentially unchanged following GSH inhalation. Seven of the studies included in this review demonstrated that GSH inhalation exerts its effects upon the lower respiratory tract (9,10,19,24–27). The upper respiratory tract also appears to benefit from GSH augmentation. Two studies involving patients with upper respiratory tract diseases showed clinical benefits from GSH inhalation treatment (20,23). The predominant mechanism responsible for GSH's therapeutic effects are probably related to its antioxidant properties that offer protection against oxidative injury, and/or assist with the normalization of the oxidant–antioxidant balance within the upper and lower respiratory tract. Even though the majority of these studies suggested that antioxidant protection was the principal reason for the favorable treatment responses, some of the studies were unable to demonstrate a change in markers of oxidation from this treatment. More data is necessary to confirm the precise nature of GSH's antioxidant properties within the upper and lower respiratory tract. Additional explanations for GSH's therapeutic effects might include an improvement in host defenses (e.g. increased cytotoxic lymphocytes), and better oxygenation (e.g. an increase in oxygen saturation). GSH inhalation produced clinically meaningful results in the majority of diseases that were studied. Specifically, GSH inhalation was shown to improve clinical markers of respiratory function that inevitably impact upon quality of life and disease progression. These improvements were the most important outcomes and features of this novel treatment.

Considerations Prior to Initiating GSH Inhalation

The urine should be tested for sulfite sensitivity. A special test strip can be dipped in the urine, and is known as the 'EM-Quant 10013 Sulfite Test.' It can be easily located through any search engine on the Internet (53). Even though instructions for sulfite testing have been published elsewhere (54), a brief description of the procedure is outlined below:

> A random (fresh) urine sample is suitable, but a first morning void may be preferable due to its higher concentration. Once the test strip is dipped in the urine (for 1 s), the reaction zone changes color to indicate the concentration of sulfites present. After 30 s, the color on the test strip is compared to a color scale on the bottle indicating the concentrations of sulfites in the urine (can detect 10, 40, 80, 180 and 400 ppm of sulfites). The resultant concentration should be multiplied by a factor of 1.5 to provide the amount of free sulfites in mg/l (ppm). The strip will not detect below 10 ppm. The urine samples should be preservative free, and the urinary pH should also be tested with pH paper. If the urine pH is below 6, then the amount of sulfites might be underestimated by the test. In such cases, consider adding sodium acetate or sodium hydroxide to raise the pH to at least 7–10 (should not exceed a pH of 12), and then repeat with a new test strip.

If the urine test were positive for sulfites (normally they are absent), the use of inhaled GSH would be strictly contraindicated.

Method of Delivery, Recommended Daily Dosages and Side Effects

With a nebulizer, a solution of GSH is made into an aerosol and is delivered to the upper respiratory tract and the lungs through a mask that covers the nose and mouth, or is delivered directly into the lungs via a mouthpiece. Any compounding pharmacist would be

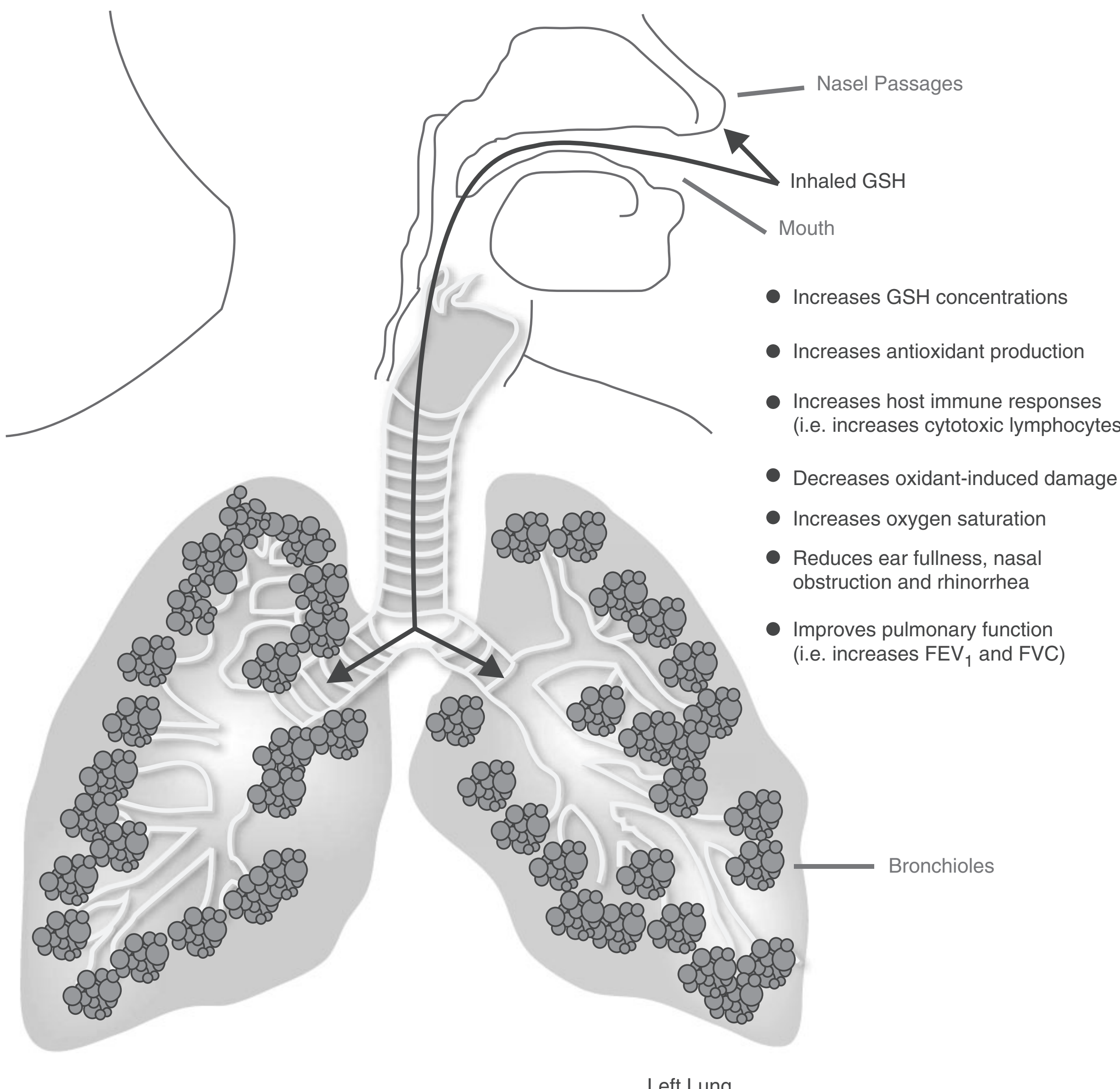

Figure 1. Inhaled GSH's mechanism of action. GSH, reduced glutathione; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity.

able to prepare the solution of GSH at the desired concentrations. The typical dosages used in the studies cited in Table 3 were 600 mg once daily, 600 mg twice daily, 900 mg daily, 1350 mg daily or a daily dose of 66 mg/kg of body weight. Better results are more likely to be achieved with doses of at least 600 mg or more each day. One of the studies used much larger doses (66 mg/kg of body weight) since the authors speculated that these would be necessary to replace half of the amount of GSH that is produced each day (e.g. a 150 lb male synthesizes 10 g daily and would need 5 g as a replacement dose) (27). When patients are unresponsive to doses in the range of 600–1350 mg per day, it might be suitable to try doses that would replace half the estimated amount of GSH that is synthesized each day. These gram doses might yield better clinical results.

In terms of side effects, GSH inhalation is very safe. Minor side effects such as mild coughing and an unpleasant odor were reported in some of the studies included in this review. These minor side effects, better described as mild nuisance problems, were not severe enough to cause any of the study participants to discontinue treatment with inhaled GSH. The only worrisome or potentially life-threatening side effect to note is bronchoconstriction, which would be more likely to occur among sulfite-sensitive asthma and MCSD patients. However, if proper precautions such as sulfite testing are done prior to treatment, this serious side effect should be avoidable.

Monitoring the Clinical Response to Inhaled GSH

For pulmonary diseases or respiratory-related conditions, baseline pulmonary function testing with a spirometer or

a simple peak flow meter is recommended prior to the first treatment. After a prescribed period of treatment time, pulmonary function tests should be repeated. This will help to establish if there are any clinical improvements from regular GSH inhalation.

Conclusions

GSH inhalation is an effective treatment for a variety of pulmonary diseases and respiratory-related conditions. Even very serious and difficult-to-treat diseases (e.g., CF, IPF) yielded benefits from this novel treatment. GSH inhalation is very safe, and rarely causes major or life-threatening side effects. The potential applications are numerous when one considers just how many pulmonary diseases and respiratory-related conditions are affected by deficient antioxidant status, poor oxygenation and/or impaired host defenses. More studies are clearly warranted.

Acknowledgments

The author would like to thank Mr Glen Carr and Mr Andrew Dick for their wonderful illustration of GSH's mechanism of action.

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Received December 22, 2006; accepted February 13, 2007

March 2, 2018

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RE: Docket FDA-2015-N-3534

Dear Ms. Hallman,

McGuff Compounding Pharmacy Services, Inc. (MCPS) is responding to the FDA's questions to the nomination of (glutathione) Reduced L-glutathione's inclusion on the 503A bulk drug substances list due by March 2, 2018.

Responses:

Q. Does MCPS still want to pursue review by the FDA and consideration by the PCAC of Reduced L-glutathione's for inclusion on the 503A bulk list?
A. Yes.

Q. Please submit in writing the disease state(s) or health condition(s) that you are proposing for FDA's review, the dosage form and strength/concentration proposed for each use, and clinical, if available and other scientific articles in support of each use. If this information is not submitted for a proposed use, FDA does not intend to review the nominated substance for that use.
A. Traditionally, pharmacies are not required to document the purported use of a prescribed drug, compounded or otherwise. Hence, we cannot report all the intended uses of compounded glutathione preparations by practitioners who have prescribed them. However, in alignment with the FDA's own guidance definition that an API or bulk drug substance is, "any substance that is intended for incorporation into a finished drug product and is intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body…," we have compiled the following information based on our own historical experience with practitioners' usages of glutathione as well as those supported by literature:

Peripheral Obstructive Arterial Disease
Dosage Form: Aqueous parenteral solution of 100 mg/mL to 200 mg/mL concentrations

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Effect of Glutathione Infusion on Leg Arterial Circulation, Cutaneous Microcirculation, and Pain-Free Walking Distance in Patients With Peripheral Obstructive Arterial Disease: A Randomized, Double-Blind, Placebo-Controlled Trial. ENRICO AROSIO, MD; SERGIO DE MARCHI, MD; MASSIMO ZANNONI, MD; MANLIO PRIOR, MD; AND ALESSANDRO LECHI, MD. Mayo Clin Proc. 2002;77:754-759.

Parkinson's Disease

Dosage Form: Aqueous intravenous solution of 100 mg/mL to 200 mg/mL concentrations

Glutathione—a review on its role and significance in Parkinson's disease. Martin H & Teismann P. The FASEB Journal. 2018;23(10):3263-3272.

The Use of Intravenous Glutathione for Symptom Management of Parkinson's Disease: A Case Report. Madalyn Otto, ND; Tracy Magerus, ND; Jeffrey Langland, PhD. ALTERNATIVE THERAPIES.

Pulmonary Diseases & Respiratory-Related Conditions

Dosage Form: Aqueous inhalation solution of 100 mg/mL to 200 mg/mL concentrations

The Treatment of Pulmonary Diseases and Respiratory-Related Conditions with Inhaled (Nebulized or Aerosolized) Glutathione. Prousky J. eCAM. 2008;5(1):27-35.

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Cancer Treatment; Enhancement of Treatment and Alleviation of Adverse Chemotherapy Effects

Dosage Form: Aqueous injection solution of 100 mg/mL to 200 mg/mL concentrations.

Neuroprotective Effect of Reduce Glutathione on Oxaliplatin-Based Chemotherapy in Advance Colorectal Cancer: A Randomized, Double-Blinded, Placebo Controlled Trial. Cascinu S et al. Journal of Clinical Oncology. 2002;20(16):3478-3483.

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Immune System Protection/Enhancement

Dosage Form: Aqueous injection solution of 100 mg/mL to 200 mg/mL concentrations

Atherosclerosis: pathogenesis and increased occurrence in individuals with HIV and *Mycobacterium tuberculosis* infection. Guilford T et al. HIV/AIDS – Research and Palliative Care. 2010;2:211-218.

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Q. Glutathione is unlikely to be stable under ordinary storage conditions when compounded as a solution, cream, or gel, which are among the nominated dosage forms. Please provide any information available about how these issues are addressed for compounded products.

A. MCPS has performed a stability study on for its Reduced L-glutathione compounded aqueous injection preparations to demonstrate formulation stability through the assigned Beyond-Use Date. The following parameters were examined and/or tested as part of the stability program:

i. Appearance, seal
ii. Appearance, vial
iii. Appearance, solution
iv. Foreign matter, visible particulate
v. pH
vi. Potency assay
vii. Endotoxin
viii. Sterility, Initial and at or after Beyond Use Date
ix. Method suitability, sterility test

x. Method suitability, Inhibition/Enhancement, Endotoxin test
xi. Container closure integrity
xii. Preservative Antimicrobial Effectiveness Test (for multi-dose vial)
xiii. Preservative concentration assay (for multi-dose vial)
xiv. Potency assay of an expired sample, after 7 days of exposure to 40-50 deg C

For dosage forms with which MCPS does have any stability data, the shelf life given follows USP guidelines for Beyond-Use Date assignments.

We would like to note that since the beginning of the year, we have received four requests to affirm our nomination of certain bulk drug substances and to provide FDA with additional information. No context has been provided for these requests; they have been sent one after another with no explanation of when the given ingredient will be considered by the Pharmacy Compounding Advisory Committee (PCAC) or what identical requests from FDA may be forthcoming. Because these requests also seek a response within a short time – typically 10 days from our receipt of FDA's emailed messages – there is very little time to react.

FDA is not being well served by this process, nor is public safety. Our organization, along with the other nominators, is being forced to gather information quickly, so the responses we are able to provide are inevitably incomplete. As FDA and PCAC are expected to act on this information, the implications for patient care and safety are significant. Information concerning dosages, delivery methods, clinical studies supporting proposed uses, etc. ought to be thorough and extensive. If PCAC is expected to make recommendations – and the Agency to consider them – based on rushed and incomplete information, good analysis and sound decision making are being shortchanged. Ultimately, public health and safety are being jeopardized.

We hasten to add that FDA's original request for nominations asked only for a substance's "proposed use" and did not ask for the disease indication or condition, nor to prioritize all uses, nor the scientific articles in support of each use. Fulfilling these requests requires further time and consideration – much more than 10 days or 2 weeks.

Based on the foregoing, we ask that FDA do the following:

- Provide at least 60 days for nominating organizations to respond
- When a request is made, indicate approximately when that bulk drug substance will be considered by the PCAC.

We will continue to provide to the best of our ability the information that is being requested by each bulk drug substance nomination questionnaire. However, for all previous responses we have submitted as well as future submissions, we reserve the right to add, modify or delete information provided in our testimony before the committee.

We look forward to your timely response, and thank you very much for your consideration.

Sincerely,

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HIV/AIDS - Research and Palliative Care

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REVIEW

Atherosclerosis: pathogenesis and increased occurrence in individuals with HIV and *Mycobacterium tuberculosis* infection

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Devin Morris[2,4]
Dennis Gray[3,4]
Vishwanath Venketaraman[3,4]

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Abstract: Atherosclerosis is a leading cause of coronary heart disease and stroke. Since 1981, more than 980,000 cases of AIDS have been reported in the United States. According to the Centers for Disease Control, more than 1 million Americans may be infected with HIV. By killing or damaging CD4+ T cells of the body's immune system, HIV progressively destroys the body's ability to fight infections. People diagnosed with AIDS often suffer from life-threatening diseases caused by opportunistic infections such as tuberculosis. HIV-infected individuals have increased risks for atherosclerosis. This review summarizes the effects of oxidized low density lipoproteins in impairing macrophage functions in individuals with atherosclerosis (with and without HIV infection) thereby enhancing the susceptibility to *Mycobacterium tuberculosis* infection.

Keywords: AIDS, HIV, *Mycobacterium tuberculosis*

Introduction

Oxidized low-density lipoprotein (ox-LDL) has been shown in several studies to be an independent marker of the progression of atherosclerosis.[1–4] The pathophysiology relates to macrophage ingestion of excess ox-LDL and the formation of foam cells, the acknowledged trigger of atherosclerosis.[5] It has been shown that both high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol contain the antioxidant enzyme glutathione peroxidase embedded in the lipoprotein, and a continuous supply of glutathione (GSH) is needed to prevent the oxidation of HDL and LDL cholesterol.[6] The observation that excessive ox-LDL ingestion by macrophage interferes with normal macrophage function may have implications in other disease conditions in which low GSH plays a role, such as HIV infection. Individuals with HIV infection are at increased risk for the progression of atherosclerosis[7] as well as infectious diseases such as tuberculosis (TB).[8] This review postulates that the compromise in macrophage function that occurs with HIV may increase the risk of both atherosclerosis as well as susceptibility to *Mycobacterium tuberculosis* (*M. tb*) infection. The increase in ox-LDL ingestion and resulting macrophage dysfunction may contribute to the increased risk for *M. tb* infection in both HIV-positive and HIV-negative individuals.

Atherosclerosis

Atherosclerosis is a leading cause of coronary heart disease and stroke, which were responsible for more than 589,000 deaths in 2005 – almost 25% of all deaths in the United States. Each year more than 1.2 million will suffer a coronary attack and almost

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DOI: 10.2147/HIV.S11977

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800,000 people will suffer a stroke. Atherosclerosis is a process in which deposits of fatty substances, cholesterol, cellular waste products, calcium and other substances build up in the inner lining of an artery. This build-up is called plaque.[7] It usually affects large- and medium-sized arteries. Some hardening of arteries often occurs when people grow older. Plaques can grow large enough to significantly reduce the blood's flow through an artery. But most of the damage occurs when they become fragile and rupture. Plaques that rupture cause blood clots to form that can block blood flow or break off and travel to another part of the body. If either happens and blocks a blood vessel that feeds the heart, it causes a heart attack. And if blood supply to the arms or legs is reduced, it can cause difficulty walking and eventually lead to gangrene. Atherosclerosis can result in myocardial infarction, and bits of plaque can lodge in arteries in the brain, causing a stroke.[7]

Glutathione

GSH is a tripeptide that, in its reduced form, protects cells against oxidizing agents, free radicals and reactive oxygen intermediates (ROI). In addition to its antioxidant role, GSH plays a vital role in maintenance of cell viability and regulating immune cell functions.[9] Synthesis of GSH occurs in 2 steps. The initial step (the rate limiting step) is the formation of a dipeptide, γ-glutamyl cysteine, a reaction that is catalyzed by γ-glutamylcysteine synthetase. Intracellular levels of L-cysteine are substantially lower than levels of L-glutamate and glycine. Therefore, GSH synthesis is limited by the availability of cysteine.[10] The second step involved in the synthesis of GSH is the formation of γ-glutamyl cysteine glycine, catalyzed by the enzyme GSH synthetase.[10] Intracellular GSH levels can be increased or decreased by treatment with N-acetyl cysteine (NAC) or buthionine sulfoximine (BSO), respectively. The most efficient way to increase the levels of cysteine in cells grown in vitro is to supply the culture medium with NAC, which is easily taken up by the cells and is nontoxic. Intracellularly, NAC is de-acetylated and cysteine is utilized for GSH synthesis. BSO specifically inhibits the activity of the γ-glutamyl-cysteinyl synthetase enzyme, which catalyzes the first step reaction in the synthesis of GSH,[9,10] leading to inhibition in the synthesis of GSH.

Oxidized LDL

Oxidation of LDL (ox-LDL) cholesterol has been shown to convert LDL cholesterol to a form that is recognized by scavenger receptors on macrophages and to contribute to foam cell formation.[6] It has been known for some time that vitamin E and β-carotene are found in the LDL complex and that decreases in vitamin E and β-carotene are early events reflecting the initial stages of lipid peroxidation formation.[11] It has only recently been shown that glutathione peroxidase (GPx) is also found to occur naturally in the LDL lipoprotein. Substituting the unique substrate for GPx, reduced GSH, with liposomal GSH can slow the formation of ox-LDL in vitro in human blood and slow atherosclerosis in vivo in the ApoE$^{(-/-)}$ mouse model of atherosclerosis.[12]

Ox-LDL and atherosclerosis

Ox-LDL has been shown in several studies to be an independent marker of the progression of atherosclerosis.[1–4] The pathophysiology relates to macrophage ingestion of excess ox-LDL and the formation of foam cells, the acknowledged trigger of atherosclerosis.[5] The mechanism of ox-LDL and the requirement for GSH to prevent oxidation of LDL cholesterol has been described in Rosenblat et al[12] using liposomal GSH as the source of GSH in both human blood, in vitro studies and in the mouse model of atherosclerosis. This study demonstrated that both LDL and HDL contain the antioxidant enzyme GSH peroxidase embedded in the lipoprotein.[12] As GSH is the single substrate for GSH peroxidase, a continuous supply of GSH in the reduced state is needed to prevent the oxidation of LDL and HDL cholesterol. This study also demonstrated that preventing the oxidation of LDL and increasing the intracellular GSH levels is able to maintain normal macrophage function. It is likely that the presence of excess amounts of ox-LDL will be an indicator of general macrophage dysfunctions as it has been shown to stimulate the release of macrophage inflammatory protein 1-alpha (MIP-1alpha)[5] as well as other immune responses. Ox-LDL is known to be ingested by macrophages and without adequate antioxidant function is prone to form foam cells (Figure 1), a transition which is also a contributing factor to the formation of atherosclerosis[14] and atherosclerotic lesions.[13–15]

Macrophages and atherosclerosis

Macrophages are derived from the circulating pool of monocytes.[16] Monocytes are produced in the bone marrow and, in the absence of specific survival signals, are programmed to undergo apoptosis in 24–48 hours.[16,17] When monocytes are recruited into local environments they differentiate into macrophages. In arteries, macrophage scavenging function may be diverted to the formation of plaque; in the lung

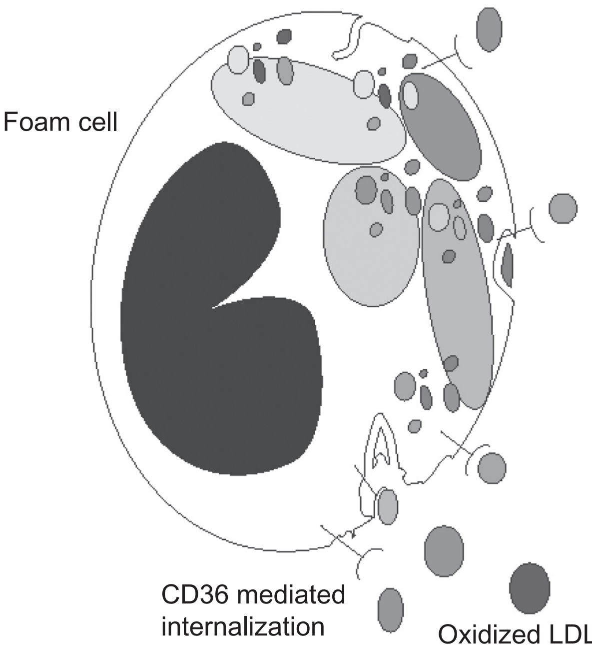

Figure 1 Receptor mediated uptake of oxidized low density lipoproteins by macrophages resulting in foam cell formation.

they become alveolar macrophages. In atherosclerosis, macrophage oxidative impairment occurs in the GSH-related antioxidant system,[5] probably related to the unregulated ingestion of ox-LDL via the CD36 receptor.[18,19]

Role of GSH in macrophage function

In the natural state, GSH levels are maintained by both the synthesis of GSH and by GSH reductase, an enzyme that catalyzes the reduction of glutathione disulfide (GSSG) to GSH, with NADPH as the reducing cofactor. Increasing the expression of GSH reductase, the enzyme which returns GSH to the biochemically reduced (functional) state, decreases atherosclerotic lesions.[20] It has been shown that lymphocyte proliferation after mitogenic lectin exposure is decreased[21] with low intracellular GSH.[21,22]

The level of reduced GSH in the extracellular lung fluid (ELF) or alveolar lining fluid has been estimated to be as much as 140-fold higher than the level of reduced GSH found in the plasma of the same individuals.[23] The antioxidant GSH is essential for the detoxification of endogenous and exogenous oxidant radicals and protection of cells residing in the airway and alveolus[24,25] and during oxidative stress such as cigarette smoking.[26] In disease states, the level of ELF GSH may decrease dramatically to the point that acute respiratory distress syndrome has only 0.05% of the amount found in the normal ELF.[27]

In the lung, intracellular antioxidants are expressed at a relatively low level in the lung epithelial tissue and are not induced by oxidative stress. The major antioxidants in the lung are extracellular and the GSH system is the major antioxidant mechanism used in the airways.[28] Alveolar macrophage cells rely on the ELF level of GSH to provide the biochemical support to maintain intracellular GSH levels[29] and protect their membranes during the respiratory burst.[30]

When macrophage GSH availability is limited, cellular functions such as phagocytosis and microbial clearance are compromised.[24] The mouse model of decreased alveolar macrophage GSH resulted in a decrease in the rate of phagocytosis of individual macrophages as well as a decrease in the overall percentage of macrophages positive for phagocytosis.[24] Similar findings have been reported in the alveolar function of guinea pigs exposed to ethanol in utero resulting in low macrophage GSH. The addition of precursors of GSH to the diet of the ethanol-exposed animals increased the GSH levels in the ELF and alveolar macrophages and maintained both the rate of internalization and the number of macrophages positive for internalization.[24] It was also shown that decreased GSH availability in the ELF and alveolar macrophages of ethanol-fed mice resulted in an increase of apoptosis of macrophages that could be reversed by maintaining GSH availability.[24]

GSH and arterial macrophage functions in atherosclerosis

GSH deficiency in the macrophage has been shown to be associated with compromise of mitochondrial function. GSH plays an integral role in protecting the mitochondria from ROI damage and it is the level of mitochondrial GSH that determines when cellular toxicity commences.[18] GSH deficiency in mitochondria contributes to macrophage dysfunction in atherosclerosis. Data from a study of the upregulation of GSH reductase in macrophages provide direct evidence that the GSH-dependent anti-oxidant system in macrophages plays a critical role in atherogenesis.[20] The study shows that overexpression of GSH in either mitochondria or the cytosol of macrophages decreases the severity of atherosclerosis in this animal model.[20]

Ox-LDL-related peroxyl radical formation is implicated in both mitochondrial dysfunction and macrophage lysis.[19] Ox-LDL toxicity is related to compromise of mitochondrial function including an increase in mitochondrial Ca^{+2}, opening of mitochondrial permeability transition pores and depolarization of mitochondrial membrane potential.

It has been observed that maintaining adequate GSH levels in macrophage will prevent the toxicity of ox-LDL in an in vitro macrophage model.[31–33] These findings help explain why a decreased amount of reduced GSH in blood has been shown to be an independent marker of progression of atherosclerosis in 2 studies.[13,14]

HIV

Worldwide, it is estimated that approximately 40 million people are infected with HIV, two-thirds of whom live in sub-Saharan Africa. Three regions, Africa, Asia, and Latin America, have the highest rates of new infections. AIDS is the fourth leading cause of death worldwide.[34] HIV is the cause of AIDS. Both HIV-1 and HIV-2 cause AIDS, but HIV-1 is found worldwide, whereas HIV-2 is found primarily in West Africa. HIV preferentially infects and kills CD4+ T lymphocytes, resulting in the loss of cell-mediated immunity and a high probability that the host will develop opportunistic infections. HIV-infected individuals have an increased risk for atherosclerosis and susceptibility to *M. tb* infection.[35]

HIV and atherosclerosis

As people with HIV live longer due to effective combination antiretroviral therapy (ART), cardiovascular disease has become an increasingly important cause of morbidity and mortality. But it remains controversial whether HIV infection contributes to accelerated atherosclerosis independent of traditional cardiovascular risk factors. In a recent cross-sectional study, more than 400 HIV-positive participants without pre-existing cardiovascular disease in the FRAM (Fat Redistribution and Metabolic Change in HIV Infection) study were compared with HIV-negative participants in the MESA (Multi-Ethnic Study of Atherosclerosis) cohort.[7] The preclinical atherosclerosis was assessed by measuring carotid intima-media thickness (IMT), or thickness of the walls of the arteries in the neck that supply the brain. IMT was evaluated at 2 sites in the artery, known as the internal/bulb. Even after adjustment for traditional cardiovascular disease risk factors, HIV infection was accompanied by more extensive atherosclerosis as measured by IMT.[7] The association of HIV infection with IMT was similar to that of traditional cardiovascular disease risk factors, such as smoking. The impact of HIV infection on preclinical atherosclerosis overrides any small differences related to antiretroviral therapy or specific drug classes. The effect of HIV is so big that no drug or class of drugs stands out as being an effective contributor.[7]

GSH and HIV-infection

Findings from our research laboratory as well as by other groups confirm that intracellular levels of GSH are decreased in patients with AIDS,[35] in whom the risk of TB is many times that of healthy individuals.[35] The factors responsible for low GSH in HIV infection are poorly understood. Infection with HIV is believed to trigger a range of metabolic changes in addition to the progressive deficits in cellular immunity and increased susceptibility to opportunistic infections that are its clinical hallmarks, and the progression to AIDS.[36–45] The decreased GSH content in immune cells of HIV-positive individuals was at least in part attributed to the decrease in plasma cysteine and increased plasma glutamate (an inhibitor of cysteine permeation via the Xc-transport system), as observed during early infection (Figure 2). The decreased intracellular GSH and plasma cysteine observed in HIV patients may also be due to chronic oxidative stress and this may lead to the progression of the disease. The decreased availability of cysteine can be overcome to some extent by the cysteine precursor NAC.[42] Herzenberg and colleagues found that NAC treatment improves the clinical situation and delays

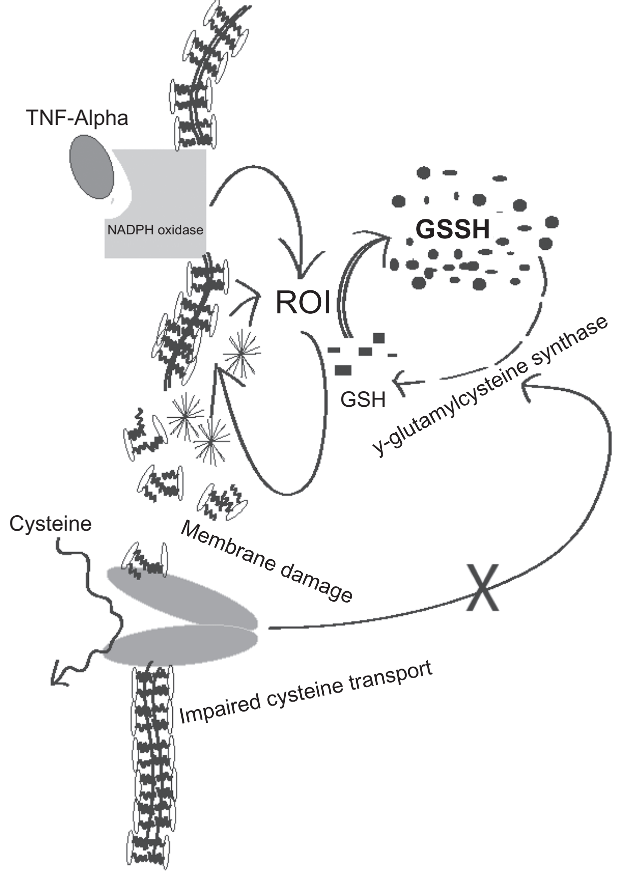

Figure 2 Possible causes for decreased intracellular levels of glutathione in individuals with HIV and/or *M. tb* infection.

the disease progression.[43] This study showed that long-term NAC administration to AIDS patients improves their hematological profile, GSH content and life expectancy.[43]

Ox-LDL and HIV

Increased incidence of cardiovascular events in HIV patients has sparked interest in elucidating the molecular mechanism behind endothelial dysfunction resulting in atherosclerotic pathology. While there is no evidence within the literature of a molecular mechanism responsible for increased cardiovascular risk within such HIV+ cohorts, animal models expressing transgenic HIV-1 offer a promising insight. One such study has shown significantly increased aortic arch lectin-like oxidized-low-density-lipoprotein receptor-1 (LOX-1) gene expression in HIV-1 transgenic rats compared to control rats.[46] LOX-1 is an endothelial receptor for ox-LDL and is hypothesized to be an early marker of endothelial dysfunction. It has been proposed that endothelial dysfunction is the first step in progression towards atherosclerotic plaque formation. This same group has also demonstrated significantly increased expression of inflammatory endothelial adhesion molecules in the same HIV-1 model. Both inducible vascular cell adhesion molecule-1 (VCAM-1) and constitutively expressed intercellular adhesion molecule-1 (ICAM-1) were both significantly elevated in HIV-1 transgenic rats compared to control rats, strongly suggesting for the first time that the HIV infection, independent of antiretroviral therapy-associated dyslipidemia, is capable of pathologic endothelial dysfunction that may prelude atherosclerotic plaque formation in major blood vessels.[46]

Tuberculosis

TB is the most prevalent infectious disease in the world.[47–54] In recent years there has been a significant increase in the incidence of TB due to the emergence of multi-drug resistant strains of *M. tb* and the increased numbers of highly susceptible immuno-compromised individuals arising from the AIDS pandemic.[8] It is also believed that in developing countries, as many as 40%–80% of individuals with AIDS are at risk of developing TB.[8,35] The innate and adaptive immune systems contribute to host defenses against *M. tb* infection.[48–54] Control of *M. tb* infection occurs both at the macroscopic and cellular levels. At the macroscopic level, the physical containment of viable mycobacteria within fibrotic granulomas contributes to prevention of overt disease.[49–50] Control of mycobacterial replication also occurs within macrophages. Resident tissue macrophages provide the first-line defense against *M. tb* infection. Macrophages and natural killer (NK) cells play an important role in innate defense against *M. tb* infection.[51–53]

Tuberculosis and GSH

Our group reported that GSH plays a key role in limiting intracellular growth of *Mycobacterium bovis* BCG in nitric oxide (NO)-deficient macrophages, such as macrophages derived from inducible nitric oxide synthase (NOS2)-knock out mice and human peripheral blood monocyte-derived macrophages (HMDM). Thus, GSH has direct antimycobacterial activity distinct from its role as an NO carrier, functioning as an effector molecule in innate defense against *M. tb* infection.[47,51,52] These results unfold a novel and potentially important innate defense mechanism adopted by human macrophages to control *M. tb* infection.[51,52] Consistent with these observations, we have also found that GSH mediates growth control of virulent *M. tb* in human blood cultures.[35,53] These results indicate that the inability of immune cells to contain *M. tb* growth may be a consequence of the inability of the immune cells to maintain adequate GSH levels during in vitro infection.[35] Furthermore, our recent studies indicate that GSH in combination with cytokines such as interleukin (IL-2) and IL-12, activate NK cells to control *M. tb* infection.[53] Most importantly, our recent studies demonstrate that GSH levels are significantly reduced in peripheral blood mononuclear cells and red blood cells isolated from TB patients and this decrease correlates with increased pro-inflammatory cytokine production and enhanced growth of *M. tb*.[54] Our group was the first to report that GSH levels are decreased in individuals with pulmonary TB and to correlate GSH levels with protective immunity.[54] Furthermore, our study provides a direct relationship between decreased GSH levels, pro-inflammatory cytokine production and enhanced growth of *M. tb* in TB patients.[54]

ROI, GSH and TB

ROI are generated from NADPH through a catalytic reaction of membrane bound enzyme NADPH oxidase, which is activated by assembling cytosolic regulatory components. The most important characteristic of ROI, either in vivo or in vitro, is peroxidation of lipids resulting in tissue damage and death of affected cells. Lipid peroxidation is a chain reaction providing a continuous supply of free radicals that initiate further peroxidation.[55] Free radical-induced lipid peroxidation causes marked alterations in the structural integrity and functions of membrane. The lipid peroxides formed at the primary site such as lungs could be transferred through

the circulation to other organs and tissues.[56–58] Jack et al[59] reported that several circulating markers of free radical activity were increased in pulmonary TB patients and some of these markers remain elevated even after completion of antimicrobial chemotherapy, indicating ongoing oxidative stress, which may contribute to decreased GSH levels. It is our prediction that in active TB, increased levels of ROI cause impaired cystine transport leading to decreased levels of GSH (Figure 2).

TNF-α, ROI and GSH

It has been shown that TNF-α stimulates ROI production. TNF-α could impair GSH-redox status by production of ROI, and impairment of the GSH-reductase *s*ystem, thereby leading to decreased regeneration of reduced GSH from oxidized GSSG (Figure 2). Moreover, enhanced ROI is likely to increase TNF-α in various cells, and depletion of reduced GSH will increase the inflammatory response to the cytokine. Nuclear factor-kappa B (NF-κB) is a DNA binding protein and a ROI-sensitive transcription factor for several cytokines, including TNF-α.[57] While NF-kB activation (leading to TNF-α production) is induced and enhanced by ROI, it can also be blocked by anti-oxidants such as vitamin E and GSH-enhancing agents such as NAC.[57] Excessive production of TNF-α and increased tissue sensitivity to this cytokine has been implicated in the immunopathology of TB, such as caseous necrotizing reactions. For example, excess TNF-α (relative to its receptors) in human bronchoalveolar lavage fluid was associated with tissue necrosis and cavity formation.[59–61] Systemic spillover of TNF-α may account for unwanted inflammatory effects like fever and wasting, which manifests clinically as cachexia, consistent with the original designation as cachetin.[65] In HIV-infected persons, the effects of TNF-α may be particularly deleterious, as this cytokine has been implicated as a stimulus for HIV expression and for activation-induced T cell apoptosis. For these reasons, several studies have examined anti-TNF treatments in persons with AIDS and in HIV+ TB.[62–65] Thus enhanced TNF-α production may represent a pathogenic loop, leading to enhanced inflammation and ROI production, leading to reduced GSH levels.

Atherosclerosis and tuberculosis

Atherosclerosis is an inflammatory disease involving the accumulation of macrophages in the intima. Wnt5a is a noncanonical member of the Wnt family of secreted glycoproteins. Recently, human macrophages have been shown to express Wnt5a upon stimulation with bacterial pathogens in vitro and in granulomatous lesions in the lung of *M. tb*-infected patients. Wnt5a expression has also been linked to Toll-like receptor-4 (TLR-4), an innate immune receptor implicated in atherosclerosis. These observations, along with the fact that Wnt5a is involved in cell migration and proliferation, led Christman et al to postulate that Wnt5a plays a role in atherosclerosis.[66] To investigate this hypothesis, Christman et al characterized Wnt5a expression in murine and human atherosclerotic lesions. Tissue sections derived from the aortic sinus to the aortic arch of apolipoprotein E-deficient mice and sections derived from the carotid arteries of patients undergoing endarterectomy were subjected to immunohistochemical analysis. All samples were found to be positive for Wnt5a with predominant staining in the areas of macrophage accumulation within the intima. In parallel, the investigators probed for the presence of TLR-4 and found coincident TLR-4 and Wnt5a expression.[66] For both the Wnt5a and TLR-4 staining, consecutive tissue sections treated with an isotype- and species-matched Ig served as a negative control and exhibited little, if any, reactivity. Quantitative RT-PCR revealed that Wnt5a mRNA expression in RAW264.7 murine macrophages can be induced by stimulation with LPS, a known ligand for TLR-4. Combined, these findings demonstrate for the first time Wnt5a expression in human and murine atherosclerotic lesions and suggest that cross-talk between TLR-4 and Wnt5a is operative in atherosclerosis.[66]

Conclusion

Macrophage functions are compromised by decreased intracellular levels of GSH when an excess of ox-LDL is present. This has been shown to increase the development and progression of atherosclerosis. Reduction of intracellular GSH is a feature of HIV, which is also associated with an increased risk of atherosclerosis. The compromise of innate immune function that occurs with low GSH in the macrophage may also increase susceptibility to infection with *M. tb*, for which individuals with HIV have an increased risk. It is possible that the compromise of macrophage function related to the ingestion of ox-LDL and the decreased macrophage levels of GSH increase the risk of contracting macrophage-related infectious disease such as *M. tb* infection in both HIV-positive and HIV-negative individuals. These observations suggest that further investigation into the relationship between low GSH, increased ox-LDL and susceptibility to infection with *M. tb* is warranted.

Acknowledgment

Corresponding author's work is supported by American Heart Association (Scientist Development Grant), Potts

Memorial Foundation, UMDNJ Foundation grant, Your Energy Systems and start-up funds from Western University of Health Sciences.

Disclosure

The authors declare no conflicts of interest.

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Glutathione—a review on its role and significance in Parkinson's disease

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ABSTRACT **Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting over a million people in the United States alone, and is characterized by rigidity, bradykinesia, resting tremor, and postural instability. Its main neuropathological feature is the loss of dopaminergic neurons of the substantia nigra pars compacta. However, the pathogenesis of this loss is not understood fully. One of the earliest biochemical changes seen in PD is a reduction in the levels of total glutathione, a key cellular antioxidant. Traditionally, it has been thought that this decrease in GSH levels is the consequence of increased oxidative stress, a process heavily implicated in PD pathogenesis. However, emerging evidence suggests that GSH depletion may itself play an active role in PD pathogenesis. This review aims to explore the contribution of GSH depletion to PD pathogenesis.—Martin, H. L., Teismann, P. Glutathione—a review on its role and significance in Parkinson's disease. *FASEB J.* 23, 3263–3272 (2009). www.fasebj.org**

Key Words: oxidative stress · complex I · ubiquitin-proteasome · inflammation · incidental Lewy body disease

Parkinson's disease (PD) is a common neurodegenerative disease that affects over a million people in the United States alone (1). Clinically, it is characterized by rigidity, bradykinesia, resting tremor, and postural instability (2). The primary neuropathogical feature of PD is the loss of the dopaminergic neurons of the substantia nigra pars compacta (SN) that project to the striatum (2). Another major pathological feature of PD is the presence of Lewy bodies in the surviving neurons. These are cytoplasmic, proteinaceous inclusions rich in α-synuclein and ubiquitinated proteins (2). The pathogenesis of PD is poorly understood, and most cases are idiopathic in onset (2). However, a number of processes have been implicated in the degeneration of the dopaminergic neurons. These processes include oxidative stress, inhibition of mitochondrial complex I, ubiquitin-proteasome dysfunction, and inflammation (3–6). One of the earliest biochemical changes seen in PD patients is a decrease in reduced glutathione (GSH) levels; GSH is a major component of cellular antioxidant defenses (7–9). A decrease in GSH levels also occurs in incidental Lewy body disease (7), which is thought to be an asymptomatic precursor to PD (10). This suggests that GSH depletion in the SN may play a more active role in PD pathogenesis than previously thought. Indeed recent studies reveal that GSH depletion may be actively involved in complex I inhibition, disruption of the ubiquitin-proteasome system, and may have effects that affect the inflammatory processes seen in PD. This review aims to examine these links and determine how potentially important GSH depletion may be in PD pathogenesis.

GLUTATHIONE FUNCTIONS AND METABOLISM

Glutathione (γ-glutamylcysteinylglycine) is the most abundant nonprotein thiol in cells (9, 11). Glutathione protects cells against exogenous and endogenous toxins, including reactive oxygen species (ROS) and reactive nitrogen species (RNS). Such radical species are removed *via* nonenzymatic reduction with GSH, whereas the removal of hydroperoxides requires enzymatic catalysis by glutathione peroxidase (**Fig. 1**) (9, 11–13). Both reactions lead to the generation of glutathione disulfide (GSSG, or oxidized glutathione), which is reduced back to GSH by glutathione reductase that uses NADPH from the pentose phosphate shunt (9). Glutathione also modifies protein sulfdryl groups by a number of reactions; reduction of protein sulfenic acids, formation of protein mixed disulfides and their subsequent reduction. Conjugation of GSH with electrophilic compounds, mediated by the glutathione-*S*-transferases (GSTs) and subsequent excretion of these conjugates from the cell, also serves to protect cells from toxins (9, 11–13).

When the redox state of a cell is altered, increased GSH usage occurs, and while the GSSG generated can be reduced back to GSH, the formation and export of GSH conjugates leads to GSH depletion. This depletion is attenuated by *de novo* synthesis of GSH (9, 13). Glutathione synthesis in neurons and the supporting glial cells, as in other cells, is a 2-step ATP-dependent

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doi: 10.1096/fj.08-125443

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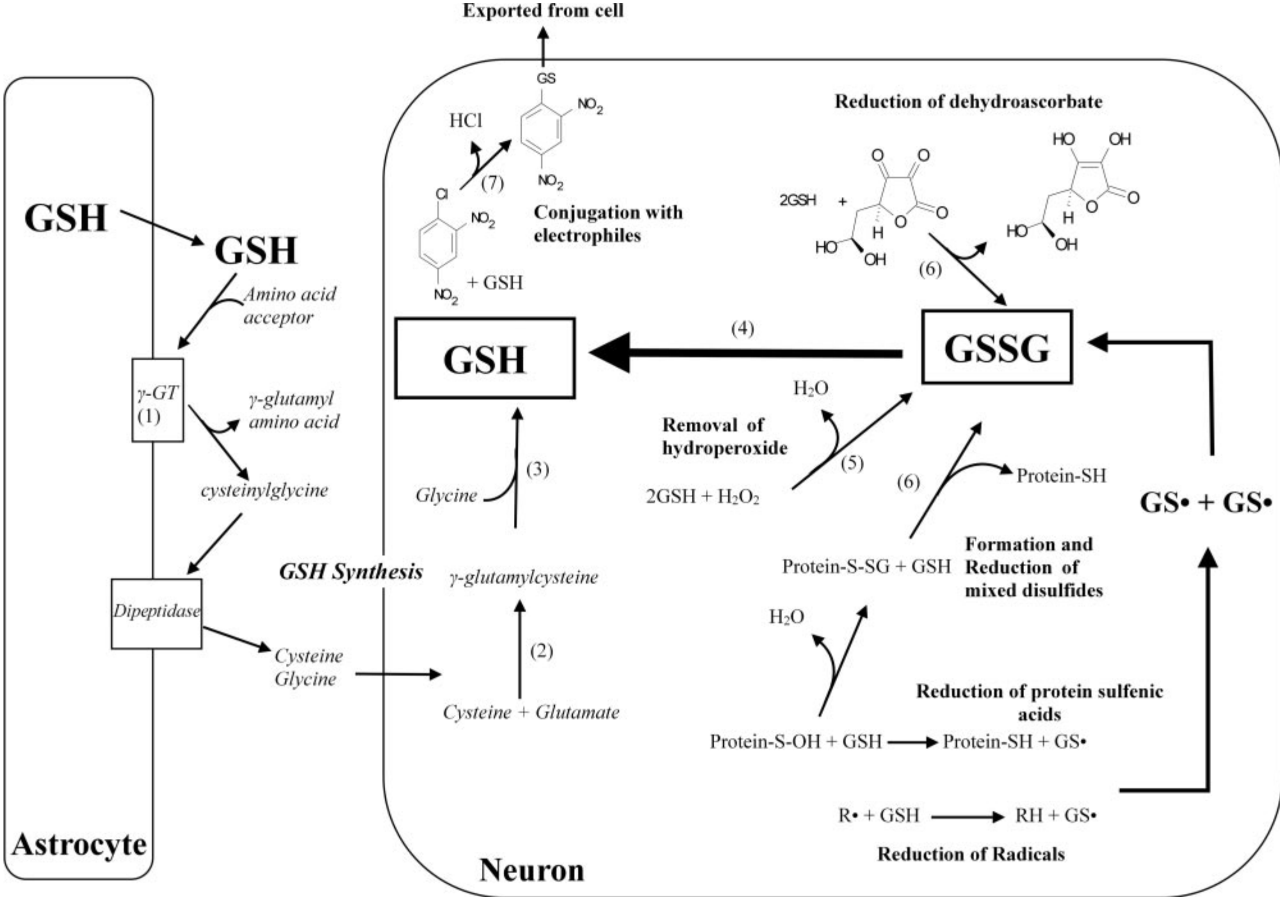

Figure 1. Glutathione synthesis and metabolism in the central nervous system. Both neurons and astrocytes have the capacity to synthesize GSH (shown in italics), but astrocytes also play important roles in substrate supply to neurons. Glutathione synthesized in astrocytes is exported in the extracellular space, where the ecto-enzyme γ-GT (*1*) transfers the glutamyl group onto an amino acid acceptor, creating cysteinylglycine, which is then broken down into its constituents, cysteine and glycine. Glutathione is synthesized in two steps: first, γ-glutamylcysteine is formed from cysteine and glutamate, catalyzed by GCL (*2*); then glycine is added by GSH synthase (*3*). Glutathione has many roles in detoxification and maintenance of redox equilibrium. The majority of these processes generate GSSG, which is reduced back to GSH by glutathione reductase and NADPH from the pentose phosphate shunt (*4*). (See refs. 9, 11–13). *1*) γ-GT; *2*) GCL; *3*) glutathione synthase; *4*) glutathione reductase; *5*) glutathione peroxidase; *6*) glutaredoxin; *7*) GST.

process (Fig. 1) (9, 11–13). The first of these steps, forming γ-glutamylcysteine from glutamate and cysteine, is rate limiting, as the critical factor is the supply of cysteine (11, 13). This step is also influenced by GSH levels, as L-glutamate:L-cysteine γ-ligase (GCL), the enzyme catalyzing this reaction, is nonallosterically inhibited by GSH in a negative feedback fashion. The second step, the addition of glycine to generate GSH, is catalyzed by glutathione synthase (11, 13). Although both neurons and glial cells can synthesize GSH, glial cells, specifically astrocytes, also have important roles to play in supplying GSH substrates to neurons. Astrocytes synthesize and export GSH, which can then undergo transpeptidation to cysteinylglycine and γ-glutamyl amino acid by the ecto-enzyme γ-glutamyl transpeptidase (γ-GT). The cysteinylglycine generated can then be utilized by neurons to manufacture GSH, probably undergoing dipeptide cleavage to its constituent amino acids first. This mechanism of substrate supply minimizes the neurotoxic effects of large amounts of extracellular cysteine, which can activate glutamate receptors (11). A full discussion of the functions of GSH and its maintenance in neuronal cells is beyond the scope of this review, and the reader is referred to Zeevalk *et al.* (11) and Dringen (12) for further information.

CAUSES OF GLUTATHIONE DEPLETION

Glutathione is a major antioxidant that functions to maintain the redox equilibrium of a cell, which can be expressed as GSSG:2GSH (14). Oxidative stress results when this redox equilibrium is altered in favor of GSSG, which can either be due to a decrease in the reducing capacity of the cell or an increase in the reduction potential (15). The former is a result of decreased levels of cellular antioxidants, predominantly GSH, while the latter comes from increased generation of ROS and RNS. The sum of these changes is increased oxidative stress. Oxidative stress can damage most of the cellular macromolecules, evident from protein and DNA adduct formation and lipid peroxi-

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dation. Such markers of oxidative stress are increased in PD patients, with raised levels of both 8-hydroxyguanine and 4-hydroxynonenal, indicative of oxidative DNA damage and lipid peroxidation, detected in the SN (16–17). Increased protein carbonyls are also seen in PD patients but not in incidental Lewy body disease, suggesting that oxidative protein damage is a late occurrence in PD pathogenesis (18).

ALTERATIONS IN THE SYNTHESIS AND METABOLISM OF GLUTATHIONE IN PD?

The GSH depletion seen in PD may result from a decrease in synthesis and recycling under normal redox conditions. To determine whether this is the case, it is necessary to assess the enzymes and substrates involved in GSH synthesis and metabolism. If alterations in GSH synthesis were the cause of the GSH depletion seen in PD, it would be anticipated that the activity of the rate-limiting enzyme in GSH synthesis, GCL, would be decreased. It has been reported that activity levels of GCL are reduced throughout the brain as a consequence of the aging process (19). This was attributed to a reduction in the levels of the light subunit of GCL (19) that modulates the affinity of the enzyme for substrates and inhibitors (13). This decrease is in agreement with the fall in GCL activity seen in immortalized N27 mesencephalic dopaminergic cells 6 and 12 h after administration of the parkinsonian toxin 1-methyl-4-phenylpyridinium (MPP^+). However, this decrease was transient, and the activity levels of GCL returned to control levels 24 h after MPP^+ treatment (20). The short-lived nature of this change is consistent with reports that the activity of GCL is unaffected in PD patients at postmortem (21). It appears unlikely that decreased GCL activity underpins GSH depletion in PD, although it may help explain why PD incidence increases with age (2). If it is true that GCL function in PD is normal, then a decrease in GSH would be anticipated to increase GCL activity due to the reduced amount of negative feedback (13); however, such an increase is not observed (20–21). This may be due to alterations in the cysteine supply that is critical for GSH synthesis (11). Indeed, circulating levels of cysteine decrease with age (22), and the incidence of PD increases with age (2), and such a decrease may contribute to the GSH depletion seen in PD. Also, the main uptake route for cysteine into neurons, the excitatory amino acid carrier 1 (EAAC1), is altered by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; metabolized to the bioactive MPP^+) treatment (23). Administration of MPTP caused EAAC1 translocation to the cell membrane of dopaminergic neurons. This finding suggests that dopaminergic neurons attempt to maintain GSH homeostasis as MPTP treatment reduces total GSH levels by ~30% (21, 23). However, nitration of EAAC1 was also increased by MPTP treatment, and this is thought to inactivate EAAC1, as the uptake of cysteine into midbrain slices treated with MPP^+ was reduced (23). This study suggests that cysteine supply to neurons could be altered in PD; this finding is supported by increased activity and levels of γ-GT both in dopaminergic cells and in PD patients as an attempt to generate neuronal GSH (21, 24).

Glutathione depletion may also result from an increased efflux of GSSG and GSH conjugates (21). However, global GST activity remains unchanged, so at least enzymatic conjugation is not increased (21), and although MPP^+ treatment caused a 30% decrease in GSH in N27 cells, no increase in GSH efflux was detected (20). It is possible that nonenzymatic oxidation of GSH to GSSG is increased, and the most likely substrates for this would be ROS/RNS. The reduction of GSSG back to GSH, in addition to *de novo* synthesis, is important in maintaining cellular levels of GSH. Levels of glutathione reductase, the enzyme responsible for this reduction (9), are reported to be increased in PD patients (25). This finding suggests that levels of GSSG may be increased, and GSSG levels have been reported to be nonsignificantly increased in PD patients at postmortem (7). This increase in glutathione reductase levels again suggests that the cells of the SN are attempting to maintain GSH levels. These findings are in concurrence with those of Drechsel *et al.* (20), showing an increase in glutathione reductase activity levels in N27 cells after MPP^+ treatment. Initially, however, glutathione reductase activity was lowered transiently before it increased, in a similar fashion to GCL activity levels (20). This decrease in glutathione reductase activity (20) can alter the GSSG:2GSH ratio, leading toward a state of oxidative stress, which may be sufficient to start the downward spiral of neurodegeneration that continues even though the activity of the GSH synthesizing and metabolizing enzymes return to normal levels.

SOURCES OF OXIDATIVE AND NITROSATIVE STRESS IN PD

The possible decrease in GSSG reduction to GSH combined with a potential decrease in *de novo* synthesis of GSH could lead to the state of cellular oxidative stress seen in Parkinson's disease. However, the late onset of idiopathic PD (2) suggests that the oxidative load of the dopaminergic neurons may need to reach a threshold before GSH depletion becomes important in the spiral of neurodegeneration. As dopaminergic neurons normally have a high oxidative load, it may not require excessive additional ROS/RNS generation to have a negative effect on these cells (3). The sources and effects of oxidative stress in PD have been widely reviewed elsewhere (most recently in refs. 26, 27), but one reason why the dopaminergic neurons might be particularly vulnerable in PD is that the metabolism of dopamine (DA) generates ROS and hydrogen peroxide (H_2O_2) (28). Nonenzymatic autooxidation of DA forms a reactive quinone, while DA is metabolized enzymatically by monoamine oxidase and catechol-*O*-methyl

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transferase, also generating H_2O_2 (28). It can also be metabolized by the peroxidase site of cyclooxygenase-2 (COX-2; also known as prostaglandin H_2 synthase), again generating reactive quinones and semiquinones (29, 30). The pharmacological or genetic ablation of COX-2 has been shown to provide neuroprotection in the MPTP mouse model of PD (31). The quinones and semiquinones formed interact with protein sulfdryl groups and GSH, thus decreasing levels of GSH (28). Detoxification of H_2O_2 requires glutathione peroxidise, and activity levels of this enzyme are mildly reduced in PD patients at postmortem (32), possibly because of the reduced GSH levels as glutathione peroxidise utilizes GSH as a cosubstrate. Hydrogen peroxide can also be converted into the more damaging hydroxyl radical in the presence of transition metals, and the levels of certain transition metals, including iron, zinc, manganese, and copper, have been shown to increase in rats after treatment with the parkinsonian toxin 6-hydroxydopamine (6-OHDA) (33). The most dramatic rise was in total iron content with an approximate increase of 2.5-fold (33) and in PD patients a rise in total iron, of a similar magnitude, was also observed (34). Thus, it can be seen that DA metabolism produces a variety of radical species that need GSH for detoxification. Consequentially, the basal demand for GSH is high in these dopaminergic neurons, so small alterations in GSH or ROS/RNS levels can have a big effect. Other important sources of ROS and RNS in PD include NADPH oxidase and nitric oxide synthase. Both of which can appear in microglia and whose genetic or pharmacological ablation provides protection against MPTP toxicity (35–40). This suggests that microglia could be a pivotal source of ROS/RNS important for GSH depletion and in PD pathogenesis. The relevance of microglia to PD pathogenesis is supported by the glial activation reaction that is seen in PD, and following MPTP administration (41–43). This glial reaction peaks before maximal dopaminergic neuron death occurs, indicating that it is not solely a response to the death of the dopaminergic neurons (38). Inhibition of microglia activation serves to attenuate MPTP neurotoxicity (44). The involvement of microglial activation in PD is not surprising considering that the SN has the highest density of microglia in the entire brain (45). The role of microglia in PD pathogenesis extends beyond ROS/RNS generation, as these cells are the immune sensors and effectors of the central nervous system (46) and therefore have important roles in the inflammatory processes seen in PD. The role of GSH in these inflammatory processes is explored in more detail later in this review.

In summary, GSH depletion and ROS/RNS generation are highly interlinked, and it is difficult to determine which comes first in PD pathogenesis. It is probable, however, that some degree of ROS/RNS generation occurs before GSH depletion. The precise source of these ROS/RNS remains elusive, although they probably come from multiple sources. The normally high oxidative load of dopaminergic cells may also make them more vulnerable to subtle increases in ROS/RNS. These increases might then be exacerbated by alterations in GSH synthesis and metabolism, especially alterations in cysteine supply. The resultant GSH depletion would then further increase the oxidative load on the dopaminergic neurons and affect the biological functioning of these cells.

GLUTATHIONE DEPLETION AND INHIBITION OF MITOCHONDRIAL COMPLEX I

Mitochondrial complex I is the first and most complicated complex of the electron transport chain (4). It oxidizes NADH and transfers the electrons to ubiqunione. Postmortem evidence from PD patients demonstrates a defect in complex I function in the SN when compared with age-matched controls (47). The role of a complex I defect in PD pathogenesis is supported further by the ability of MPP^+, 6-OHDA, and rotenone to induce dopaminergic neuron death, as these compounds are known to inhibit complex I (48, 49). It has been suggested that a prolonged yet mild inhibition of complex I would lead to increased ROS production and a subsequent decrease in GSH levels (4), implying that GSH depletion is secondary to complex I inhibition. However, MPTP and rotenone are also reported to reduce GSH levels by ~30 and 50%, respectively (23, 50), and GSH depletion is seen in incidental Lewy body disease, a presymptomatic precursor to PD, in the absence of complex I inhibition (7). Consequentially, it remains unclear whether GSH depletion or complex I inhibition comes first. However, a number of studies have shown that GSH depletion can impair complex I function and may even precede complex I inhibition. Hsu *et al.* (51) have demonstrated that reducing GSH by ~50% in dopaminergic PC12 cells results in a 50% reduction in complex I activity as measured by reduction of 2,6-dichlorophenolindophenol. These cells were genetically engineered to permit inducible inhibition of GCL, and by using this method to reduce GSH levels, the changes seen in GSH and GSSG mimic those seen in PD patients (8, 52). These effects of GSH depletion on complex I activity were replicated in N27 cells using a pharmacological inhibitor of GCL, L-buthionine-*S*-sulfoximine (BSO), for 7 d to mimic more chronic GSH depletion as is the case in PD (53). Both these studies further explored this effect and implicated the complex I inhibition seen as being RNS dependent, which strongly suggests that *S*-nitrosation of complex I occurs in GSH-depleted conditions. This is in concurrence with Clementi *et al.* (54), who have shown that persistent inhibition of complex I occurs with prolonged exposure to nitric oxide and that this condition is accelerated by GSH depletion. This inhibition was reversible with the addition of reducing agents such as DTT, exogenous GSH, or the presence of light. This reversibility, especially the photolability, implies that a nitrosothiol group had been formed (54). Dahm *et al.* (55) have also demonstrated that

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nitrosative stress leads to increased nitrosothiol group formation in mitochondrial proteins. They have proposed that this formation involves *S*-nitrosoglutathione, and these nitrosothiols would normally be removed by reduced GSH. Bharath and Andersen (56) have also shown an increase in nitrotyrosine immunoreactivity in complex I-enriched fractions from GSH-depleted cells. These studies indicate that GSH normally functions to protect complex I from nitrosative damage, so the GSH depletion seen in PD could lead to complex I inhibition.

Another mechanism by which GSH depletion is thought to have a negative effect on complex I activity is *via* the increase in γ-GT activity seen in PD (21, 24). It is hypothesized that such an increase would result in increased intraneuronal levels of cysteine which can react with DA to form 5-cysteinyl dopamine quinones, metabolites of which can inhibit complex I (57). However, inhibition of γ-GT exacerbates the GSH depletion-induced reduction in complex I activity that was alleviated by the addition of cysteine (24). These results suggest that the increases seen in γ-GT activity are a compensatory event attempting to increase GSH levels that would otherwise lead to a reduction in complex I activity (24).

It appears that these GSH depletion-induced reductions in complex I activity are primarily confined to dopaminergic cells, as Seaton *et al.* (58) did not find any in changes in complex I activity in the cerebral cortices of rats treated with BSO. Also, in contrast to dopaminergic cells, GSH depletion in glial cultures causes up-regulation of complex I activity and enhanced levels of mRNA of the ND6 subunit of complex I (59). This suggests that the ability of GSH depletion to inhibit complex I is specific to dopaminergic cells and may, at least in part, account for their vulnerability in PD. If complex I inhibition is secondary to GSH depletion, it raises the question as to the mechanism by which GSH is depleted, as it is generally assumed that oxidative stress resulting from complex I inhibition and the downstream effects of this gives rise to the decreased GSH levels. As reviewed in the previous section, GSH depletion and oxidative stress are inextricably linked, and so the GSH depletion that affects complex I activity may result from multiple processes. Inhibition of complex I activity has several downstream effects, including increased ROS generation that compounds GSH depletion and a reduction in ATP generation by the electron transport chain. Both these effects affect the other pathogenic processes that result in PD.

GLUTATHIONE DEPLETION AND DYSFUNCTION OF THE UBIQUITIN-PROTEASOME SYSTEM (UPS)

The UPS targets misfolded and damaged proteins to the 26S proteasome for degradation by the addition of ubiquitin monomers (**Fig. 2**) (5). Evidence for UPS dysfunction in PD comes from the rare familial forms of the disease that can be linked to specific genes whose products are involved in the UPS–Parkin, for example, is an E3 ubiquitin ligase (5). Also, the Lewy bodies seen in PD contain high amounts of ubiquitinated proteins (2). Glutathione depletion may affect the UPS either in a direct manner or indirectly as a result of complex I inhibition and subsequent ATP depletion, as ubiquitination is an ATP-dependent process (5). In fact, GSH depletion directly affects the UPS at both the level of E1 ubiquitin ligase and at the level of proteasome. Jha *et al.*

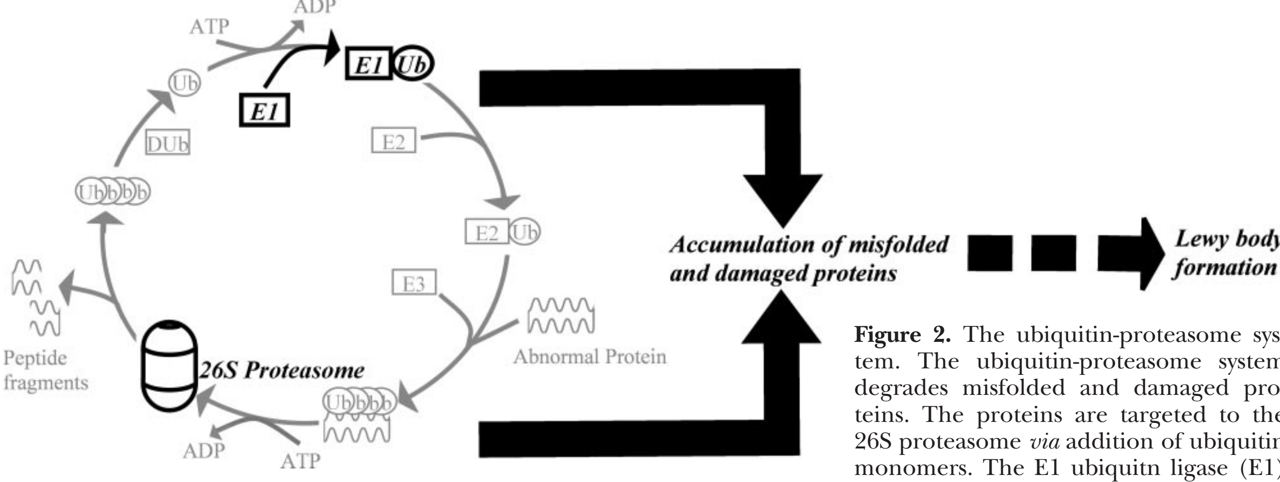

Figure 2. The ubiquitin-proteasome system. The ubiquitin-proteasome system degrades misfolded and damaged proteins. The proteins are targeted to the 26S proteasome *via* addition of ubiquitin monomers. The E1 ubiquitn ligase (E1) conjugates with an ubiquitin monomer in an ATP-dependent process. The ubquitin monomer is then transferred to E2 ubiquitin ligase (E2) before being attached to an abnormal protein by E3 ubiquitin ligase (E3). Once the abnormal protein has a polyubqiuitin chain, it is degraded by the 26S proteasome, which consists of a 20S catalytic subunit and two regulatory subunits, into small peptide fragments. The polyubiquitin chain is recycled by deubiquinating enzymes. The effects of GSH depletion on the ubiquitin-proteasome system are shown in black, with decreased ubiquitn tagging of misfolded and damaged proteins due to reduced E1 activity as well as reduced degradation of ubquitin tagged proteins because of decreased proteasome activity. These reduction ubquitin-proteasome system activities lead to the buildup of misfolded and damaged proteins and Lewy body formation (5, 60–62). Ub, ubiquitin; Dub, deubiquinating enzyme.

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(60) have shown that ubiquitination of proteins decreases in GSH-depleted PC12 cells. This is due to an inability of E1 to bind ubiquitin when GSH levels are <50%. Restoration of GSH to normal levels restores E1 ubiquitination. The reversibility of this event implies that it is due to a thiol oxidation event, as one of the major functions of GSH is to maintain thiol groups in a reduced, functional state (9, 60). It is known that E1 ubiquitin ligase contains a sulfhydryl group that functions to add ubiquitin to its targets, and this group also conjugates with GSH (61).

Glutathione depletion also has effects on the 26S proteasome itself. Caneda-Ferron *et al.* (62) have shown that GSH depletion precedes inhibition of chymotrypsin-like proteasome activity in SH-SY5Y cultures treated with 2 mM MPP^+ or 0.5 mM DA. Indeed, in the cultures treated with DA, this initially increased GSH levels, and proteasome inhibition did not occur in these cultures until GSH levels had dropped below 50% of the controls, providing strong evidence that GSH depletion needs to occur first for proteasome inhibition in this model. These compounds also inhibited activity in purified human erythrocyte 20S (the catalytic subunit of the 26S proteasome) extracts, indicating that they have a direct effect on the proteasome (62). It is probable that in whole cells the effects of MPP^+ and DA on the UPS are both direct and indirect *via* complex I inhibition and ATP depletion. The role of potential interplay between complex I inhibitors and the proteasome has been explored by Höglinger *et al.* (63) in rat primary mesencephalic cultures. They have demonstrated that administration of a proteasome inhibitor, epoxomicin, and complex I inhibitors, MPP^+ and rotenone, act synergistically to increase toxicity. A 1 μM dose of MPP^+ combined with 100 nM of epoxomicin gave approximately the same toxicity as a 10 μM dose of MPP^+ alone. This effect was specific to complex I inhibitors. Unfortunately, although this study assessed changes in ROS and ATP production induced by such combination treatments, alterations in GSH levels were not assessed. It would be interesting to see whether the effects reported were preceded by a reduction in GSH levels.

These studies, taken together, implicate GSH depletion in UPS dysfunction, with direct effects on E1 ubiquitin ligase and the proteasome as well as more indirect effects on UPS function due to inhibition of complex I and ATP depletion. The effects of GSH depletion on components of the UPS are unlikely to be the sole cause of UPS dysfunction. Misfolded and damaged proteins are also liable to contribute to the dysfunction, including α-synuclein, the major component of Lewy bodies (64–66). It is highly probable that some of this damage is oxidative, including proteins modified by lipid peroxidation products, such as 4-hydroxynoneal and acrolein, and those with oxidized thiol groups that would normally be maintained in a reduced state by GSH. Glutathione may, therefore, have a more important role in maintaining effective clearance of misfolded and damaged proteins than previously thought, and the GSH depletion seen in PD would negatively affect this process.

GLUTATHIONE DEPLETION AND INFLAMMATION

The effects of GSH depletion on the inflammatory processes seen in PD have not been well studied, but evidence suggests that GSH and cytokine regulation are highly interlinked. Cellular redox status plays an important role in the regulation of interleukin (IL)-1, IL-6, and tumor necrosis factor-α transcription, and in the regulation of the signaling pathways triggered by these cytokines (67). These cytokines play a role in PD pathogenesis, as evidenced by raised levels of these proinflammatory cytokines in the cerebrospinal fluid, peripheral blood, and brains of PD patients (68–71). Yet the role of GSH in proinflammatory signaling is complex and not fully understood. Evidence suggests that GSH depletion may actually suppress immune responses (72–75). Examination of IL-1 signaling illustrates the complex nature of the influence of GSH on proinflammatory signaling. IL-1 signaling has three main stages: complex formation, activation of nuclear factor κB (NF-κB), and NF-κB nuclear translocation leading to gene transcription (67). All of these stages are inhibited by thiol-oxidizing compounds (76), the effects of which would normally be reversed by GSH. The relationship between NF-κB activation and GSH is not solely one of inhibition by thiol oxidizers. Indeed NF-κB is activated in conditions of oxidative stress (76), and this activation is inhibited by overexpression of glutathione peroxidases, indicating an important role for GSH. Glutathione also regulates NF-κB-mediated signaling, as glutathionylation of the p50 subunit prevents DNA binding (76). This suggests that the precise redox state is crucial for NF-κB activation, and so GSH depletion is likely to have mixed effects on this process. The complexity of redox state influences, and therefore GSH levels, on inflammatory mediators is highlighted by a report that the inflammatory response induced by lipopolysaccharide, in primary mesencephalic cultures, prevented GSH depletion-induced cell death (77). However, in this study and in alveolar macrophages, IL-1β and GSH depletion acted synergistically to increase cell death (77, 78). Thus, it appears that the effects of GSH depletion on inflammatory processes depend on cell type and the spectrum of inflammatory mediators produced.

Glutathione depletion can also affect the inflammatory processes occurring in PD by affecting the c-Jun N-terminal kinase (JNK) pathway, which has many roles in inflammation and immunity (reviewed in ref. 79). The JNK pathway can be activated by upstream effectors, including apoptosis signal-regulating kinase (ASK1) which normally associates with glutaredoxin. However, in a GSH-depleted environment, glutraredoxin dissociates from ASK1, which then activates JNK (11, 80). Indeed, both JNK2 and JNK3 null mice showed signif-

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icant protection against MPTP-induced dopaminergic neuron loss in the SN, while the greatest protection was seen in the double-knockout animals (81). Activation of JNK can lead to COX-2 induction (81), which, as discussed above, can oxidize dopamine in its peroxidase site, generating reactive quinones. Cyclooxygenase-2 is a major source of prostaglandins, notably prostaglandin E_2, but also 15-deoxy-Δ-12,14-prostaglandin-J_2 *via* further metabolic steps. 15-deoxy-Δ-12,14-prostaglandin-J_2 can up-regulate COX-2 in a GSH-dependent manner (82). Pretreatment with BSO increased the up-regulation of COX-2 by 15-deoxy-Δ-12,14-prostaglandin-J_2 in human mesangial cells, while the GSH precursor *N*-acetylcysteine (NAC) decreased COX-2 up-regulation. It appears also that COX-2 activation is enhanced in GSH-depleted conditions. Whether this situation extends into PD pathogenesis remains to be seen, but long-term users of nonsteroidal antiinflammatory drugs that inhibit COX-2 have a reduced risk of developing PD (82), which suggests that COX-2 is important.

These studies suggest that GSH depletion may modulate the inflammation seen in PD; however, this area requires further exploration. It will be of interest, for instance, to investigate the effects of GSH depletion on IL-1 signaling in neuronal tissues, as this may differ from the effects seen in alveolar macrophages. Also, examination of the role of other JNK-activated inflammatory pathways, such as cytokine production (79), may provide useful insights into PD pathogenesis.

TREATMENT STRATEGIES

It has become clear that decreased GSH levels are linked to a significant number of the cellular processes known to be affected in PD (**Fig. 3**). This finding, combined with the fact that GSH depletion occurs early in PD, suggests that such depletion may be a critical factor in dopaminergic neurodegeneration and that replenishment of GSH may provide an option for patient treatment. However, it needs to be ascertained whether GSH replenishment would be an effective treatment at the stage of degeneration seen when PD patients are diagnosed, as this is often later than the stage when interventions are given in PD models. Administration of a precursor may provide a better

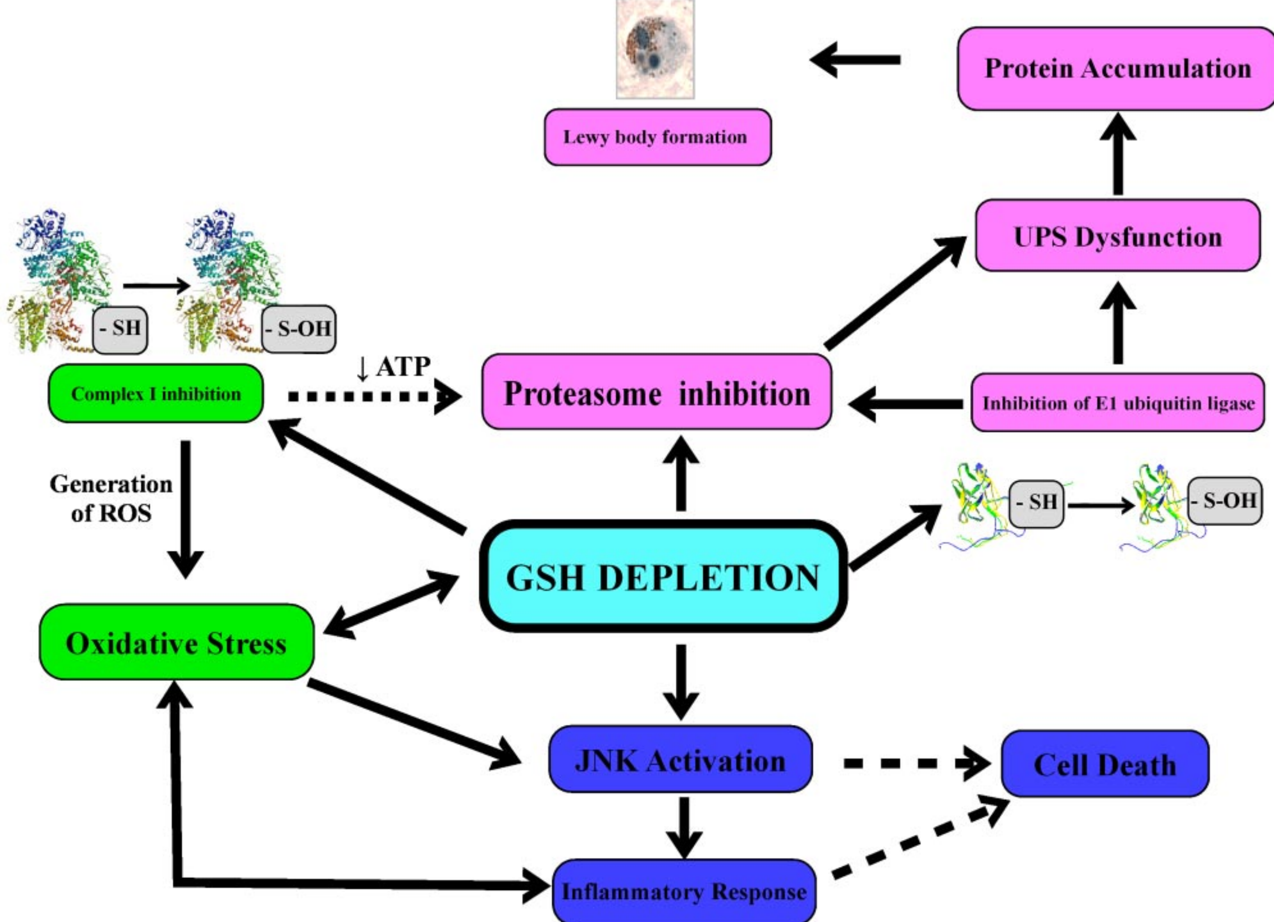

Figure 3. Effects of glutathione depletion. Glutathione depletion can inhibit complex I, E1 ubiquitin ligase (E1), and proteasome activity. It can also exacerbate oxidative stress and activate the JNK pathway, leading to an inflammatory response. These effects have the potential to cause dopaminergic neuron death and accumulation of protein into Lewy bodies. These effects suggest that GSH may have an important role to play in the pathogenesis of Parkinson's disease (32, 92–93). Dashed lines indicate potential links.

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treatment, because GSH itself does not penetrate neurons, since they do not possess a GSH transporter (11). This is supported by the failure of subcutaneous GSH ethyl ester administration to rats to increase brain GSH levels (83). The most common GSH precursor, *N*-acetylcysteine (NAC), does not cross the blood-brain barrier in significant amounts (84). However, intraperitoneal administration of NAC to rats and gerbils has provided protection against oxidative damage to brain proteins and lipids (85, 86). Recent work by Zeevalk *et al.* (87) suggests that administration of γ-glutamylcysteine and cysteinylglycine can alleviate GSH depletion in mesencephalic cultures and provide neuroprotection against MPP^+ and oxidative stress. They have also demonstrated that these dipeptides can be utilized when attached to nanoparticles formed from human serum albumin, giving a potential administration route as dipeptide access to the brain is highly regulated by the blood-brain barrier. Indeed intraperitoneal administration to mice of γ-glutamylcysteine ethyl ester, another dipeptide precursor of GSH in a lipid soluble form that can penetrate the blood-brain barrier, provided a degree of protection from MPTP-induced loss of dopaminergic neurons (88). Despite these promising results, only one small, 9-patient study was reported by Sechi *et al.* (89). Intravenous administration of GSH showed promising results with the clinical disability of patients reduced by ~40%. The biochemical mechanisms of these effects are unclear, especially as these patients are likely to have already suffered significant dopaminergic neuron loss to present with clinical symptoms. It can be seen that replenishing GSH may have therapeutic benefits, but delivery to the dopaminergic neurons is very challenging. However, a brain-penetrable version of NAC, *N*-acetylcysteine amide (also known as AD4), has recently been developed (90). It has been shown to protect PC12 cells from rotenone-induced toxicity, and this protection was extended to the *in vivo* situation (91). These results suggest that a PD treatment that replenishes GSH levels is a clinical possibility, and to this end it will be interesting to see the outcomes of randomized, blinded placebo-controlled studies with GSH and its precursors.

CONCLUSIONS

Although GSH depletion has long been known to be an early change in PD, only recently have the biochemical effects of such depletion become apparent, notably the inhibition of complex I activity and UPS. Despite the advances in our knowledge of the role of GSH in PD pathogenesis, GSH depletion and oxidative stress in this process remain inextricably linked, with one leading to the other and *vice versa*. This means identifying the primary pathogenic event in PD remains as elusive as ever. If the cause of the GSH depletion could be delineated, this finding may provide an alternative route to maintaining GSH levels in PD patients, alleviating the neurodegenerative spiral that occurs in a GSH-depleted environment. However, the importance of GSH in the maintenance of reduced thiol groups has become clear as the inhibition of complex I and UPS dysfunction can be induced, at least in part, by oxidative and nitrosative modification of thiol groups. Such knowledge provides a potential therapeutic target, but further work must determine whether alleviation of GSH depletion slows the degeneration seen in PD. The recent advances in our understanding of the role of GSH in PD could provide exciting and promising potential for new treatments for PD patients. However, the benefits of GSH treatment for patients remain unclear and will require further studies. FJ

We thank Professor Peter McCaffery for his assistance and help in the preparation of this manuscript.

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Received for publication February 9, 2009.
Accepted for publication May 28, 2009.

Downloaded from www.fasebj.org by (76.224.100.48) on February 27, 2018. The FASEB Journal Vol. 23, No. 10, pp. 3263-3272.

Original Article

Effect of Glutathione Infusion on Leg Arterial Circulation, Cutaneous Microcirculation, and Pain-Free Walking Distance in Patients With Peripheral Obstructive Arterial Disease: A Randomized, Double-Blind, Placebo-Controlled Trial

ENRICO AROSIO, MD; SERGIO DE MARCHI, MD; MASSIMO ZANNONI, MD; MANLIO PRIOR, MD; AND ALESSANDRO LECHI, MD

• ***Objective*: To assess the effects of glutathione on pain-free walking distance (PFWD) and hemodynamic parameters in patients with peripheral artery disease.**

• ***Patients and Methods*: Forty patients with Fontaine stage II peripheral artery disease who were seen between September 2000 and March 2001 at the vascular laboratory and ward of the Division of Vascular Medicine and Rehabilitation at Verona University were studied in a double-blind, placebo-controlled trial. The patients were randomly assigned (20 per group) to treatment with intravenous glutathione twice a day or saline solution twice a day for 5 days. Treatments were administered in a double-blind manner. The 2 groups of patients underwent measurement of PFWD by strain-gauge plethysmography and laser Doppler flowmetry (with postischemic test) of the symptomatic leg at rest and after treadmill test. All measurements and tests were repeated 12 hours after the last infusion.**

• ***Results*: Between the 2 groups, hemodynamic tests showed no differences in baseline values and at rest after treatment. At rest, no differences were observed between basal and posttreatment values; findings in the saline group were similar during tests before and after the infusion period. In the glutathione group, we observed increases in PFWD (196±15 vs 143±11 m; *P*<.04), macrocirculatory flow after treadmill test with plethysmography at the end of treatment (9.3±2 vs 2.8±0.5 mL per 100 mL/min; *P*<.002), and postischemic hyperemia with laser Doppler flowmetry, registered as perfusion units (PU), at the end of infusions (14.4±3.2 vs 6.18±1.5 PU; *P*<.005), with a greater area under the curve after treatment (705±103 vs 508±45 PU/s; *P*<.001) and reduced time to flow motion (32±4 vs 48±11 seconds; *P*<.05).**

• ***Conclusion*: In patients with peripheral artery disease, glutathione prolongs PFWD and shows an improvement of macrocirculatory and microcirculatory parameters.**

Mayo Clin Proc. 2002;77:754-759

PFWD = pain-free walking distance; PU = perfusion units

Oxidative stress is considered an important condition in the atherosclerotic process, favoring cellular and vascular damage. Several studies have been performed to evaluate the use of antioxidants in the reduction of vascular events[1,2] and protection from development of atherosclerotic damage.[3] Other large-scale trials on secondary prevention of cardiovascular disease are ongoing. Currently, few data are available regarding peripheral occlusive disease, although some studies were performed in the past.[4]

Impairment of endothelial function is considered a critical step in the pathogenesis of atherosclerosis,[5,6] mainly as a result of altered production of and response to nitric oxide.[7-9] Also, platelet function is modified by oxidative stress,[10] resulting in increased adhesion and aggregation; free oxygen radicals may cause platelet activation through the regulation of protein kinase C, with an increase in platelet aggregation and fibrinogen-binding site exposure.[11]

Some authors have also shown an overexpression of adhesion molecules (intercellular adhesion molecule 1 and vascular cell adhesion molecule 1) and an increased release of free radicals in endothelial cells if exposed in vitro to shear stress,[12,13] with an alteration of leukocyte-endothelial cell interaction.[14,15]

Peroxinitrite is a metabolite of nitric oxide considered a toxic agent that leads to vascular and myocardial dysfunction.[16] This effect has been shown in particular in inflammatory-like conditions, such as ischemia-reperfusion injury.

Reduced glutathione is an intracellular tripeptide thiol that is present in high concentration inside cells. It is, with ascorbic acid, the major water-soluble antioxidant and plays a central role in the regulation of the cellular redox state.[17] Emerging data show the importance of glutathione in the modulation of endothelium-derived nitric oxide.[18] Patients with atherosclerosis are characterized by impaired nitric oxide–mediated arterial relaxation.[19,20] In these patients, treatment with agents that increase intracellular syn-

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Table 1. **Characteristics of the Study Population***

Characteristic	Glutathione group	Saline group
Age (y)	55-70	52-73
Body mass index (kg/m^2)	24±3	26±4
Total cholesterol (mg/dL)	188±33	184±28
Triglycerides (mg/dL)	162±24	167±28
HDL-C (mg/dL)	48±8	47±10
HDL-C/total cholesterol ratio	5.4±1.1	5.9±1.3
Glucose (mg/dL)	99±10	100±10

*Data are presented as mean ± SD. HDL-C = high-density lipoprotein cholesterol.

thesis of glutathione has caused an increase in nitric oxide bioactivity.[21]

Glutathione peroxidase increases the inhibitor effect of nitric oxide on platelet aggregation by reducing hydroperoxides.[22] Moreover, other authors have shown that glutathione favorably influences the coronary circulation.[23] In fact, the intracoronary infusion of reduced glutathione improves endothelial vasomotor response in subjects with coronary risk factors and potentiates the vasodilator effect of nitroglycerin.[23]

PATIENTS AND METHODS

Study Population

Between September 2000 and March 2001, we studied 40 consecutive patients who were seen at the vascular laboratory and ward of the Division of Vascular Medicine and Rehabilitation at Verona University with Fontaine stage II stable obstructive arterial disease of the lower limbs (35 men and 5 women; age range, 55-70 years). Arterial disease was defined on the basis of pain-free walking distance (PFWD) (measured during a treadmill test with 0% slope and 4.0-km speed),[24] Doppler velocimetry (for calculation of ankle-brachial index), and digital arteriography of the lower limbs (Tables 1 and 2). Ischemic heart disease, diabetes, and smoking (no smoking at least in the 6 months preceding the study) were the exclusion criteria; 5 of the recruited patients were affected by mild hypertension and were treated with clonidine (0.250 mg twice a day) (Table 1). Subjects with arthrosic pathologic findings of the lower limbs were also excluded. Vasoactive drugs were not allowed, and all patients were treated with aspirin (300 mg/d) in the last 3 months before the beginning of the study. All patients examined gave oral informed consent before being included in the protocol.

Table 2. **Heart Rate (HR), Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), Ankle-Brachial Index (ABI), and Pain-Free Walking Distance (PFWD) Before and After Infusions in the 2 Groups***

	Glutathione group		Saline group	
	Before	After	Before	After
HR (beats/min)	76±11	77±12	76±11	77±12
SBP (mm Hg)	148±15	145±16	146±16	144±16
DBP (mm Hg)	82±7	77±9	85±7	78±8
ABI at rest	0.74±0.09	0.77±0.06	0.76±0.10	0.77±0.07
PFWD (m)	113±11	221±15†	119±18	159±26

*Data are presented as mean ± SD.
†$P<.04$ vs basal.

Study Protocol

The study was performed during patient hospitalization at 9 AM in a comfortable environment and at a constant temperature (22°C±1°C). After a 15-minute period of stabilization with the patient supine, hemodynamic measurements (plethysmography and laser Doppler flowmetry) were taken. Then the patients underwent treadmill tests (4.0 km/h, 0% slope) until onset of the first pain at the calf so we could determine the PFWD. The treadmill test was performed at least 6 times in 2 consecutive days (3 times per day, in the morning and with an interval of 30 minutes, under the control of a therapist) to obtain stable values (<3% of variance) in the last 3 tests.

Sixty seconds after determination of PFWD, the plethysmographic measurements were once again taken; this time was chosen because the patient was already supine on the examination bed and the instrument was positioned on the leg. Postexercise plethysmography is a reliable method for detecting and analyzing peripheral obstructive disease.[25] Sixty seconds were precisely counted for each patient, and this time reflects only part of the actual hyperemic curve,[25,26] since in healthy patients we could recognize the last part of the curve 60 seconds after exercise. Furthermore, the vasodilation phenomenon is evident at that time, and the evaluation of the ankle-brachial index is conducted 1 minute after the exercise,[27] when the decrease in pressure due to vasodilation may still be well documented.

Twenty patients were randomized to receive an infusion of glutathione, 0.646 g in 250 mL of 0.9% sodium chloride solution twice a day for 5 consecutive days, and 20 patients were treated with infusion of saline solution, 250 mL of 0.9% sodium chloride twice a day; the treatments were administered in a double-blind manner.

Hemodynamic Measurements

Blood flow at the large arteries of the lower limb was measured by means of strain-gauge plethysmography (Periquant 3800 venous-occlusion plethsmography, Guttman Elektronik, Eurasburg, Germany).[28] Strain gauges were applied 8 cm below the tuberositas tibiae and the cuff at the middle third of the thigh in the symptomatic leg. At least 10 measurements were performed, and the mean value was calculated (expressed in milliliters per 100 mL of tissue per minute).

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Cutaneous microcirculation of the foot was studied using laser Doppler flowmetry (Periflux PF 3, Perimed, Järfälla, Sweden). This device has a helioneon laser that emits light at a wavelength of 632.8 nm, which is transmitted by optic fiber to a probe. The device can measure the dermal microcirculatory flow through analysis of the backscattered light, and it expresses the flow in perfusion units (PU). The volume sample is a hemisphere with a radius of 1 mm (with white skin). The probe was fixed, using an adhesive support, to the dorsal aspect of the foot, at the middle third, of the symptomatic leg. The precise position of the probe was marked on the skin.

Rest measurements were followed by postischemic hyperemia measurements,[29,30] which were obtained by placing a sphygmomanometer cuff on the middle portion of the calf of the symptomatic leg and inflating it with oversystolic pressure (Doppler ultrasonography was used to determine it). Cuff pressure was maintained for 3 minutes and then interrupted by rapid deflation of the cuff. The values supplied by the laser Doppler flowmetry were expressed in PU, and we used the mean value for rest evaluation. For postischemic hyperemia evaluation, we used the peak flow and the area under the curve. The area under the curve is calculated by the device software and describes the area under the hyperemic curve of the monitor trace from the peak to the return to rest values. We also performed the spectral analysis of flow motion by means of Fourier transformation and calculated the time to flow motion after ischemic stimulus.[31] Flow motion is periodic fluctuation of laser Doppler flow due to local regulation of microcirculation (myogenic, neurogenic, and cardiorespiratory). This pattern is a characteristic of the preserved cutaneous microcirculatory system and in Fontaine stage II is normally preserved. The device can calculate the time between the end of ischemia, which abolishes these periodic fluctuations, and the recovery of flow motion[31]; flow motion can be detected in segments of the trace of at least 1 minute, and the analysis of adjacent segments permits the detection of flow motion recovery. This periodic variation of flow describes the integrity of microcirculatory vasomotor regulation; after ischemia, the sooner flow motion reappears, the more efficient these mechanisms are.

Dynamic Tests

We measured the arterial flow at rest and 60 seconds after the treadmill test. Microcirculatory cutaneous blood flow was evaluated at rest and after ischemia. Both procedures were performed before and 12 hours after the last administration of therapy.

Statistical Analysis

The data are expressed as mean ± SD, and the statistical analysis was performed by using analysis of variance (SPSS statistical software, SPSS Italia srl, Bologna, Italy) followed by post hoc *t* test. $P<.05$ was considered statistically significant.

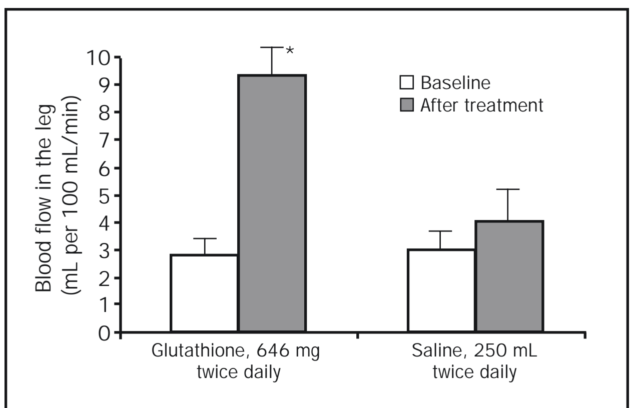

Figure 1. Hyperemia after treadmill tests by means of strain-gauge plethysmography before and after treatments. Asterisk indicates $P<.002$ vs baseline and vs saline treatment. Error bars indicate SD.

RESULTS

No differences were observed between the 2 groups regarding heart rate, blood pressure, and PFWD at basal conditions (Table 2). Hemodynamic parameters were unvaried at rest in 2 stages of the study in both groups. At the end of the infusion period, we observed an increase in PFWD in patients treated with glutathione (221±15 vs 113±11 m; $P<.04$) (Table 2); only a slight, nonsignificant increase in PFWD was registered in patients who had received saline solution.

Regarding plethysmography, no variations were detected at rest before and after infusions in the 2 groups. In all patients, in basal conditions a slight (but nonsignificant) increase in after-treadmill flow was observed. After infusions, only the glutathione group showed a statistically significant increase in plethysmographic values after treadmill testing compared with rest measurements (9.3±2 vs 2.8±0.5 mL per 100 mL/min; $P<.002$) (Figure 1); this value is higher than that in the saline group at the same stage of the study ($P<.002$).

In all patients, no variations of PU were observed at rest with laser Doppler flowmetry. Both groups showed a hyperemic response to ischemic tests before and after infusions. At the end of the infusion period, we observed an increased hyperemic response at peak flow in the glutathione group vs basal conditions and vs the saline group (14.4±3.2 vs 6.18±1.5 PU; $P<.005$) (Figure 2). The glutathione group presented a greater area under the curve, describing reactive hyperemia, after treatment (705±103 vs 508±45 PU/s; $P<.001$); no variations were observed in the control group. A characteristic flow motion pattern was observed in all

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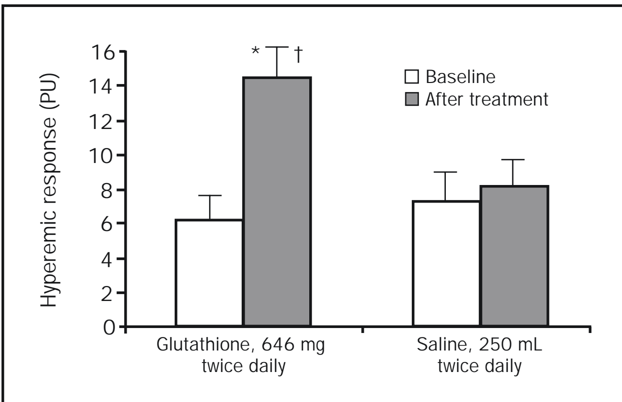

Figure 2. Cutaneous postischemic hyperemia (laser Doppler flowmetry) at baseline and after treatments. Asterisk indicates $P<.005$ vs baseline glutathione; dagger indicates $P<.05$ vs saline after treatment. Error bars indicate SD. PU = perfusion units.

patients, with a fundamental frequency of 3.6±1.0 cicle/min; the same pattern was recognized after treatments. Time to flow motion after ischemia was shortened by treatment with glutathione (32±4 vs 48±11 seconds; $P<.05$) (Figure 3).

In addition, in the glutathione group, 4 patients had a PFWD that was prolonged but less than the others and no difference in postexercise blood flow in the leg after glutathione administration (Figure 4). The response to therapy is recognizable in microcirculatory flow analysis. These patients had no different clinical features.

DISCUSSION

The main clinical result of our study is the improvement in PFWD after therapy with glutathione. Because PFWD represents a physiologic parameter, we considered it more representative of a patient's physical status than absolute walking distance even if it can vary more (a series of tests was performed at the recruiting phase of the study).[32] Furthermore, we used the PFWD test to determine postexercise hyperemia. The improvement of PFWD could be explained by the favorable modifications of the hemodynamic parameters studied through dynamic tests.

Postexercise hyperemia is a physiologic, adaptive response of circulation to increased request for oxygen by muscles, and it is guaranteed by normal arterial tree patency. The mechanisms of exercise-induced vasodilation are complex, and endothelium plays a role mainly in postexercise blood flow regulation.[33] Moreover, collateral vessels are activated in this condition of exercise. In our study patients we did not register any significant hyperemia at baseline due to the important atherosclerotic damage responsible for both multiple occlusions of large and medium vessels and reduced endothelium-dependent vasodilation. The output in the small vessels was reduced as well, since lesions under the popliteal artery were documented in these patients. As a consequence, the hyperemia is, in fact, limited in "amplitude" and is diluted during a long interval so that in our patients it was not detectable.

The increase in plethysmographic values after exercise in the glutathione group depends on the increase of total flow in the lower limb after exercise. This is allowed presumably by a better output in the distal arterial segments, an improved vasodilation elicited by exercise, and, we speculate, an activation of collateral vessels. In atherosclerotic patients, endothelial dysfunction plays a central role in vascular impairment and progression of disease. Some authors have documented an improvement in endothelial dysfunction,[21] with restored flow-mediated arterial dilation and endothelium-dependent dilation in response to acetylcholine after infusion of glutathione in coronary vessels.[23] The improvement in arterial flow is therefore also dependent on restored endothelial function.[34] We believe that these patients were able to sustain a larger fraction of their peak flow with glutathione.

In atherosclerotic patients, in particular during exercise, we considered the hypothesis of an increased production of peroxinitrite due to the presence of superoxide anions, with toxic consequences on vessels.[16] Glutathione could prevent this toxic injury, ensuring better vascular function with "easier overcoming" of oxidative stress.

Postischemic hyperemia depends not only on the integrity of anatomical vascular structures but also on the vessel wall capacity to respond to stimuli from tissue metabolism and neuronal stimulation. Therefore, the greater hyperemia registered with laser Doppler flowmetry after treatment with glutathione but not with placebo indicates an im-

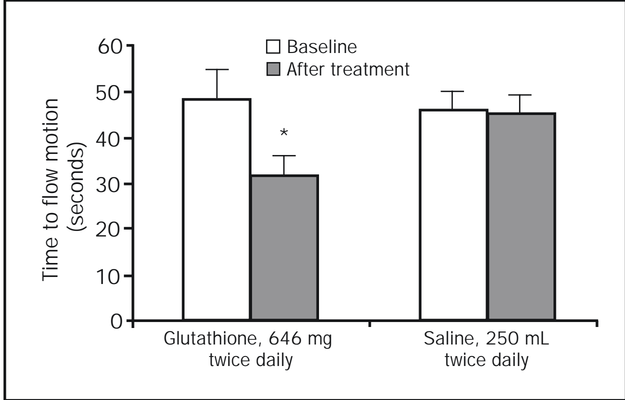

Figure 3. Time to flow motion detected by means of laser Doppler flowmetry before and after treatments. Asterisk indicates $P<.05$ after glutathione vs baseline. Error bars indicate SD.

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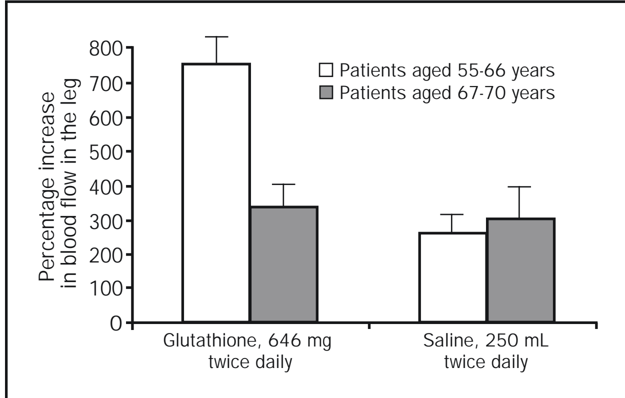

Figure 4. Percentage increase in blood flow in the leg with plethysmography after exercise compared with correspondent flow at rest in 2 subgroups of patients. Error bars indicate SD.

proved capability of vessels to respond to an ischemic stimulus. Another minor observation is the reduced time to flow motion. The shortening of time to flow motion detected in patients treated with glutathione is due to the improved function of the mechanisms responsible for flow motion (myogenic and neurogenic vasoregulation of the microcirculatory vascular bed).[31,35,36]

An additional observation regards the nonresponders to therapy. There were 4 nonresponders in the glutathione group (age range, 67-70 years) and 3 in the saline group. These patients had a slight improvement in PFWD and no change in postexercise hyperemia (Figure 4). Microcirculatory parameters were homogeneous to the others in the group. The group is too small to give the findings statistical significance; a further study should be conducted to analyze age-related response to this treatment.

In conclusion, the results of our study suggest that antioxidative treatment (glutathione) can improve PFWD in patients with peripheral arterial disease. Since glutathione improves postexercise hyperemia and postischemic hyperemia, we suggest that it produces a better vasoregulation in response to exercise and ischemia and a partially restored microcirculatory-mediated dilation in patients with obstructive peripheral artery disease, leading to the clinical effect of improving PFWD.

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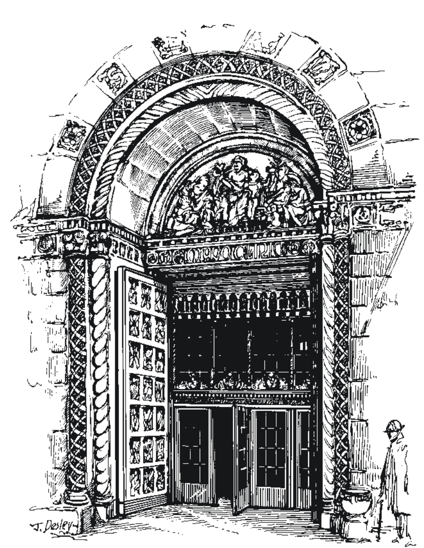

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Advance Access Publication 17 May 2007

eCAM 2008;5(1)27–35
doi:10.1093/ecam/nem040

Review

The Treatment of Pulmonary Diseases and Respiratory-Related Conditions with Inhaled (Nebulized or Aerosolized) Glutathione

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Reduced glutathione or simply glutathione (γ-glutamylcysteinylglycine; GSH) is found in the cytosol of most cells of the body. GSH in the epithelial lining fluid (ELF) of the lower respiratory tract is thought to be the first line of defense against oxidative stress. Inhalation (nebulized or aerosolized) is the only known method that increases GSH's levels in the ELF. A review of the literature was conducted to examine the clinical effectiveness of inhaled GSH as a treatment for various pulmonary diseases and respiratory-related conditions. This report also discusses clinical and theoretical indications for GSH inhalation, potential concerns with this treatment, its presumed mechanisms of action, optimal doses to be administered and other important details. Reasons for inhaled GSH's effectiveness include its role as a potent antioxidant, and possibly improved oxygenation and host defenses. Theoretical uses of this treatment include Farmer's lung, pre- and postexercise, multiple chemical sensitivity disorder and cigarette smoking. GSH inhalation should not be used as a treatment for primary lung cancer. Testing for sulfites in the urine is recommended prior to GSH inhalation. Minor side effects such as transient coughing and an unpleasant odor are common with this treatment. Major side effects such as bronchoconstriction have only occurred among asthma patients presumed to be sulfite-sensitive. The potential applications of inhaled GSH are numerous when one considers just how many pulmonary diseases and respiratory-related conditions are affected by deficient antioxidant status or an over production of oxidants, poor oxygenation and/or impaired host defenses. More studies are clearly warranted.

Keywords: aerosolized glutathione (GSH) – antioxidant – inhaled GSH – nebulized GSH – reduced GSH

Introduction

Reduced glutathione or simply glutathione (γ-glutamylcysteinylglycine; GSH) is found in the cytosol of most cells of the body (1). It is a tripeptide consisting of glycine, cysteine and glutamate. GSH functions in several enzyme systems within the body that assist with the quenching of free radicals and the detoxification of fat-soluble compounds (Table 1) (2–5). It also plays a significant metabolic role in supporting many different biochemical processes (e.g. amino acid transport, deoxyribonucleic acid synthesis and immune system augmentation) considered to be important mediators of health status (6).

Glutathione in the epithelial lining fluid (ELF) of the lower respiratory tract is thought to be the first line of defense against oxidative stress (6). The ELF concentration of GSH is 140 times that of serum concentrations with a redox ratio of $>9:1$ (7). In fact, alternations in alveolar and lung GSH metabolism are widely recognized as a central feature among many inflammatory lung diseases (8–14). In healthy lungs, the oxidant burden is balanced by local antioxidant defenses. However, in lung

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Table 1. Enzyme systems involving glutathione

Enzyme system	Function
Glutathione synthetase	Gamma-glutamyl cycle.
Riboflavin-containing glutathione reductase	Catalyzes the conversion of oxidized glutathione (glutathione disulfide; GSSG) to its reduced form.
GSH transferase isoenzymes	Conjugation of GSH with fat-soluble substances for liver detoxification and the detoxification of environmental carcinogens, such as those found in tobacco smoke.
Selenium-containing glutathione peroxidase (GPX)	Protects cells from hydrogen peroxides and lipid hydroperoxides. If not neutralized, these peroxides will damage cellular membranes and other vital cellular components.
Leukotriene C_4 synthase	Conjugation of leukotriene A_4 with GSH, resulting in the generation of leukotrienes C_4. Gamma-glutamyl transpeptidase then metabolizes leukotrienes C_4 to leukotrienes D_4.

diseases cellular damage and injury is mediated by an increased oxidant burden and/or decreased antioxidant defenses.

In inflammatory lung diseases, supplementation with exogenous sources of GSH would be necessary to reduce the oxidant load and/or correct for antioxidant deficiencies within the lungs. A few published clinical studies have shown the oral administration of GSH to be ineffective at increasing plasma levels when given to healthy subjects (15), or when used for the treatment of hepatic cirrhosis (16). If the oral administration of GSH cannot raise plasma levels in healthy and diseased patients, it is doubtful that this method of delivery would have any appreciable effects at increasing GSH concentrations within the lungs.

Intravenous administration might be effective since it bypasses the gastrointestinal tract, immediately enters the blood stream, and presumably would saturate body tissues such as the lungs. Unfortunately, the results of a study did not show intravenous administration to be effective at increasing GSH levels within the ELF (17). When 600 mg of GSH was delivered intravenously to sheep, the levels in the venous plasma, lung lymph and ELF increased only for a very brief period of time. However, when the same amount of GSH was delivered through inhalation (nebulized or aerosolized), the baseline GSH level in the ELF ($45.7 \pm 10\,\mu M$) increased 7-fold at 30-min ($337 \pm 64\,\mu M$), remained above the baseline level 1 h later ($P < 0.001$) and returned toward baseline levels by 2 h. Despite this short-term increase in GSH concentrations within the ELF, the inhalation method did not significantly increase the amount of GSH in the lung lymph, venous plasma and urine during the 2 h study period. The authors of this report concluded that inhalation specifically increased GSH levels at the lung epithelial surface.

Given that inhalation is the only known method that increases GSH levels in the ELF for a significant duration, a review of the literature was conducted to examine the clinical effectiveness of inhaled GSH as a treatment for various pulmonary diseases and respiratory-related conditions. Only reports involving human subjects were included in the analysis. The clinical and theoretical indications for GSH inhalation were summarized and potential concerns with this treatment reported. Other pertinent details such as its presumed mechanisms of action and optimal doses to be administered were compiled and evaluated.

Methods

Literature Search

Computer searches were conducted of English and non-English language articles in the Biomedical Reference Collection (1984 to August 2006), CINAHL (1982 to August 2006), MEDLINE (1965 to August 2006) and Nursing and Allied Health Collection (1985 to August 2006) databases. Articles were searched with the key search terms 'Nebulized Glutathione,' and 'Glutathione' in combination with 'Aerosol' OR 'Inhalation.' These keywords were also searched with words related to pulmonary and/or respiratory disease. To supplement the search, references of the articles found from the initial search were reviewed. Hand searching of relevant journals was also completed as part of the search.

Selection of Articles

To be included in the final review, articles had to report on the use and administration of inhaled GSH for pulmonary diseases and respiratory-related conditions in human subjects. Only peer-reviewed articles were reviewed.

Quality Assessment

An evidence grade was determined for each article. These evidence grades were adapted from the hierarchy of evidence developed by the Oxford Centre of Evidence Based Medicine (Table 2) (18).

Table 2. Grades of evidence

A	Systematic reviews of randomized controlled trials and/or randomized controlled trials with or without double-blind placebo control.
B	Systematic reviews of observational studies and/or high-quality observational studies including cohort and case-control studies and/or cohort 'outcomes' research and/or nonrandomized controlled trials.
C	Case-series, case-reports, and/or poor-quality cohort and case-control studies.
D	Expert opinion without explicit critical appraisal or based on physiology, bench research or 'first principles.'

Results

A total of 12 reports were screened (9,10,17,19–27). Only one report was excluded because it involved the use of inhaled GSH in sheep (17). In total, 11 articles were found to meet the inclusion criteria and were included in this review (9,10,19–27). Table 3 displays the characteristics of the studies included in this review.

Discussion

Based exclusively on the published evidence included in this review, inhaled GSH is potentially indicated for the following clinical conditions: cystic fibrosis (CF), chronic otitis media with effusion (OME), HIV seropositive individuals, idiopathic pulmonary fibrosis (IPF) and chronic rhinitis. These conditions were chosen since the published studies were of good quality, received A and B evidence grades, and their respective results demonstrated benefits from the use of GSH inhalation.

Inhaled GSH cannot be recommended as a potential treatment for emphysema since the quality of evidence is lacking at the present time. The emphysema case report had notable limitations since serial spirometry was not documented, and the placebo effect could not be ruled-out (22). However, this does not necessarily indicate that GSH inhalation would be of no benefit for emphysema patients. There is experimental and human data demonstrating a link between GSH, oxidant-derived damage and possible protection against the development of emphysema. An *in vitro* study demonstrated that GSH could retard the oxidant-mediated down-regulation of α-1-proteinase inhibitor activity in smokers' emphysema (28). This finding is important since one of the principal pathophysiological mechanisms of emphysema is the down-regulation of this enzyme by means of oxidative damage (29). Moreover, in a recent review of lung GSH and cigarette smoke-induced airway disease, increased GSH in the ELF of chronic smokers was presumed to be a protective adaptive mechanism against the development of chronic obstructive pulmonary disease (COPD) (30). Considering that not all chronic smokers go on to develop COPD, the authors in that review pointed out that genetic variations in the molecular mechanisms that regulate GSH metabolism might explain why some individuals are better protected against the development of COPD. It thus appears that emphysema patients are subjected to progressive tissue damage due, in part, to the consequences of GSH deficiency and/or genetic variations in GSH metabolism. Since GSH inhalation would presumably offer both antioxidant protection and GSH replenishment, this method of treatment would potentially benefit emphysema patients.

Asthma is another condition where inhaled GSH cannot be recommended since this treatment caused notable side effects (e.g. breathlessness, bronchoconstriction and cough) in the cited study (21). These side effects were linked primarily to the production of sulfites that occurred when GSH was in solution. GSH inhalation should continue to be explored as a potential treatment for asthma. None of the asthma patients in the study had their urine tested for sulfites. A positive test for sulfites would have eliminated these patients from entering the study. Accordingly, the results might have been much more favorable if patients without sulfite sensitivities were included.

This issue of asthma and sulfite sensitivities is an important one for clinicians to be mindful of. Sulfites are found in beer, wine, restaurant salad bars, seafood, potatoes, processed foods and many pharmaceuticals (31). Many asthma patients report being sensitive to sulfites. In an Australian study, ~30% of asthmatic patients reported being sensitive to sulfites in wine (32). A more recent and rigorous scientific study, however, demonstrated that asthma patients can tolerate varying amounts of sulfites in wines ranging from 20, 75 or 150 parts per million (ppm) (33). Only a small minority of patients in this study (4 of 24 self-reported wine-sensitive asthmatics) exhibited reactions when challenged with 300 ppm of sulfites. One report indicated that 4–8% of asthmatics are sensitive to sulfites (34). Other reports have estimated the incidence of sulfite sensitivity to be around 5–11% (35,36). Even though the exact percentage of sulfite-sensitive asthmatics is difficult to ascertain, sulfite sensitivity is an important factor to assess when using or evaluating research done on inhaled GSH.

Future Research Directions

There are additional clinical conditions that might benefit from this type of treatment, but further studies are necessary. One such condition is Farmer's lung (FL), which is a hypersensitivity pneumonitis caused by the inhalation of thermophilic actinomycetes and spores of *Aspergillus* specie (11). A study was undertaken to investigate the effect of pulmonary GSH levels after hay exposure in patients with FL and in asymptomatic farmers (AF) (11). Fifteen symptomatic patients with FL

Table 3. Summary of articles demonstrating the effectiveness of inhaled glutathione for the treatment of pulmonary diseases and respiratory-related conditions

Reference	Condition	*N*	Dosages of inhaled GSH	Outcome	Evidence grade
(21)	Asthma	Eight asthma patients [mean age, 29 ± 7 (standard deviation; SD) years]	600 mg once weekly for 3 months	A subset of patients with clinically stable mild asthma experienced a bronchoconstrictor effect when treated with inhaled GSH.	A: Randomized placebo-controlled trial
(23)	Chronic otitis media with effusion (chronic OME)	30 patients (3–12 years of age; mean age, 5.8 years) and 30 controls (3–12 years of age; mean age, 6.1 years)	600mg of GSH in 4 ml of saline subdivided into five 2-min sessions by nasal aerosol every 3–4 waking h for 2 weeks	GSH should be considered for the nonsurgical management of chronic OME.	A: Randomized placebo-controlled trial
(24)	Cystic fibrosis (CF)	Nine patients [mean age, 16.1 ± 1.44 (SD) years] received the S-nitrosoglutathione (GSNO) and 11 patients [mean age, 19.9 ± 3.45 (SD) years] received the phosphate-buffered saline (PBS) solution	0.05 ml/kg of 10 mM GSNO	The treatment group showed a modest improvement in oxygenation that was thought to be independent of the physiological effects of nitric oxide.	A: Randomized placebo-controlled trial
(27)	CF	19 patients (6–19 years of age) were randomized to treatment [mean age, 13.3 ± 4.1 (SD) years] or placebo groups [mean age, 12.9 ± 4.9 (SD) years]	Total daily dose administered to the patients in the treatment group was 66 mg/kg of body weight	GSH can improve clinical parameters in CF patients, and that effective treatment should include the correction of GSH deficiency.	A: Randomized placebo-controlled trial
(9)	Idiopathic pulmonary fibrosis (IPF)	10 patients with IPF [mean age, 46 ± 3 (SD) years] and 19 normal nonsmokers [mean age, 36 ± 3 (SD) years]	600 mg twice daily for 3 days	Inhaled GSH might be beneficial among IPF patients by reversing the oxidant–antioxidant imbalance.	B: Nonrandomized controlled trial
(19)	Human immunodeficiency virus (HIV) seropositive individuals	14 HIV seropositive individuals [mean age, 32 ± 2 (SD) years]	600 mg twice daily for 3 days	It is a reasonable therapeutic strategy to augment the deficient GSH levels of the lower respiratory tracts of HIV seropositive individuals.	B: Cohort 'outcomes' research
(20)	Chronic rhinitis	13 patients with chronic rhinitis and 13 healthy subjects (4–15 years of age for all subjects; mean age, 8.2 years)	600 mg daily for 14 days	Statistically significant improvement in nasal obstruction, rhinorrhea and ear fullness.	B: Nonrandomized controlled trial
(10)	CF	Seven CF patients [mean age, 25 ± 1 (SD) years]	600 mg of GSH for 3 days	Inhalation therapy with GSH does normalize the respiratory epithelial surface oxidant–antioxidant balance in CF patients.	B: Cohort 'outcomes' research
(25)	CF	21 patients with CF (16–37 years of age for all subjects)	300 or 450 mg three times daily for 14 days	Inhaled GSH can permeate the lower airways of the lungs and improve important parameters of lung function in CF patients despite not having any effect upon markers of oxidative injury.	B: Cohort 'outcomes' research

(continued)

Table 3. Continued

Reference	Condition	*N*	Dosages of inhaled GSH	Outcome	Evidence grade
(26)	CF	17 patients with CF (18–29 years of age for all subjects; mean age, 24 years)	450 mg three times daily for 14 days	Inhaled GSH did not affect the oxidative status of the patients who were tested, but it did favorably modulate their immune responses.	B: Cohort 'outcomes' research
(22)	Emphysema	One (95 year-old male)	120 mg of GSH in office, then 120 mg twice daily for 3 days, and continuation of treatment (dose unknown) for 2 years	When the patient returned for a follow-up visit, he no longer required the use of his wheelchair and oxygen. The striking results were unexpected and unlikely to be due to placebo alone.	C: Case report

[mean age, 42 ± 1 (SD) year] were compared with 10 AF [mean age, 43 ± 1 (SD) year] serving as the control group. All patients had baseline lung function testing and testing at various time intervals following hay exposures. The authors of this study concluded that FL and AF patients have characteristically different intrapulmonary levels of GSH, and that the pathogenesis of FL is likely related to GSH regulatory mechanisms. They also speculated that AF patients have a better ability to upregulate their pulmonary GSH levels, which would protect them against active disease. Clinical testing of inhaled GSH in patients with FL is warranted.

The administration of GSH inhalation before and/or immediately following exercise is another potential application of this novel treatment. Exercise is a known inducer of oxidative stress leading to free radical production, which can encourage lipid peroxidation and tissue damage among individuals with deficient and/or impaired antioxidant systems. As stated in the beginning of this report, selenium is a cofactor in the GPX enzyme that protects cells from hydrogen peroxides and lipid hydroperoxides. When under situations of oxidative stress, the GPX enzyme will markedly increase in the lungs as an antioxidant adaptive response (37). By supplying more GSH to the lung tissues, more of this enzyme might be available to help reduce the production of free radicals associated with exercise. Although these assumptions are very speculative, it does seem possible and even logical that GSH inhalation would benefit those who regularly exercise by increasing exercise tolerance, and by maintaining and/or replenishing the antioxidant systems within the lungs.

Multiple chemical sensitivity disorder (MCSD) is another condition that might be clinically responsive to this treatment. Patients with this disorder are known to have bronchial hyperreactivity and even exhibit asthma-like symptoms (38). Unlike asthma, MCSD is not associated with atopy and immunoglobulin E (IgE)-mediated allergic mechanisms (39). The prevailing theory explaining the cause of MCSD is a fusion between two separate theories—the neural sensitization and nitric oxide/peroxynitrite theories (40). This fusion theory, proposed by Pall, links long term potentiation of *N*-methyl-d-aspartate (NMDA) receptors at the synapses of nerve cells by glutamate and aspartate to an increased production of nitric oxide and its oxidant product, peroxynitrite (40,41). Treatment with antioxidants may improve symptoms of MCSD by reducing the peroxynitrite elevations and other biochemical dysfunctions that are associated with such elevations (40,41). Glutathione inhalation may be ideal since the primary route by which patients with MCSD get triggered is through smelling and breathing. Sulfite sensitivity would have to be considered since inhaled GSH could provoke adverse events. This treatment might be capable of providing antioxidant protection to both the upper and lower respiratory airways, which would theoretically help to reduce the symptoms of MCSD and the production of peroxynitrite. More research studies are necessary.

Two final conditions, cigarette smoking and lung cancer, are worth mentioning since they are intimately related to each other and are affected by GSH and its related enzymes. These conditions are influenced by the glutathione S-transferase (GST) group of enzymes that are found in significant quantities in the bronchioles and alveoli of the lungs (42), and in very high concentrations in the bronchial epithelium (43). Among smokers, a lack of the GST mu enzyme was thought to be associated with a greater risk of lung cancer, especially if there was a cancer and/or lung cancer history among the relatives of the patients in this study (44). Since the GST mu enzyme detoxify carcinogens in tobacco, any deficiency of this enzyme was presumed to be associated with an increased risk of lung cancer. However, a more recent study pertaining to the GST group of enzymes found no such association (45). In this meta-analysis, polymorphisms in the GST genes had no associations or weakly positive associations with risk factors for lung cancer. Despite the

need for more research, GSH inhalation might be beneficial for smokers to augment their GST enzymes, which would help facilitate the detoxification of carcinogens. Even though the best intervention for these patients would be smoking cessation, many patients lack the necessary willpower to quit. For these patients, regular GSH inhalation might reduce oxidants generated from cigarette smoke ($\sim 10^{14}$ free radicals/puff) (46), and the epithelial lung injury associated with smoking (47).

For lung cancer patients, the use of GSH inhalation is not recommended. Cancer cells use multiple mechanisms (e.g. altered transport of a drug, inhibition of drug-induced apoptosis and elevation of cellular GSH) to circumvent the cytotoxic effects of chemotherapeutic agents (48). Early research studies showed that GSH was able to reduce cytotoxicity to chemotherapeutic compounds by boosting the metabolism of drugs to less active compounds, or by the detoxification of free radicals (49,50). More recently, research has revealed that the levels of a specific GST enzyme increases among cancer cells with higher differentiation grades, and that these drug-resistant gene products are found in lung carcinomas at the time of surgical resection (51). There is also speculation that GSH might be capable of repairing drug-induced injury at the DNA level (48). A recent review article has described the involvement of glutathione in the detoxification or inactivation of platinum drugs—the most commonly employed drugs for the treatment of advanced stage lung cancer patients (52). Based on this information, it would be unwise and illogical to use GSH inhalation while lung cancer patients are undergoing active chemotherapy treatment.

Mechanism of Action

Inhalation of GSH results in a mechanism of action confined to the upper airways and lungs (Fig. 1), and will not influence plasma levels to a significant degree. In the studies that measured both lung and plasma levels of GSH, the plasma levels remained essentially unchanged following GSH inhalation. Seven of the studies included in this review demonstrated that GSH inhalation exerts its effects upon the lower respiratory tract (9,10,19,24–27). The upper respiratory tract also appears to benefit from GSH augmentation. Two studies involving patients with upper respiratory tract diseases showed clinical benefits from GSH inhalation treatment (20,23). The predominant mechanism responsible for GSH's therapeutic effects are probably related to its antioxidant properties that offer protection against oxidative injury, and/or assist with the normalization of the oxidant–antioxidant balance within the upper and lower respiratory tract. Even though the majority of these studies suggested that antioxidant protection was the principal reason for the favorable treatment responses, some of the studies were unable to demonstrate a change in markers of oxidation from this treatment. More data is necessary to confirm the precise nature of GSH's antioxidant properties within the upper and lower respiratory tract. Additional explanations for GSH's therapeutic effects might include an improvement in host defenses (e.g. increased cytotoxic lymphocytes), and better oxygenation (e.g. an increase in oxygen saturation). GSH inhalation produced clinically meaningful results in the majority of diseases that were studied. Specifically, GSH inhalation was shown to improve clinical markers of respiratory function that inevitably impact upon quality of life and disease progression. These improvements were the most important outcomes and features of this novel treatment.

Considerations Prior to Initiating GSH Inhalation

The urine should be tested for sulfite sensitivity. A special test strip can be dipped in the urine, and is known as the 'EM-Quant 10013 Sulfite Test.' It can be easily located through any search engine on the Internet (53). Even though instructions for sulfite testing have been published elsewhere (54), a brief description of the procedure is outlined below:

> A random (fresh) urine sample is suitable, but a first morning void may be preferable due to its higher concentration. Once the test strip is dipped in the urine (for 1 s), the reaction zone changes color to indicate the concentration of sulfites present. After 30 s, the color on the test strip is compared to a color scale on the bottle indicating the concentrations of sulfites in the urine (can detect 10, 40, 80, 180 and 400 ppm of sulfites). The resultant concentration should be multiplied by a factor of 1.5 to provide the amount of free sulfites in mg/l (ppm). The strip will not detect below 10 ppm. The urine samples should be preservative free, and the urinary pH should also be tested with pH paper. If the urine pH is below 6, then the amount of sulfites might be underestimated by the test. In such cases, consider adding sodium acetate or sodium hydroxide to raise the pH to at least 7–10 (should not exceed a pH of 12), and then repeat with a new test strip.

If the urine test were positive for sulfites (normally they are absent), the use of inhaled GSH would be strictly contraindicated.

Method of Delivery, Recommended Daily Dosages and Side Effects

With a nebulizer, a solution of GSH is made into an aerosol and is delivered to the upper respiratory tract and the lungs through a mask that covers the nose and mouth, or is delivered directly into the lungs via a mouthpiece. Any compounding pharmacist would be

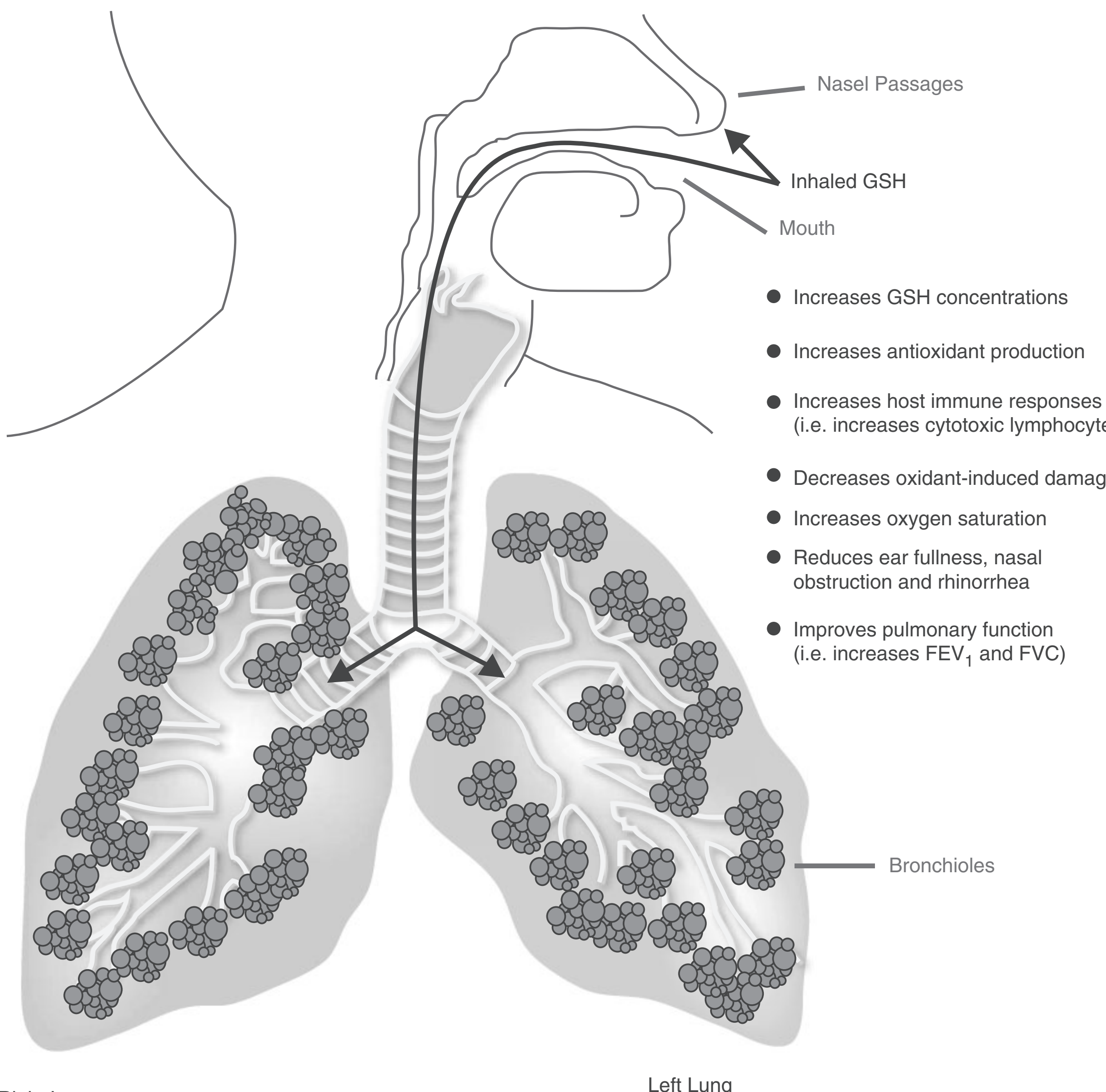

Figure 1. Inhaled GSH's mechanism of action. GSH, reduced glutathione; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity.

able to prepare the solution of GSH at the desired concentrations. The typical dosages used in the studies cited in Table 3 were 600 mg once daily, 600 mg twice daily, 900 mg daily, 1350 mg daily or a daily dose of 66 mg/kg of body weight. Better results are more likely to be achieved with doses of at least 600 mg or more each day. One of the studies used much larger doses (66 mg/kg of body weight) since the authors speculated that these would be necessary to replace half of the amount of GSH that is produced each day (e.g. a 150 lb male synthesizes 10 g daily and would need 5 g as a replacement dose) (27). When patients are unresponsive to doses in the range of 600–1350 mg per day, it might be suitable to try doses that would replace half the estimated amount of GSH that is synthesized each day. These gram doses might yield better clinical results.

In terms of side effects, GSH inhalation is very safe. Minor side effects such as mild coughing and an unpleasant odor were reported in some of the studies included in this review. These minor side effects, better described as mild nuisance problems, were not severe enough to cause any of the study participants to discontinue treatment with inhaled GSH. The only worrisome or potentially life-threatening side effect to note is bronchoconstriction, which would be more likely to occur among sulfite-sensitive asthma and MCSD patients. However, if proper precautions such as sulfite testing are done prior to treatment, this serious side effect should be avoidable.

Monitoring the Clinical Response to Inhaled GSH

For pulmonary diseases or respiratory-related conditions, baseline pulmonary function testing with a spirometer or a simple peak flow meter is recommended prior to the

first treatment. After a prescribed period of treatment time, pulmonary function tests should be repeated. This will help to establish if there are any clinical improvements from regular GSH inhalation.

Conclusions

GSH inhalation is an effective treatment for a variety of pulmonary diseases and respiratory-related conditions. Even very serious and difficult-to-treat diseases (e.g., CF, IPF) yielded benefits from this novel treatment. GSH inhalation is very safe, and rarely causes major or life-threatening side effects. The potential applications are numerous when one considers just how many pulmonary diseases and respiratory-related conditions are affected by deficient antioxidant status, poor oxygenation and/or impaired host defenses. More studies are clearly warranted.

Acknowledgments

The author would like to thank Mr Glen Carr and Mr Andrew Dick for their wonderful illustration of GSH's mechanism of action.

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Received December 22, 2006; accepted February 13, 2007

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CASE REPORT

The Use of Intravenous Glutathione for Symptom Management of Parkinson's Disease: A Case Report

Madalyn Otto, ND; Tracy Magerus, ND; Jeffrey Langland, PhD

ABSTRACT

Intravenous glutathione has been suggested empirically to improve Parkinson's disease (PD) symptoms of tremor and rigidity, but there is limited supporting research. This case report demonstrates both subjective and objective symptom improvement of a conventionally-treated patient suffering from PD when adjunctive intravenous glutathione was administered. In addition to suggesting clinical benefit, this case also suggests an effective therapeutic frequency of therapy and a minimal therapeutic dose. The consistent pattern of improvement following glutathione injections asserts that this therapy may improve symptoms common to PD patients and can offer additional quality of life that would be otherwise unattainable to these patients. (*Altern Ther Health Med.* [E-pub ahead of print.])

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Parkinson's disease (PD) is a clinical diagnosis based on the presentation of motor and nonmotor features including resting tremor, bradykinesia, and rigidity. Other motor symptoms include hypomimia, dysphagia, micrographia, dysarthria, autonomic dysfunction, cognitive changes and sleep disorder. The primary symptom of bradykinesia in PD is best correlated to the loss of dopaminergic neurons in the substantia nigra of the brain.[1] Deterioration of the substantia nigra and loss of dopamine is the hallmark biochemical feature of this disease and contributes to the impaired motor function seen in disease progression. There are no biomarkers or imaging for diagnosis outside of clinical presentation. Left untreated, PD causes severe disability and even death within 5 to 10 years in more than half of patients. Increased function and longevity in recent decades are attributed to the administration of levodopa and the medications with which it is combined.[2,3] There is currently no medication regimen that can arrest or reverse the disease process.

Although the etiology of dopaminergic neuronal destruction in PD remains unknown, there are contributing factors and possible mechanisms underlying its development. One of the most common of these hypotheses is excessive oxidative processes acting on the central nervous system (CNS) producing large amounts of free radicals, thereby depleting its antioxidant capacity, particularly of glutathione.[4,5] The oxidative stress hypothesis is evidenced by a decrease in glutathione without a corresponding deficiency in glutathione transferase, which might otherwise suggest a disorder of glutathione synthesis rather than depletion.[6] Studies have revealed a 40% reduction in CNS glutathione in patients with early PD supporting its role in the pathogenesis of this disease. Glutathione deficiency in PD patients seems to occur significantly in the substantia nigra and not in other brain regions.[7] As a result of these findings, small clinical trials have been conducted to evaluate the use and efficacy of glutathione in patients with Parkinson's disease. The results of these studies suggest a positive effect of this therapy.[8-10]

Due to the fact that the hallmark biochemical feature of PD is a loss of dopaminergic neurons, the mainstay conventional treatment for PD continues to be carbidopa/levodopa. Levodopa is a dopamine precursor that crosses the blood-brain barrier to be converted to dopamine, thereby replacing brain

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Table 1. Treatment Regimen in the Course of Adjunctive Alternative Treatment

Drug	Stalevo (L/C/E)	Azilect	NAC	Silybum	GSH IV	GF diet
Dose	100/25/200 mg, 5×/d	1 mg QD	100 mg BID	3 caps	1400 mg in NS IV 2-3 ×/wk	85% to 95% of the time

Note: Conventional treatment included Stalevo (levadopa/carbidopa/entacapone) 200 mg/50 mg/200 mg taken 5 times daily and Azilect (rasagiline) (1 mg QHS). Natural treatments included oral *N*-acetyl-cysteine (100 mg BID), oral *Silybum marianum*, and IV glutathione injections performed 2-3 times weekly and dosed at 1400 mg GSH diluted in normal saline. Gluten-free diet was also implemented by the patient with adherence of 85% to 95%.

Abbreviations: NAC, *N*-acetyl-cysteine; GSH, glutathione; IV, intravenous; GF, gluten free.

levels of this neurotransmitter. Carbidopa is a decarboxylase inhibitor that maximizes levodopa conversion to dopamine in the CNS rather than in peripheral tissues. Both drugs are used only to treat the symptoms of the disease and do not address the etiology of dopaminergic neuronal death. Initial PD treatment generally includes levodopa, often combined with carbidopa. Levodopa has a particularly short half-life (90 to 20 minutes), which seems to increase the risk of side effects, namely motor fluctuations.[11] As the disease advances, the duration of improvement decreases substantially. At this time, a monoamine oxidase (MAO-B) inhibitor or a catechol-*O*-methyltransferase (COMT) inhibitor is commonly added to the regimen. MAO inhibitors do not slow the progression of the disease better than levodopa does, but rather they slow the enzymes that inactivate dopamine, maintaining the carbidopa/levodopa effect for longer when used adjunctively.[11] There is no evidence that progression to motor fluctuation is decreased.[2] The patient in this case report had been prescribed Azilect (rasagiline), an MAO-B inhibitor, during his conventional course of treatment. COMT inhibitors inhibit peripheral levodopa metabolism, thereby enhancing its action in the CNS. The drug Stalevo, which the patient in this case report had been previously prescribed, is a combination of carbidopa/levodopa and entacapone (a COMT inhibitor) and will be referred to by its brand name (Stalevo) from this point forward.

The following case demonstrates not only efficacy of intravenous (IV) glutathione as an adjunctive treatment for the management of PD symptoms, but also the pattern of symptom improvement following treatment.

PRESENTING CONCERNS

The patient was a 61-year-old white male, nonsmoker, working in commercial real estate management. He was diagnosed with PD 5 years prior at age 56 years. The patient began carbidopa/levodopa (Sinemet) treatment at the time of diagnosis. Azilect was added 2 months later. He had no other significant health concerns but had a first-degree relative also diagnosed with PD. At the time of diagnosis, he was experiencing a stiffened gait, dysarthria, micrographia, and hypomimia. As the disease progressed, he also suffered from cognitive symptoms of mental fatigue, lack of motivation, and poor memory.

At the time that alternative treatment was sought 4 years after diagnosis, the patient had been taking a consistent dose of Stalevo (200 mg/50 mg/200 mg 5 times per day) and Azilect (1 mg QD) for 1 year prior, which had been helping to manage his symptoms.

INTERVENTION

The patient sought alternative therapies to be added to his conventional medication regimen to help better control his mental and motor PD symptoms. Immediately prior to seeking alternative medical care, the patient self-prescribed a gluten-free diet after reading that it may be helpful for Parkinson's symptoms. He continued this diet with estimated 85% to 95% adherence throughout treatment. Later that same month, the patient was assessed at our office and additional adjunctive treatments were initiated (Table 1). Glutathione injections were administered twice weekly at a 1400-mg dose diluted in normal saline for the first few months of treatment, administered at 8:00 AM every 3 to 4 days. The patient eventually received IV therapy 3 times per week, as this was found to be the optimal frequency of dosing to achieve the greatest consistent effect. The patient was also prescribed oral *N*-acetyl-cysteine (NAC), a precursor to glutathione as well as *Silybum*, an herbal hepatoprotective agent used in multidrug patients.[12] He continued his 5-times-per-day oral dosing of Stalevo along with Azilect throughout the course of treatment.

OUTCOMES

The patient's primary symptoms during treatment included dysarthria, micrographia, and hypomimia along with cognitive symptoms of mental fatigue, lack of motivation, and poor memory. Because the patient describes the latter (cognitive) symptoms interchangeably, they will be discussed as a combined symptom of mental function from this point forward. Outcome assessment of alternative therapies was discerned based on patient's subjective symptom changes and objective findings from the patient's friends and family. Symptoms were subjectively rated by the patient on a scale of 1 to 10 to describe lowest to highest level of function (1, unable to function; 10, the highest level of function before having PD) in the course of conventional treatment and throughout the course of alternative treatments.

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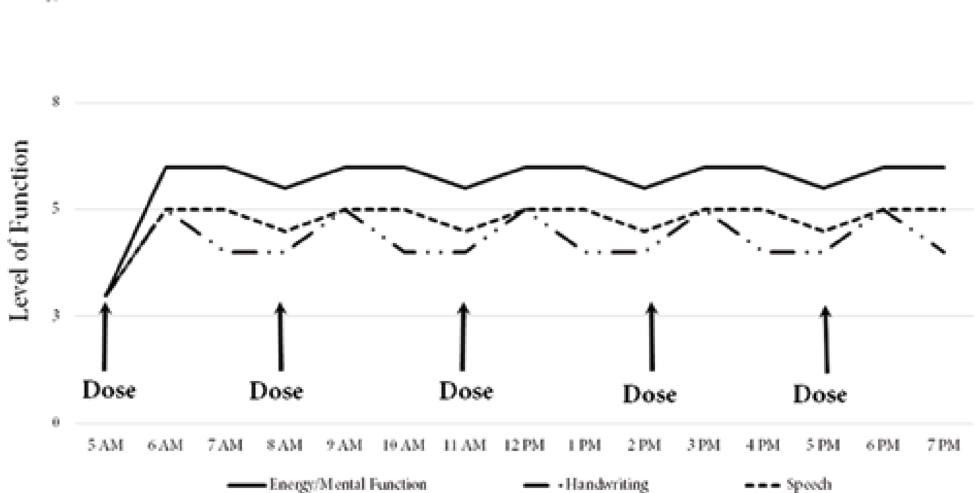
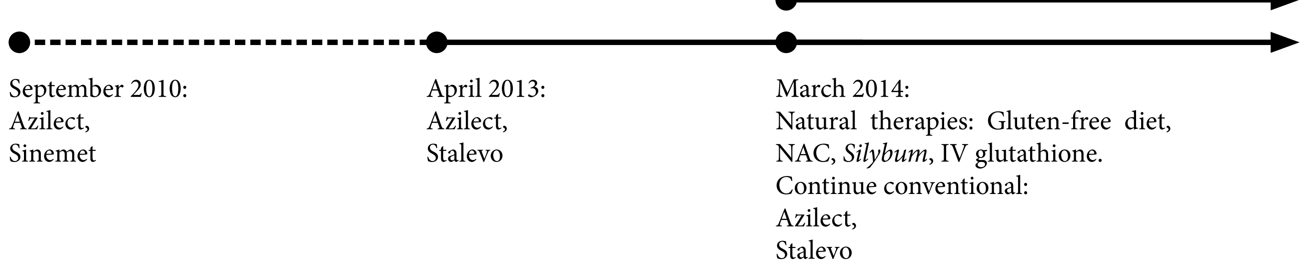
Figure 1. Level of Motor and Mental Function Throughout the Day When Taking Stalevo 5× per Day



Note: Figure 1 shows symptom management on Stalevo and Azilect alone. Graph illustrates a daily snapshot of mental functioning, speech, and handwriting when administered conventional therapies alone. The patient's dosing schedule of Stalevo (levodopa/carbidopa/entacapone) was 5 times daily and Azilect was 1 time daily. Within 1 hour of a dose of Stalevo, patient experienced maximum benefit in symptoms followed by a plateau and regression until the following dose. Maximum improvement occurred 1 hour after a dose of Stalevo. Maximum regression occurred upon waking or after the longest period of time between doses. Mental function was rated by the patient at 3/10 upon waking, improved to a maximum of 6/10 after dosing Stalevo, and regressed to 5/10 by the time the next dose was taken. The patient defines 1/10 as an inability to function and 10/10 is defined as his level of function before he had Parkinson's disease.

Table 2. Timeline of Therapy Intervention



Note: Patient began carbidopa/levodopa (Sinemet) treatment upon diagnosis in 2010. He was additionally prescribed Azilect 2 months later. In 2013, he was switched to Stalevo in addition to Azilect and maintained this regimen until the time of this publication. In 2014, natural therapies were added including oral NAC and *Silybum*, a gluten-free diet, and biweekly IV glutathione injections.

Abbreviations: NAC, *N*-acetyl-cysteine; IV, intravenous.

While taking Stalevo and Azilect alone, the patient rated his mental and motor function to be a maximum of 4-6/10 (Figure 1) as long as he maintained a 5-times-per-day dosing schedule of Stalevo. If he missed a single dose, he noted that his motor and mental function decreased. Figure 1 represents the level of function the patient experienced on a typical day when taking Stalevo and Azilect alone. Despite this moderate improvement, he continued to have significant difficulty with his occupational and home tasks. It was because of this that the patient sought additional treatments.

All alternative treatments were started during the same month (Table 2). The patient reported that he did not perceive a notable change with a gluten-free diet or with NAC and *Silybum* supplementation. He was not completely compliant with the diet and did not notice fluctuations in symptoms with dietary indiscretions.

During the first 3 weeks of alternative treatment including IV glutathione therapy, the patient experienced no change in symptoms. After 3 weeks of alternative treatments, he began to experience a significant improvement in his

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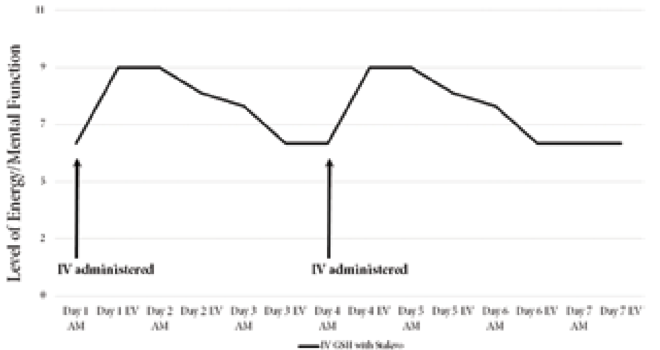
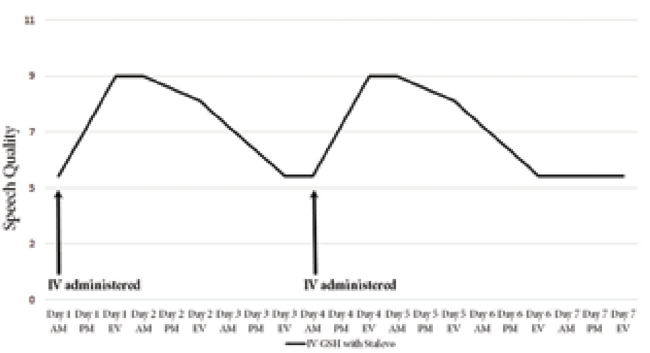
Figure 2. Level of Mental Function During the Week When Receiving Glutathione Injections



Note: Figure 2 shows mental function following IV glutathione injections. Graph illustrates symptom improvement in the course of a week in relation to IV glutathione injections. Glutathione injections were administered at 8:00 AM on days 1 and 4. Approximately 8 hours after an injection, patient felt that his mental functioning improved to 9/10 that would maintain fully for approximately 24 hours before regression would occur. Rating scale is defined as follows: 1/10, inability to function; 10/10, level of function before diagnosis of Parkinson's disease.

Abbreviations: IV, intravenous; GSH, glutathione.

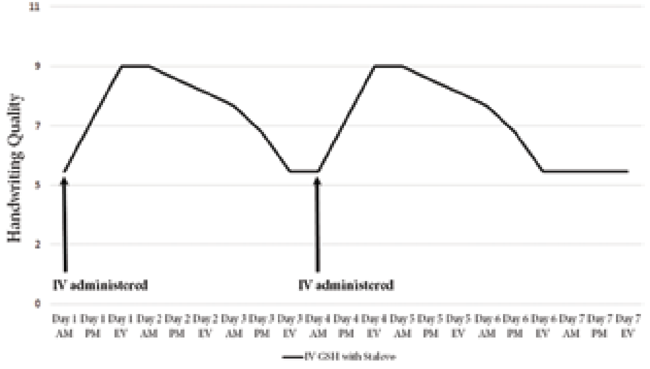
Figure 3. Level of Speech Quality During the Week When Receiving Glutathione Injections



Note: Figure 3 shows speech function following IV glutathione injections. Graph illustrates symptom improvement in the course of a week in relation to IV glutathione injections. Glutathione injections were administered at 8:00 AM on days 1 and 4. Approximately 8 hours after an injection, patient felt that his speech improved to 9/10 that would maintain fully for approximately 24 hours before regression would occur. Rating scale is defined as follows: 1/10, inability to articulate speech; 10/10, quality of speech before diagnosis of Parkinson's disease.

Abbreviations: IV, intravenous; GSH, glutathione.

Figure 4. Level of Mental Function During the Week When Receiving Glutathione Injections



Note: Figure 4 shows handwriting ability following IV glutathione injections. Graph illustrates symptom improvement in the course of a week in relation to IV glutathione injections. Glutathione injections were administered at 8:00 AM on days 1 and 4. Approximately 8 hours after an injection, patient felt that his speech improved to 9/10 that would maintain fully for approximately 24 hours before regression would occur. Rating scale is defined as follows: 1/10, inability to write legibly; 10/10, quality of handwriting before diagnosis of Parkinson's disease.

Abbreviations: IV, intravenous; GSH, glutathione.

symptoms following IV glutathione treatment that surpassed the benefit of Stalevo and Azilect alone. Figures 2, 3, and 4 represent a typical week of mental and motor function observed following twice-weekly IV glutathione along with other medications. Glutathione injections were administered at 8:00 AM on days 1 and 4. Approximately 8 hours after an injection, the patient would feel a surge of improvement in all measured symptoms that would maintain fully for approximately 24 hours before any decline was seen. He rated his level of function at 9/10 for all primary symptoms immediately following IV treatment. Symptoms would return to the baseline function of Stalevo/Azilect-only treatment (4-6/10) within 36 hours or until the patient received another injection.

This pattern was consistent for all his primary symptoms and was reproduced with each subsequent injection in the period of 1 year. If an injection or series of injections was missed due to schedule conflict or vacation, the patient reported that his level of function would regress to the baseline Stalevo/Azilect level seen in Figure 1. As mentioned in the Intervention section, the patient was initially administered glutathione injections twice weekly but eventually received injections 3 times per week as this was found to be the optimal frequency of dosing to achieve the greatest consistent effect. The patient reported that within a few months of consistent IV glutathione treatments, his coworkers and friends began to remark that his facial expression improved dramatically. This symptom had

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previously caused social and occupational difficulties and remained improved in the course of treatment. Since the improvement of masked facies occurred after several months' duration of the alternative treatment regimen, the patient reports that this is when he experienced maximal benefit.

DISCUSSION

This case study not only suggests that the efficacy of IV glutathione as an adjunctive treatment in a condition that is very difficult to treat, but it also demonstrates the pattern and timing of symptom improvement following treatment. This offers insight to a treatment protocol that could produce the greatest improvement for patients with PD. All-natural interventions including diet changes (self-prescribed), oral nutrient therapy, and IV therapy were initiated at the same time. Because all treatments were maintained, it is unclear the extent of the role each played or whether they were necessary to see clinical benefit. Although several treatments were initiated together, maximum improvement consistently correlated with IV glutathione treatments. In addition, delaying injections consistently correlated with regression in function. This suggests that glutathione was the primary cause of symptom improvement. The patient in this case had a more advanced case of PD and was still significantly affected by the introduction of IV glutathione. Noticeably, after receiving IV glutathione treatments for 1 year with sustained improvement, no adverse effects were reported. This protocol represents a treatment for patients with PD patients who have progressed past the early stages of the disease.

Two previous trials that have been conducted to evaluate the use of IV glutathione clinically in patients with PD have shown promising results. One study was conducted to evaluate the effect of IV glutathione in 9 early stage, untreated patients with PD. All patients experienced significant reduction in symptoms for 2 to 4 months posttreatment with no adverse effects.[8] A later randomized, placebo-controlled, double-blind trial investigated the effect of IV glutathione in medicated patients with PD in the course of 4 weeks and found only mild symptom improvement in patients with virtually no adverse effects.[9] These trials both illustrate the possible subjective improvement of PD symptoms, but neither treated patients for more than 30 days.

The patient in this case did not experience any subjective benefits until the third week of treatment and experienced benefit in facial expression after several months of treatment. This finding suggests that there may be a loading dose required before seeing a therapeutic effect and suggests that there is a repletion period before maximal effect is observed. Neither previous clinical study remarked on a pattern in the symptom improvement, which is the highlighted clinical feature in this case report. The establishment of this pattern can help guide a clinician in prescribing an optimal therapeutic regimen using this therapy. An important finding in both trials as well as this case report is that IV glutathione therapy appears extremely safe. No serious side effects or adverse events occurred.

As shown in this case, administration of IV glutathione in addition to daily conventional pharmacotherapy yielded symptom improvement of PD superior to that from pharmacotherapy alone. This case report suggests that IV glutathione should be investigated on a larger scale as a treatment option for PD both in early and later stages of the disease. Trials should consider a loading-dose time period of at least 2 weeks before symptoms should be expected to improve as well as a repletion period where maximal improvement may be expected after 2 months of consistent treatment at a frequency of 2 to 3 injections per week. This treatment represents a very low risk approach with superior beneficial outcomes.

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Reprinted from
Volume 20, Number 16 **August 15, 2002**

JOURNAL OF CLINICAL ONCOLOGY

Neuroprotective Effect of Reduced Glutathione on Oxaliplatin-Based Chemotherapy in Advanced Colorectal Cancer: A Randomized, Double-Blind, Placebo-Controlled Trial

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Neuroprotective Effect of Reduced Glutathione on Oxaliplatin-Based Chemotherapy in Advanced Colorectal Cancer: A Randomized, Double-Blind, Placebo-Controlled Trial

By Stefano Cascinu, Vincenzo Catalano, Luigi Cordella, Roberto Labianca, Paolo Giordani, Anna Maria Baldelli, Giordano D. Beretta, Emilio Ubiali, and Giuseppina Catalano

Purpose: **We performed a randomized, double-blind, placebo-controlled trial to assess the efficacy of glutathione (GSH) in the prevention of oxaliplatin-induced neurotoxicity.**

Patients and Methods: **Fifty-two patients treated with a bimonthly oxaliplatin-based regimen were randomized to receive GSH (1,500 mg/m² over a 15-minute infusion period before oxaliplatin) or normal saline solution. Clinical neurologic evaluation and electrophysiologic investigations were performed at baseline and after four (oxaliplatin dose, 400 mg/m²), eight (oxaliplatin dose, 800 mg/m²), and 12 (oxaliplatin dose, 1,200 mg/m²) cycles of treatment.**

Results: **At the fourth cycle, seven patients showed clinically evident neuropathy in the GSH arm, whereas 11 patients in the placebo arm did. After the eighth cycle, nine of 21 assessable patients in the GSH arm suffered from neurotoxicity compared with 15 of 19 in the placebo arm. With regard to grade 2 to 4 National Cancer Institute common toxicity criteria, 11 patients experienced neuropathy in the placebo arm compared with only two patients in the GSH arm (*P* = .003). After 12 cycles, grade 2 to 4 neurotoxicity was observed in three patients in the GSH arm and in eight patients in the placebo arm (*P* = .004). The neurophysiologic investigations (sural sensory nerve conduction) showed a statistically significant reduction of the values in the placebo arm but not in the GSH arm. The response rate was 26.9% in the GSH arm and 23.1% in the placebo arm, showing no reduction in activity of oxaliplatin.**

Conclusion: **This study provides evidence that GSH is a promising drug for the prevention of oxaliplatin-induced neuropathy, and that it does not reduce the clinical activity of oxaliplatin.**

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OXALIPLATIN, A new cytotoxic agent from the diaminocyclohexane platinum family, has a spectrum of activity and toxicity different from that of cisplatin and carboplatin, and it has demonstrated a lack of cross-resistance with other platinum compounds.[1] The role of oxaliplatin in colorectal cancer has been well established. In combination with fluorouracil (5FU), it represents an effective first-line therapy, and its addition to 5FU regimens also represents an active salvage therapy.[2-5] Furthermore, a combination of oxaliplatin and 5FU has proven beneficial in enabling surgical removal of hepatic resections in patients with previously unresectable liver metastases.[6] The coming years will probably expand the therapeutic potential of oxaliplatin in several other cancers, such as breast, ovarian, non–small-cell lung, prostate, and stomach cancers.[7-9]

The most common toxicity resulting from oxaliplatin therapy is neurotoxicity. There are two distinct types of neurotoxicity. There are cold-sensitive paresthesias, which are unique among the platinum complexes studied to date. They occur at low total cumulative doses, are always reversible, and do not require discontinuation of therapy. However, there is also a peripheral sensory neuropathy with symptoms similar to those seen with cisplatin. This form of neurotoxicity is the most important for its clinical implications. The risk of developing severe disturbance of neurologic function is related to the cumulative dose, generally becoming a clinical problem when the cumulative dose approximates 800 mg/m². It is reversible, but it may last for several months and can even require discontinuation of treatment.[10] The mechanism of neurotoxicity induced by platinum drugs has been proposed to involve the accumulation of platinum within the peripheral nervous system, especially in the dorsal root ganglia.[11] However, unlike the case with cisplatin, for oxaliplatin it seems that the greater retention of platinum is due to a slower clearance rather than a greater accumulation of oxaliplatin.[12] These data suggest that a strategy optimal for reducing the neurotoxicity associated with oxaliplatin may be the use of agents such as glutathione

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Submitted July 16, 2001; accepted February 20, 2002.

This article was published ahead of print at www.jco.org.

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0732-183X/02/2016-3478/$20.00

DOI: 10.1200/JCO.2002.07.061

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(GSH), which is able to prevent the initial accumulation of platinum adducts in the dorsal root ganglia.[13]

Clinical trials conducted to assess the neuroprotective efficacy of GSH in patients treated with cisplatin reported a lower incidence of neurotoxicity compared with placebo, without any negative interference in oncolytic activity.[14-17] On the basis of these premises, to assess the efficacy of GSH in preventing oxaliplatin-induced neuropathy, a double-blind, placebo-controlled trial was performed in patients with advanced colorectal cancer. All were treated with the same oxaliplatin-based regimen and were given either GSH or placebo.

PATIENTS AND METHODS

Patients with a histologically verified advanced colorectal carcinoma were eligible for the study. Other eligibility criteria included Eastern Cooperative Oncology Group performance status of 0 to 2 and normal bone marrow function (leukocyte count > 4,000/μL, platelet count > 100,000/μL), liver function (serum bilirubin < 1.5 mg/dL), renal function (creatinine < 1.5 mg/dL), and cardiac function (stable heart rhythm, no active angina, and no clinical evidence of congestive heart failure). Previous chemotherapy with 5FU, adjuvant or not, was allowed. Patients were excluded if they had established clinical neuropathy, diabetes mellitus, alcoholic disease, other neurologic disease, or brain involvement. Patients who received vitamin B_1, B_6, or B_{12} supplements or who followed other vitamin diets were also excluded.

Informed consent was obtained from all participants after the nature of the study had been fully explained. The protocol was approved by the institutional review board.

The chemotherapeutic regimen consisted of oxaliplatin 100 mg/m^2 on day 1, given as a 2-hour infusion in 250 mL of dextrose 5%, concurrent with 6-*S*-stereoisomer of leucovorin 250 mg/m^2 as a 2-hour infusion followed by a 24-hour infusion of 5FU 1,500 mg/m^2/d for 2 consecutive days. Therapy was repeated every 2 weeks. GSH was given at a dose of 1,500 mg/m^2 in 100 mL of normal saline over a 15-minute period immediately before each oxaliplatin administration, while normal saline solution was administered to placebo-randomized patients. Routine antiemetic prophylaxis with dexamethasone 8 mg and 5-hydroxytryptamine-3 receptor antagonist was used for both treatment arms.

Response was evaluated after four cycles of therapy according to the standard World Health Organization criteria.[18] Patients who showed responsive or stable disease received four further cycles of chemotherapy. Toxicity was assessed after every 2-week cycle using the National Cancer Institute's (NCI) common toxicity criteria (CTC).[19] Chemotherapy was delayed until recovery if the neutrophil count decreased to less than 1,500/μL or the platelet count decreased to less than 100,000/μL. 5FU and oxaliplatin doses were reduced when NCI CTC grade 3 diarrhea, dermatitis, or stomatitis occurred. In the case of NCI grade 2 sensory neuropathy, the oxaliplatin dose was reduced to 75% of the previous dose; in the case of NCI grade 3 sensory neuropathy, oxaliplatin was omitted from the regimen until recovery. Patients who experienced NCI CTC grade 4 toxicity, apart from alopecia, were withdrawn from the study.

A complete standardized neurologic examination, including an evaluation of strength and deep tendon reflexes, was performed by the neurologists (L.C. and E.U.) involved in the study. Special care was devoted to the presence of symptoms of peripheral nervous system involvement and to the assessment of position and vibratory sensations. The degree of neurotoxicity was expressed according to the NCI CTC.[19] The neurophysiologic evaluation was based on the bilateral determination with surface electrodes of the sensory nerve conduction in the sural nerves. All neurophysiologic examinations were performed under constant conditions of skin temperature (34°C). The same examiners, blinded with respect to the group to which each patient belonged, always performed the neurologic and electrophysiologic evaluations. All the patients were examined before entry onto the study and after four, eight, and 12 cycles of chemotherapy within 2 weeks of the end of treatment.

The study was defined as a double-blind, randomized, phase III trial in which at least 25 patients were assigned to each of the two treatment arms. The sample size was determined to detect a 40% difference in the occurrence of grade 2 to 4 (NCI CTC) neurotoxicity between the two treatment arms, with alpha and beta errors of 0.05 and 0.1, respectively. Grade 2 to 4 toxicities were chosen because, in our experience, these degrees seem to impair the quality of life of patients.

Using cards from a computer-generated list in sealed envelopes, randomization was performed by a person not involved in the care or evaluation of the patients. The personnel who evaluated the efficacy and tolerability of the treatment did not know the drug administered because administration was performed by other staff members.

Analysis of variance with repeated measures and a supplementary two-sided paired *t* test were used to compare the neurophysiologic results of the two groups after four cycles (oxaliplatin cumulative dose, 400 mg/m^2) and eight cycles (oxaliplatin cumulative dose, 800 mg/m^2) of chemotherapy. A χ^2 test with Yates' correction and the Wilcoxon test were used to assess the difference in terms of clinical neurotoxicity between the two groups, both as overall incidence and as a score.[20] This score was derived from the sum of the degree of the worst neurologic toxicity, according to the NCI scale, for each patient divided by the number of assessable patients for each dose step (400 mg/m^2, 800 mg/m^2, and 1,200 mg/m^2).

RESULTS

Fifty-two patients were entered onto the study: 26 were assigned to the placebo arm and 26 to the GSH arm. The patients' characteristics are listed in Table 1. Twelve patients in the placebo arm and 11 in the GSH arm received a 5FU/leucovorin regimen as adjuvant treatment. Seventeen patients in the placebo arm and 19 in the GSH arm were treated with 5FU and leucovorin as first-line treatment at the time of relapse. At baseline, the distribution of the other clinicopathologic variables was comparable between the two groups, except for a major incidence of women in the GSH arm (P = .09). No patient was excluded from the study, and an intention-to-treat analysis was performed.

In the placebo arm, seven patients did not complete the second step of treatment (eight cycles): five showed progressive disease, and two patients complained of persistent grade 3 or 4 neurotoxicity. In the GSH arm, five patients did not complete the treatment: four showed progressive disease, and one refused further therapy without clinical signs of neurotoxicity or disease progression.

In the placebo arm, a total of 172 cycles were administered (median, eight); the median dose-intensity of oxali-

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Table 1. Patient Characteristics

	Placebo Arm	GSH Arm
No. of patients	26	26
Age, years		
Median	65	65
Range	50-76	40-77
Sex male/female	19/7	12/14
ECOG performance status		
0	20	17
1	6	9
Primary site		
Colon	15	12
Rectum	11	14
Site of metastases		
Liver	18	16
Abdomen	8	10
Peritoneum	4	3
Lung	10	6
Lymph nodes	3	5
Others	3	1
No. of sites		
1	9	14
> 2	17	12
Previous treatment		
No	9	6
Yes	17	19
Adjuvant therapy		
No	14	15
Yes	12	11

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

platin was 38.8 mg/m^2/wk, and the median cumulative dose of oxaliplatin was 783 mg/m^2. In the GSH arm, a total of 175 cycles was administered (median, eight); the median dose-intensity of oxaliplatin was 39.2 mg/m^2/wk, while the median cumulative dose of oxaliplatin was 782 mg/m^2. The reduced dose-intensity of oxaliplatin was mainly due to neurotoxicity in the placebo arm and to other toxicities in the GSH arm. No statistical difference in number of cycles, dose-intensity, or cumulative dose of oxaliplatin between the two groups was observed. At baseline, no patient suffered from clinical neuropathy in either arm.

At the time of the second neurologic examination (four cycles), seven patients had a clinical neuropathy (grade 1 or 2) in the GSH arm (27%; 95% confidence interval [CI], 9.8% to 44%) compared with 11 patients in the placebo arm (42%; 95% CI, 23% to 61%) (Table 2).

After eight cycles of chemotherapy, nine patients (43%; 95% CI, 22% to 64%) had clinical neuropathy in the GSH arm (score, 0.52) compared with 15 patients (79%; 95% CI, 60% to 80%) in the placebo arm (score, 1.68) (P = .04). Remarkably, the incidence of moderate to severe (grade 2 to 4 NCI CTC) clinical neurotoxicity was present in 11 of 19 assessable patients (58%; 95% CI, 35% to 80%) in the placebo arm, compared with only two of 21 assessable patients (9.5%; 95% CI, 0% to 22%) in the GSH arm (P = .003). Furthermore, grade 3 or 4 neurotoxicity was not present in the GSH arm, while it was reported in five patients (26%) in the placebo arm (P = .01).

Only 18 patients received 12 cycles of treatment, 10 in the GSH arm and eight in the placebo arm. Grade 2 to 4 neurotoxicity was observed in only three patients in the GSH arm and in eight patients in the placebo arm (P = .004).

The most frequent neurologic symptoms were distal paresthesia, numbness in the legs, and ataxia, while the physical examinations generally showed decrease or loss of deep tendon reflexes.

The neurophysiologic evaluation showed that no changes in mean latency and sensory amplitude potentials of sural nerves occurred in the GSH and placebo arms after four cycles of chemotherapy. On the contrary, after eight cycles of chemotherapy in the GSH arm, no changes in mean latency and sensory amplitude potentials of the sural nerves had occurred; in the placebo arm, these parameters were significantly affected (Tables 3 and 4). Patients did not continue to receive GSH after oxaliplatin had been stopped, and none of the patients experienced a rebound of their neurologic symptoms.

The other chemotherapy toxicities are reported in Table 5. There were no chemotherapy-related deaths. The main toxicities were neutropenia, diarrhea, stomatitis,

Table 2. Clinical Evaluation of Neurotoxicity

Neurotoxicity NCI NCTC Grade	After 4 Cycles		After 8 Cycles		After 12 Cycles	
	Placebo (n = 26)*	GSH (n = 26)*	Placebo (n = 19)*	GSH (n = 21)*	Placebo (n = 8)*	GSH (n = 10)*
0	15	19	4	12	–	1
1	9	6	4	7	–	6
2	2	1	6	2	2	2
3	–	–	4	–	4	1
4	–	–	1	–	2	–
Score	–	–	1.68	0.52	3	1.3

*Number of assessable patients.

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Table 3. Electrophysiologic Results in the Placebo Arm

Sural Nerve	Basal	After 4 Cycles	P	After 8 Cycles	P
Latency, msec	3.07 ± 0.33	2.90 ± 0.69	NS	3.19 ± 1.70	.03
SAP, μV	10.98 ± 6.92	9.80 ± 5.35	NS	7.20 ± 5.05	.05
CV, m/sec	45.91 ± 4.59	44.03 ± 10.19	NS	39.33 ± 11.66	.01

Abbreviations: SAP, sensory amplitude potential; CV, conduction velocity; NS, not significant.

nausea and vomiting, and transient hepatic failure. They were generally mild, and no statistically significant difference in incidence and severity of toxicities was found between the two groups (Table 5).

No complete response was observed in either arm. A partial response was observed in seven patients (26.9%; 95% CI, 9.8% to 43.9%) in the GSH group and in six patients (23.1%; 95% CI, 6.8% to 39.2%) in the placebo arm, for an overall response rate of 25.0% (95% CI, 13.2% to 36.7%) (Table 6).

After a median overall follow-up period of 11.5 months (range, 3 to 30 months), the median progression-free survival was 7 months (range, 2 to 12 months) for patients in the GSH arm and 7 months (range, 2 to 16 months) for those in the placebo arm. Median survival time was 16 months and 17 months in the GSH and placebo arms, respectively.

DISCUSSION

The mechanism of neurotoxicity induced by platinum drugs has been proposed to involve the accumulation of platinum within the peripheral nerve system.[11,12] The major site of damage seems to be the dorsal root ganglia, which is consistent with the platinum accumulation studies. In fact, biodistribution studies have shown that the platinum concentrations are greater in the dorsal root ganglia followed by the dorsal root and peripheral nerves.[12] Damage to the dorsal root ganglia seems to result in axonopathy of peripheral nerves, especially in the large myelinated fibers responsible for sensory nerve conduction. In a rat model, the sciatic nerves showed marked axonal atrophy and a decrease in the number of large sensory axons, whereas the motor axons remained unaffected.[21]

The neurotoxicity associated with oxaliplatin is similar in nature to that associated with cisplatin. However, unlike the case with cisplatin, the pathologic presence of oxaliplatin in the dorsal root ganglia is due to a relative slower clearance of the drug rather than to an increased accumulation.[13] These data suggest that an optimal strategy for reducing the neurotoxicity associated with oxaliplatin may be the use of agents such as GSH, which may be able to prevent the initial accumulation of platinum adducts in dorsal root ganglia.[13]

A major role of GSH in the prevention of platinum-induced neurotoxicity has been suggested by recent experimental findings. Park et al[22] showed that reactive oxygen species generated by platinum compounds play an important role in platinum-induced neuronal apoptotic cell death via activation of the p53 signaling pathway. Preincubation of nerves from a mouse dorsal root ganglion with *N*-acetylcysteine, a precursor of GSH, blocks or attenuate the accumulation of p53 protein in response to platinum, resulting in a block of platinum-induced apoptosis and in a neuroprotective effect.[22] Finally, preclinical and clinical experiences provided evidence that GSH was effective for the prevention of cisplatin-induced neurotoxicity without reducing the clinical activity of cisplatin.[14-17]

On the basis of these premises, we performed this double-blind, placebo-controlled, randomized trial using the same GSH schedule as reported in our previous work.[16] Our results indicate that GSH can exert a beneficial effect on oxaliplatin neurotoxicity. In fact, we have shown that GSH given concurrently with oxaliplatin is able to reduce the symptoms and signs of neuropathy significantly. In addition, neurophysiologic investigations based on the evaluation of latency and amplitude of the sensory nerve conduction, the most common indexes impaired in platinum neuropathy, supported the neuroprotective effects of GSH.

These findings may have important clinical implications. In fact, in several cases, despite good clinical activity, treatment with oxaliplatin must be discontinued because of the onset of neurotoxicity. The concomitant use of GSH

Table 4. Electrophysiologic Results in the GSH Arm

Sural Nerve	Basal	After 4 Cycles	P	After 8 Cycles	P
Latency, msec	2.98 ± 0.97	3.17 ± 0.76	NS	3.08 ± 0.99	NS
SAP, μV	9.09 ± 6.34	10.89 ± 7.89	NS	8.71 ± 5.50	NS
CV, m/sec	39.87 ± 13.0	39.48 ± 13.04	NS	39.13 ± 11.63	NS

Abbreviations: SAP, sensory amplitude potential; CV, conduction velocity; NS, not significant.

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Table 5. Worst Grade of Toxicity by Each Patient (absolute numbers)

Toxicity (NCI CTC)/Grade	Placebo Arm	GSH Arm
Anemia		
1/2	2	5
3/4	0	0
Neutropenia		
1/2	7	7
3/4	4	1
Thrombocytopenia		
1/2	5	4
3/4	0	0
Nausea		
1/2	9	10
3	0	0
Vomiting		
1/2	7	10
3/4	0	0
Diarrhea		
1/2	6	6
3/4	0	2
Stomatitis		
1/2	6	6
3/4	0	0

Table 6. Tumor Response and Survival

	GSH Arm	Placebo Arm
Patients enrolled, n	26	26
CR	0	0
PR, %	26.9	23.1
SD, %	57.7	53.8
PD, %	15.4	23.1
OR, %	26.9	23.1
95% CI, %	9.8-43.9	6.8-39.2
PFS, months	7+	7
Survival, months	16	17

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; OR, overall response; PFS, progressive-free survival.

may allow the administration of an effective treatment for a more prolonged time. In fact, in the placebo arm, none of the patients could receive further oxaliplatin treatment because of the development of neurotoxicity; in the GSH arm, seven patients did not develop any sign of clinical neurotoxicity and could continue on treatment. In the coming years, there will be an expanding use of oxaliplatin in several other cancers as well as in the adjuvant setting, as indicated by two ongoing randomized trials in colon cancer in Europe (Multicenter International Study of Oxaliplatin 5FU-LV in the Adjuvant Treatment of Colon Cancer [MOSAIC] trial) and the United States (National Surgical Adjuvant Breast and Bowel Project C-07), oxaliplatin-induced neuropathy will be a growing, relevant clinical problem.

Regarding toxicity and possible interference with oxaliplatin antitumor activity by GSH, as previously reported in other studies on cisplatin, we did not observe either.

The results we achieved with this double-blind, placebo-controlled trial have provided evidence indicating that GSH is a promising drug for the prevention of oxaliplatin-induced neuropathy. Other attempts to reduce neurotoxicity associated with oxaliplatin included the development of regimens alternating the combination of oxaliplatin/5FU with 5FU alone in order to allow a long-term period of treatment but reducing the total cumulative dose of oxaliplatin, or the use of other possible chemoprotectants, such as gabapentin. Preliminary data with this drug seem to be promising.[23] In seven patients, neuropathy disappeared and did not recur with additional chemotherapeutic courses. However, in some patients, increased doses of gabapentin were needed; so far, a prolonged administration of this drug may be precluded because of its potential side effects. In contrast, the lack of toxicity and interference with oxaliplatin activity, as well as its low economic cost, makes GSH an ideal new drug for the prevention of oxaliplatin-induced neuropathy in colorectal cancer patients.

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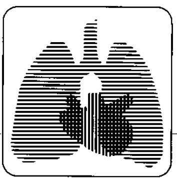

preliminary report

A Pilot Study of the Effect of Inhaled Buffered Reduced Glutathione on the Clinical Status of Patients With Cystic Fibrosis*

Clark Bishop, MD, FCCP; Valerie M. Hudson, PhD; Sterling C. Hilton, PhD; Cathleen Wilde, BS

Study objectives: **To assess the impact of inhaled, buffered reduced glutathione (GSH) on clinical indicators of cystic fibrosis (CF) pathophysiology.**

Design and patients: **A randomized, double-blind, placebo-controlled pilot study was conducted over an 8-week period. Nineteen subjects, age 6 to 19 years, with CF status documented by positive sweat chloride test results (> 60 mEq/L) were recruited for the trial. After matching on age and sex, 10 patients were randomly assigned to the treatment group and 9 patients to the placebo group. Primary outcomes were FEV_1, FVC, forced expiratory flow at 25 to 75% of vital capacity, and peak flow; secondary outcomes were body mass index, 6-min walk distance, and self-reported cough frequency, mucus production/viscosity/color, wellness, improvement, and stamina.**

Interventions and analysis: **Treatment was buffered GSH, and placebo was sodium chloride with a hint of quinine. The total daily dose of buffered GSH was approximately 66 mg/kg of body weight, and the total daily dose of placebo was approximately 15 mg/kg of body weight (quinine, 25 to 30 μg/kg). Doses were distributed across four inhalation sessions per day and spaced 3- to 4-h apart. General linear mixed models were used to analyze the data. The final sample size was nine subjects in the treatment group and seven subjects in the placebo group.**

Results: **Mean change for peak flow was − 6.5 L/min for the placebo group and + 33.7 L/min for the GSH group ($p = 0.04$), and self-reported average improvement on a scale from 1 to 5 (1 being much worse and 5 being much better) was 2.8 for placebo and 4.7 for GSH ($p = 0.004$). Of the 13 primary and secondary outcomes examined, 11 outcomes favored the treatment group over the placebo group ($p = 0.002$), indicating a general tendency of improvement in the GSH group. No adverse events in the treatment group were noted.**

Conclusion: **This pilot study indicates the promise of nebulized buffered GSH to ameliorate CF disease, and longer, larger, and improved studies of inhaled GSH are warranted.**

(CHEST 2005; 127:308–317)

Key words: aerosolized; bronchiectasis; cystic fibrosis; glutathione; lung function; mucolysis; oxidative stress

Abbreviations: BMI = body mass index; CF = cystic fibrosis; CFTR = cystic fibrosis transmembrane conductance regulator; ELF = epithelial lining fluid; $FEF_{25–75}$ = forced expiratory flow at 25 to 75% of vital capacity; GSH = reduced glutathione; GSH+GSSG = total glutathione; GSSG = glutathione disulfide; IL = interleukin; mOsm = milliosmol

Cystic Fibrosis (CF) is a genetic disease affecting some 30,000 individuals in North America, and > 200,000 worldwide. CF is caused by the autosomal recessive mutation of the CF transmembrane conductance regulator (CFTR) protein, which is located on chromosome 7. In nonaffected individuals, the CFTR protein is transcribed and then migrates to and creates a channel in the cell membrane; this channel allows for the egress from the cell of certain anions. In patients with CF, this channel is missing or defective.[1,2]

CF pathology is characterized by excessive inflam-

mation in the respiratory and GI systems. Focusing on the respiratory system, patients with CF have thick mucus secretions, and colonization with bacteria occurs at an early age. The usual course of CF in

For editorial comment see page 12

the respiratory system is chronic infection that leads to airways obstruction, bronchiectasis, and eventual respiratory failure, with approximately 95% of patients with CF eventually dying from respiratory failure. Although much CF research has been on the altered chloride efflux from the CFTR channel,[1,2] other anions, such as bicarbonate (HCO_3^-) and reduced glutathione (GSH) [L-gamma-glutamyl-L-cysteinyl-glycine] also pass through the CFTR to exit the cell.[3,4]

While CF pathology is well documented, the means by which the CF mutation causes disease is not well understood. It has been hypothesized that the altered distribution of GSH in patients with CF provides a significant link between CF genetics and CF pathology.[5] The mechanism of this potential link can be understood by first understanding the function of GSH in normal individuals.

GSH in Epithelial Lining Fluid

GSH performs several important functions in the epithelial lining fluid (ELF), particularly of the lung. First, GSH functions as the primary water-soluble antioxidant in the ELF, by directly or enzymatically scavenging hydrogen peroxide, hyperchlorous acid, and other free radicals.[6] In this process, GSH is oxidized to glutathione disulfide (GSSG). Body compartments normally exposed to high oxidative stress have correspondingly high levels of GSH on the epithelial surface, and a high redox ratio (GSH/GSSG).[7] For example, the ELF of the lungs contains 140 times the serum concentration of GSH, and the redox ratio is > 9:1.[8] Second, GSH helps to maintain proper mucus viscosity and facilitates cell signaling through the cleavage of disulfide bonds.[5,9,10] Third, after oxidation to GSSG, GSH reversibly bind to certain proteins, protecting them from irreversible damage in times of oxidative and nitrosative stress.[11]

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All funding provided by the Utah Valley Institute of Cystic Fibrosis.

Manuscript received March 1, 2004; revision accepted May 14, 2004.

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In patients with CF, research[3,12–14] has shown that lung epithelial cells have normal total glutathione (GSH+GSSG) but exhibit significantly diminished efflux of GSH through CFTR channels at the apical surface. This diminished efflux results in profoundly decreased GSH+GSSG as well as decreased redox ratio in the ELF of the CF lung; total glutathione can be 10 to 50% of normal, and the redox ratio can be as low as 3:1[15,16]; therefore, patients with CF receive neither the full antioxidant nor mucolytic benefits of GSH.

GSH in the Immune System

GSH also plays multiple, pivotal roles in the normal immune system. First, inflammation is closely tied to both the GSH/GSSG ratio and GSH+GSSG.[5,17] A decrease in either amount, even in the absence of infection, leads to the transcription of nuclear factor-κB, with a cascade of proinflammatory cytokines, such as tumor necrosis factor-α, interleukin (IL)-8, IL-6, IL-1a, and others following.[17–20] Second, normal intracellular levels of GSH are essential for important immune system cell functions such as chemotaxis, phagocytosis, appropriate apoptosis, oxidant burst, microtubule integrity, cell signaling, antigen presentation, release of lysosomal enzymes, and other bactericidal activities.[9,21–23] Third, the GSH system profoundly affects the nitric oxide system, with GSH deficiency inducing a reduction in available nitric oxide for use in bactericidal, bronchodilation, and cell-signaling functions.[24–26]

In CF, reduced levels of GSH+GSSG and a diminished GSH redox ratio could help explain the chronic and excessive inflammation in the respiratory system. Although epithelial cells have a defective or missing CFTR channel, immune cells, such as neutrophils, possess redundant anion channels, including those of the multidrug resistance-associated protein family, which allows for the egress of GSH from these cells[27]; however, research[28] has shown that neutrophils in CF also have diminished levels of GSH. It is hypothesized that part of the altered functionality of the neutrophils could be explained by the constant demand placed on them by the diminished GSH levels in the ELF.[5]

Therefore, chronic GSH deficiency in the CF lung could explain the paradox of an overactive immune system (chronic inflammation) that is nevertheless ineffective in eradicating bacteria (chronic infection). This paradox together with the loss of antioxidant protection and thickened mucus lead to a CF lung that is seriously compromised by genetically induced GSH transport dysfunction (Fig 1).

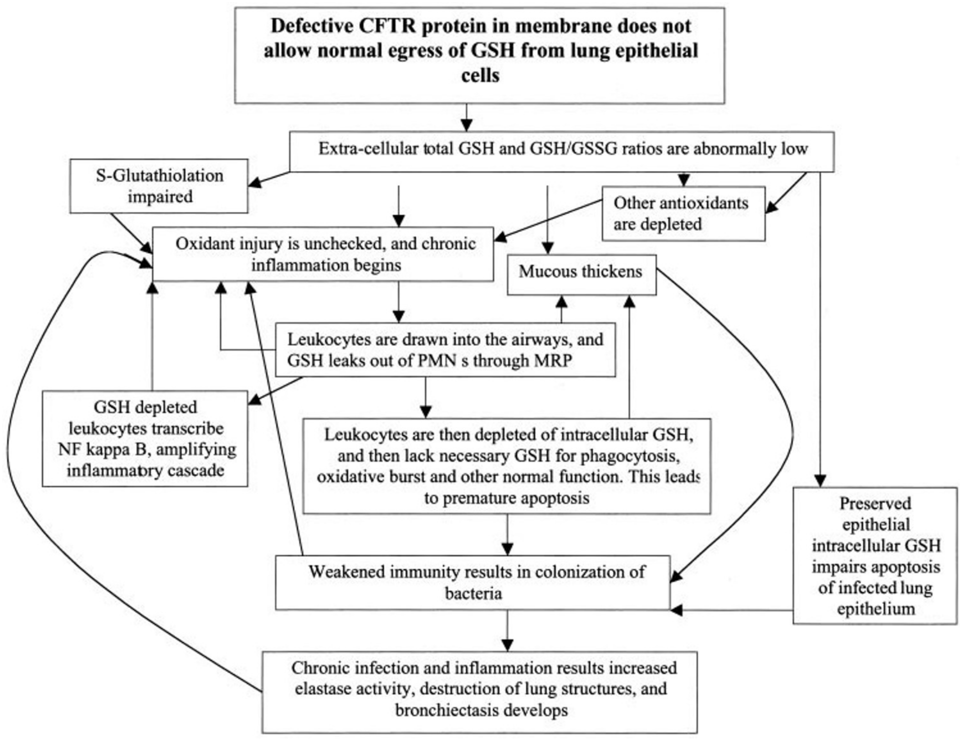

FIGURE 1. Role of ELF GSH depletion in the pathophysiology of CF. PMN = polymorphonuclear leukocytes; NF = nuclear factor; MRP = multidrug resistance-associated protein.

While it has been known since 1987[29] that GSH is depleted in respiratory ELF in CF, some[30] have argued that the low levels of GSH are secondary to chronic inflammation. Research[3,12–14] allows us to say that the decreased GSH in the ELF of the CF lung, while aggravated by inflammation and infection characteristic of CF, is nevertheless caused in the first place by CFTR mutation. This new understanding constitutes an important link between CF genetics and CF pathophysiology, and it raises a question about the therapeutic potential of augmenting GSH by exogenous means.

Efforts to augment ELF GSH levels in the CF lung have been undertaken. In addition to one *in vitro* trial using CF human bronchial epithelial cells lines,[31] there have been nine human trials on inhaled GSH, and one trial using sheep.[32–40] No serious adverse effects were reported in any of the nine human *in vivo* trials. Two of the nine human *in vivo* trials involved patients CF. The first trial[31] demonstrated GSH in the lower airway following nebulization, and the study demonstrated decreased hypersensitivity of CF leukocytes to inflammatory stimuli. However, this trial was not placebo controlled, and it was only of 3 days in duration, with a maximum dose of 600 mg bid, and few clinical variables were examined. The second trial[40] also demonstrated GSH in the lower airways following nebulization, and found increased FEV_1 and altered lymphocyte profile. But again, this trial was not placebo controlled, and the longest duration of use was 2 weeks, also with a fairly small dose (450 mg tid).

Given the theoretical promise of GSH therapy in patients with CF and the previous findings of inhaled GSH,[31–40] we conducted a placebo-controlled pilot study of longer duration with a higher daily dose of a buffered GSH solution. The objective was to assess the impact of GSH on clinical indicators of CF pathophysiology. Specific hypotheses included the following: (1) primary indicators involving lung function, FEV_1, FVC, forced expiratory flow at 25 to 75% of vital capacity ($FEF_{25–75}$), and peak flow, would be positively affected by this intervention; (2) secondary outcomes representing other markers of CF disease would be positively affected, including body mass index (BMI), 6-min walk distance, and self-reported cough frequency, mucus production/viscosity/color, wellness, improvement, and stamina; and (3) no adverse effects would be attributable to this intervention (no pattern of worsening of bacterial cultures/counts, or other types of adverse events). All outcomes and their scales are defined in Table 1, and qualitative scales of subjective measures were all categorized as secondary outcomes. The study rationale, design, methods, and protocol were reviewed

Table 1—*Baseline Demographic and Clinical Characteristics of the Two Treatment Groups**

Variables	Placebo (n = 9)	GSH (n = 10)
Clinical characteristics		
FVC	87.2 (23.60)	89.3 (17.18)
FEV_1	79.7 (24.72)	81.6 (18.17)
FEF_{25-75}	67.7 (33.82)	68.4 (24.49)
Peak flow	285.5 (118.46)	379.5 (105.46)
BMI	17.9 (2.34)	18.5 (2.13)
6-min walk distance, feet	2,823.0 (414.33)	3,101.1 (578.30)
Usual stamina	2.8 (0.63)	3.1 (0.60)
General wellness	2.7 (0.48)	3.1 (0.60)
Cough frequency	2.7 (0.67)	2.3 (0.87)
Sputum amount	1.8 (0.79)	1.7 (1.22)
Sputum viscosity	2.0 (0.47)	1.7 (1.12)
Sputum color	2.7 (0.75)	2.5 (1.54)
Demographics		
Age, yr	12.9 (4.9)	13.3 (4.1)
Weight, lb	88.5 (31.4)	92.3 (29.1)
Male gender, %	60	67

*Data are presented as mean (SD) unless otherwise indicated.

and approved by the Utah Valley Regional Medical Center Institutional Review Board, Provo, UT.

Materials and Methods

Subjects

Nineteen subjects aged 6 to 19 years with CF status documented by positive sweat chloride test results (> 60 mEq/L) were recruited for the trial. Patients were recruited between March 2002 and June 2002, through personal contact, newspaper advertisement, and Internet groups. Participants were not paid, but were reimbursed for travel expenses > 60 miles round-trip. Patients were excluded for the following reasons: (1) positive culture finding for *Burkholderia cepacia* at any time, (2) history of hemoptysis or pneumothorax, (3) FEV_1 < 30% of predicted, or (4) severe asthma. Informed consent was obtained after explaining the study and its purpose to the participants and parents. Patients of legal age provided informed consent, and parents of minor children provided informed consent. Minor children added their own assent, after an age-appropriate explanation of the trial was given. Sample size for the trial was based on a power analysis of FEV_1 with an anticipated effect size of 15% improvement.

Experimental Protocol

Randomization: Patients were first paired by age and sex, and then each member of the pair was randomly assigned to the treatment or placebo groups. Just before the trial began, one patient in the placebo group dropped out. No member of the clinical team was involved in the coding or assignment to treatment/placebo groups, and both patients and the clinical team remained blinded to treatment group assignment throughout the trial. Nonclinical researchers involved in any coding were only provided patient identification numbers, not patient names.

Trial Structure: The trial duration was 8 weeks, with enrollment occurring between late June and early July 2002 depending on patient availability for the first examination. Each patient was seen by the clinical team at the Utah Valley Regional Medical Center at the beginning and end of the trial. Primary and secondary outcomes (Table 1) were collected at these visits with the exception of peak flow; patients were asked to perform peak flows at least twice daily throughout the trial using a peak flowmeter (Personal Best; Respironics; Murrysville, PA). Baseline bacterial culture samples and counts were either collected at the first visit or extracted from medical records for the 3 months prior to the first visit; bacterial cultures and counts were also collected at the end of the trial.

Patients were instructed to continue taking all currently prescribed medication and treatments, and to phone or e-mail immediately if there were any adverse events. Study protocol outlined that all adverse events were to be promptly reported to the principal investigator who would assess the clinical situation and recommend any necessary deviation from the treatment regimen. Also, clinical researchers phoned or e-mailed patients biweekly to monitor side effects and to encourage compliance, and they asked patients midway through the trial of their perceptions of any change in clinical status. Finally, patients were asked to record daily in a logbook changes in status, timing of treatments, and peak flows. A subjective assessment of compliance was made after the trial ended based on logbook entries.

During clinic visits, patients were segregated according to bacterial status. Patients with mucoid *Pseudomonas aeruginosa* or methicillin-resistant *Staphylococcus aureus* were segregated from each other and other patients, *ie*, seen in separate rooms with all equipment and rooms cleaned between patients. In addition, patients with methicillin-resistant *S aureus* were gowned, and their testing was done at the end of the day.

Materials and Dosage

GSH The treatment group received capsules containing reduced GSH buffered with sodium bicarbonate. Each capsule contained 300 mg of GSH and 72 mg of 100% pure sodium bicarbonate. The capsules were formulated by Theranaturals, Inc. (Orem, UT). The GSH was manufactured using good manufacturing practice by the Kohjin Company (Tokyo, Japan). Nelson Laboratories (Salt Lake City, UT), an independent testing laboratory, performed limulus amebocyte lysate tests for endotoxins on the lot of GSH used in the study. No endotoxins were found by tests with sensitivity thresholds < 0.015 endotoxin units per milligram. This lot of GSH was also tested and found negative for *S aureus*, *P aeruginosa*, Salmonella species, *Escherichia coli*, and fungi. The purity of this lot of GSH was assayed using high-pressure liquid chromotography, with purity measuring 98.8% GSH with heavy metals not > 5 ppm, iron not > 5 ppm, and arsenic not > 1 ppm. The pH of the buffered GSH in solution tested at 5.2, and average osmolality of one capsule dissolved in 3.75 mL of sterile water was measured at 469 milliosmol (mOsm)/kg, producing a slightly hypertonic solution of 1.4% (physiologic osmolality being 280 to 310 mOsm/kg).

Placebo: The placebo group received capsules containing sodium chloride with a hint of quinine. Since GSH has a distinct taste and odor, quinine was added to the placebo in order to create a distinct taste and odor. Each capsule contained approximately 68 mg (range, 68 to 102 mg) or 1.16 mmol of sodium chloride, and 125 μg or 0.4 μmol/L of quinine. The capsules were formulated by Theranaturals, Inc. The pH of the placebo in solution tested at 4.9, and average osmolality of one capsule dissolved in 3.75 mL of sterile water was measured at 1,360 mOsm/kg, producing approximately a 4% hypertonic saline solution.

Dosage: Total daily dose of buffered GSH for each patient was approximately 66 mg/kg of body weight, and total daily dose of

placebo for each patient was approximately 15 mg/kg of body weight (25 to 30 μg/kg of quinine). These doses were distributed across four inhalation sessions per day, and patients were asked to space these sessions 3- to 4-h apart. For the first week of treatment, patients were instructed to use one fourth of the recommended total dosage, and in the second week to use one half of the recommended total dosage. After the second week, patients were instructed to use the full daily total dosage. The primary purpose of this incremental dosage regimen was to monitor for adverse events at lower dosage levels.

Each patient was individually instructed regarding the number of capsules to use for each session and to dilute each capsule using 3.75 mL of sterile water. All patients received a portable compressor/nebulizer (Omron NE-C21 Comp-Air Elite; Omron Healthcare; Vernon Hills, IL), whose particle size tested at 6 μm. For a given session during the day, patients were instructed to make the solution, place it in the nebulizer, and inhale it using a face mask until it was gone. Patients were instructed to always make a fresh solution for each session. Laboratory experiments show that freshly made buffered GSH solution is stable for up to 5 h with GSH in solution, being 97 ± 0.1% in the reduced form.[29]

Data Analysis

Differences between posttrial and baseline outcomes were analyzed using a general linear mixed model that allowed for correlation between outcomes within the age/sex pair used for randomization. For peak flow, *baseline* was defined as the average of all measurements taken the first 5 days of the trial, and *post* was defined as the average of all measurement taken the last 5 days on treatment. All analyses were conducted on a modified intent-to-treat basis, *ie*, regardless of compliance to the treatment regimen, all patient outcomes were included in the analysis except when patients were hospitalized or were missing data. Patients who were hospitalized were excluded from analysis because their posttrial lung function outcomes would be clearly influenced by their hospitalization treatment. Also, when patients were missing data for a particular outcome, they were excluded from the analysis of that outcome; however, missing data were minimal. Two patients in the GSH group did not record daily peak flow data; and at the posttrial clinic visit, one patient in the placebo group did not provide data on sputum viscosity and color, and two patients in the GSH group did not provide data on sputum color.

Participant Flow

The recruitment process and patient experience is summarized in Figure 2. Fifty-eight patients inquired about the trial, 23 patients were excluded on the basis of the exclusion criteria noted above, and 16 patients chose to not participate. Nineteen patients were enrolled in the trial: 10 in the treatment group and 9 in the placebo group. After the trial commenced, three patients (two receiving placebo and one receiving GSH) were hospitalized due to nonacute pulmonary exacerbations that were unrelated to participation in the study. Since hospitalized patients were excluded from data analyses, study results are based on data from seven patients receiving placebo and nine patients receiving GSH.

Results

Baseline patient demographic and clinical characteristics are given in Table 2. As expected after

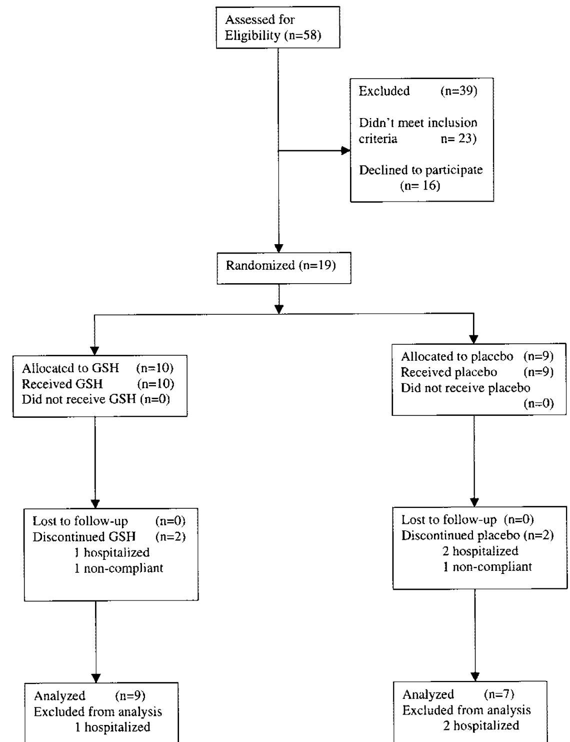

FIGURE 2. Patient flow.

randomization, no statistically significant differences were found between the placebo and baseline groups for any of the baseline demographic and clinical characteristics. The variance component for the pairs was estimated to be zero in most analyses, which suggests that there was very little variability between the pairs, and that age/sex matching was probably unnecessary in this trial. This, of course, may not be the case in trials conducted for longer periods of time and with more patients.

Adverse events recorded during the trial are provided in Table 3. Three patients were hospitalized (two patients receiving placebo, and one patient receiving GSH) due to nonimprovement of conditions present at baseline. During the trial, one participant complained to the principal investigator of chest pain, and another complained of cough and nasal irritation. Both were instructed to reduce their dosage to one half the number of capsules and diluent. Both patients were in the placebo group, and these were the only patients to verbally report any adverse event during the trial. The other adverse events reported in Table 3 were extracted from comments written in the daily logbooks. In general, the symptoms listed in Table 3 are commonly found in individuals with CF, and no apparent differences in these symptoms were found between the two treatment groups. Also, an analysis of the number

Table 2—*Primary and Secondary Outcomes Measured at Baseline and End of Trial, and Scales on Which They Are Measured*

Outcomes	Scales
Primary	
FEV_1	% predicted of FEV_1
FVC	% predicted of FVC
FEF_{25-75}	% predicted of FEF_{25-75} of FVC
Peak flow*	Liters per minute using flow meter
Secondary	
BMI	
6-min walk test	Distance (feet) walked in 6 min
Sputum color	Self-report on following scale: 1 = clear, 2 = white, 3 = yellow, 4 = green, 5 = brown, 6 = blood streaked
Sputum amount	Self-report on scale: 1 = scant, 2 = < 1 teaspoon, 3 = > 1 teaspoon
Sputum viscosity	Self-report on scale: 1 = very thin, 2 = slightly sticky, 3 = very sticky
Cough frequency	Self-report on scale: 1 = no cough, 2 = infrequent, 3 = several times a day, 4 = every hour
General wellness	Self-report on scale: 1 = poor, 2 = fair, 3 = good, 4 = excellent
Usual stamina	Self-report on scale: 1 = poor, 2 = fair, 3 = good, 4 = excellent
Improvement†	Self-report on scale: 1 = significantly worse, 2 = a bit worse, 3 = about the same, 4 = a bit better, 5 = significantly better

*Measured by patient at least twice daily throughout trial period. Baseline measure is average of first 5 days of trial; end of trial measure is average of last 5 days of trial.
†Only measured at end of trial.

and severity of species identified in bacterial cultures indicated no significant differences between the treatment and placebo groups over the course of the trial.

The mean differences (postbaseline) between the GSH and placebo groups for both primary and secondary outcomes are presented in Table 4. Also found in Table 4 are 95% confidence intervals for the effect size and p values corresponding to hypothesis tests of no effect size. Changes in sample size due to missing data are noted. The effect size for all four primary outcomes favored the GSH group; however, none were statistically different except for peak flow ($p = 0.04$). For peak flow, the placebo group essentially had a small average decline of − 6.5 L/min, and the GSH group improved on average by 33.7 L/min. For the FEF_{25-75} measure, there was a slight decline (− 5.0%) in the placebo group, and a slight improvement (1.2%) in the GSH group. For the FVC and FEV_1 measures, both groups declined on average over the 8-week period; however, the declines were slightly less for the GSH group compared to the placebo group.

Table 3—*Type of Adverse Events During Trial for Each Treatment Group**

Events	Placebo (n = 9)	GSH (n = 10)
Hospitalization for nonacute pulmonary exacerbations	2	1
Rhinitis/sinusitis	3	2
Cough	3	4
Pharyngitis	4	4
Stomach pain/cramps	4	1
Headache	2	4
Chest tightness/bronchospasm	3	1
Nose bleed	3	2
Shortness of breath	2	1

*Data are presented as No. of patients.

For the nine secondary outcomes, the self-reported overall improvement score was statistically different ($p = 0.004$) between the treatment groups. The placebo group reported feeling on average "about the same," while the GSH group reported feeling on average "much better." Two of the secondary outcomes, general wellness ($p = 0.09$) and cough frequency ($p = 0.20$), tended toward statistical significance. The GSH group improved on average 0.4 on a 4-point scale more than the placebo in terms of self-reported general wellness, and the self-reported frequency of cough declined slightly in the GSH group but remained the same in the placebo group. The remaining six outcomes were not significantly different between the two groups, although four of the six outcomes favored the GSH group.

One GSH patient was grossly noncompliant, *ie*, stopped the treatment after the first 5 days of the trial. In order to obtain a clearer picture of the potential effect of GSH, the data were analyzed excluding this patient. These results are not reported since they are very similar to those in Table 4 except for two noteworthy differences: (1) the difference in average cough frequency declined by 0.6 (scale 1 to

Table 4—*Modified Intent-to-Treat Analysis: Estimated Average Differences Between End of Trial and Baseline Measures for Two Treatment Groups, and Estimated Effect Size Associated With Test of No Effect Size*

Outcomes	Placebo (n = 7)		GSH (n = 9)		Effect Size (GSH − placebo)	95% Confidence Interval	p Value
	0	(SD)	0	(SD)			
FVC	− 3.3	(4.3)	− 2.7	(9.7)	0.6†	− 7.8–9.1	0.88
FEV_1	− 3.7	(3.8)	− 2.8	(10.4)	0.9†	− 7.4–9.3	0.81
$FEF_{25–75}$	− 5.0	(11.7)	1.2	(14.8)	6.2†	− 8.0–20.9	0.38
Peak flow	− 6.5	(32.0)	33.7	(35.2) [n = 7]	40.2†	1.0–79.4	0.04
BMI	0.0	(0.6)	0.1	(1.1)	0.1†	− 0.9–1.0	0.90
6-min walk distance	30.9	(158.1)	57.8	(123.7)	26.9†	− 124.0–178.0	0.71
Usual stamina	0.6	(0.5)	0.4	(1.0)	− 0.2	− 1.0–0.8	0.77
General wellness	0.4	(0.5)	0.8	(0.7)	0.4†	− 0.1–1.0	0.09
Cough frequency	0.0	(0.6)	− 0.4*	(0.7)	− 0.4*†	− 1.1–0.3	0.20
Sputum amount	0.0	(0.6)	0.1*	(0.7)	0.1*	− 0.5–0.7	0.71
Sputum viscosity	0.2	(1.2) [n = 6]	− 0.2*	(0.9)	− 0.4*†	− 1.4–0.6	0.42
Sputum color	0.7	(2.2) [n = 6]	− 0.1*	(1.1) [n = 7]	− 0.8*†	− 2.8–1.2	0.39
Improvement	2.8	(1.2)	4.7	(0.6)	1.9†	0.8–3.0	0.004

*A negative value indicates an improved condition.
†Favors the GSH group: p = 0.002 that 11 of 13 patients would favor GSH if there were no positive GSH effect.

4) in the GSH group, and was statistically different (p = 0.03) from the placebo group; and (2) the average difference in $FEF_{25–75}$ between the GSH and placebo groups increased to 8.4%, which tended toward statistical significance (p = 0.24).

Discussion

This pilot study yielded encouraging results that warrant a closer examination by means of larger, longer clinical trials. Small airway function improved in the GSH group, as seen in the significant improvement in peak flows and the tendency toward significance of $FEF_{25–75}$ in the ancillary compliance analysis. Because two subjects in the GSH group did not record peak flow data, the peak flow comparison is comparable to the compliance analysis. While the effect size in peak flow is relatively small (40.2 L/m), improvement in small airway function is noteworthy because research[41] in CF pathophysiology suggests that changes in peripheral air flow precede changes in FEV_1 and FVC in this disease. In addition to small airway function, two self-reported secondary indicators significantly improved in the GSH treatment group: subjective sense of improvement (p = 0.004), and subjective assessment of cough frequency in the ancillary compliance analysis (p = 0.03). A measure of subjective general wellness tended toward significant improvement as well (p = 0.09). Finally, none of the outcomes significantly favored the control group over the GSH group.

This pilot study demonstrates significant differences in a few important end points, and the results are consistent with the hypothesis that GSH does improve clinical indicators in CF patients. Why then were not more indicators, especially FEV_1 and FVC, significantly different between the two groups? There are two opposing possibilities that are worth considering. First, inhaled GSH may not be an effective treatment for CF symptoms. If this is the case, then the significant differences found in this study represent statistical type I errors, and further study of inhaled GSH is unnecessary. However, this explanation is improbable given the small p values and the multiple significant differences that were observed in this study. The second possible explanation for finding few significant differences in this study is that inhaled GSH is an effective treatment for CF symptoms, but limitations in this pilot study prevented the actual effect of GSH to occur and to be detected. In order to fully consider this second explanation, it is important to discuss the limitations of the study.

An important limitation of this study is its short duration. The study lasted only 8 weeks, and patients were only at full dosage for the last 6 weeks of the trial. It is possible that more time receiving treatment is required before detectable improvements in lung function occur. Another limitation is the small sample size. Measures of lung function exhibit natural variability; therefore, it is possible that if group differences exist, they will only be detectable with larger samples. Also, the patients in this study were fairly healthy patients (average FEV_1 was 80.7%, and average FVC was 88.3%), which limits the amount of improvement that can be observed. Another limitation is the placebo solution. The smallest amount of the placebo mixture that could be reliably encapsulated at the facilities available to us was 100 mg. This resulted in a 4% hypertonic saline solution, and the

use of hypertonic saline of 3 to 12% has been demonstrated to have therapeutic value to patients with CF.[42–44] Therefore, it is possible that the observed effects in these data are smaller than they would be if a true placebo solution had been used.

Another important limitation of this study is that the optimal dose of inhaled GSH is still unknown. Most of the previous *in vivo* studies[31–40] of inhaled GSH used 600 mg/d; however, neither explanation nor justification for this dose has been given. We used the following rationale to arrive at the dosage levels in this study. Given that research[8,15,28] has shown reduced levels of GSH in blood serum, lymphocytes, and WBCs, and others[13,16] have shown a 50% reduction in export of GSH, and given that Meister[45] has shown that a normal 150-lb male subject synthesizes 10 g of GSH per day, our dosage schedule was based on a 50% replacement of daily, synthesized GSH in the body. While previous *in vivo* trials[31,40] have demonstrated adequate delivery and a half-life of several hours of inhaled GSH, not all of the prepared solution reaches the lung due to leakage and other factors.[40] Also, given the recent findings[46] that normal mice respond to oxidative stimuli with a threefold increase in ELF GSH, and that CFTR knockout mice lack this response, it is possible that even higher doses are required to achieve effective treatment. Clearly, further dosing studies are necessary in order to identify the optimal dose.

If inhaled GSH is effective and the study limitations discussed above muted or prevented the actual effect of GSH to occur and/or to be detected, then only a few outcomes might show a significant GSH effect while a general tendency or syndrome of improvement might exist collectively across the outcomes. A simple way to conceptualize a general tendency or syndrome of improvement is to assume that there is no GSH effect. Under this assumption, one would expect the estimated effect size to favor the control and GSH groups with equal probability. Between the primary and secondary outcomes, there are 13 clinical end points, of which 11 favored the GSH treatment group. Under the assumption of no GSH effect, the probability of observing 11 of 13 effects favoring the GSH group is 0.002. This probability is extremely small, which suggests that in this 8-week period we are seeing a syndrome of improvement in the GSH treatment group affecting a broad spectrum of symptoms, including airway function, sputum characteristics, weight gain, cough frequency, and stamina measured in distance walked, among others (Table 4, column 3).

This general syndrome of improvement is consistent with the significant difference ($p = 0.004$) between the groups in their subjective assessment of improvement: the GSH group reported feeling much better, while the control group reported feeling about the same. It is also consistent with information garnered from exit interviews with study participants. Several patients in the GSH group reported an amelioration of other symptoms that we did not investigate, including improvement in sinus inflammation and nasal mucus, improved stool characteristics, and decreased need for pancreatic enzymes. Given that some significant differences were found despite the study limitations and given that the data show a general tendency toward improvement, it seems that inhaled GSH has the potential to be an effective treatment for many CF symptoms. Certainly there is sufficient promise that longer and larger studies of inhaled GSH are warranted.

In general, the GSH treatment was well tolerated by the patients, and none of the patients in the GSH group notified the principal investigator of serious adverse events. Based on verbal complaints and logbook entries, the GSH and placebo groups appeared to have similar frequency and severity of reported adverse events. Also, there were no significant changes or differences in the number and severity of species identified in bacterial cultures between the treatment and placebo groups (the noncompliant GSH patient cultured rare *Burkholderia gladioli* at the end of the 8-week trial; the pathogenicity of *B gladioli* in patients with CF is unknown); subsequent culture findings from this patient have been negative for *B gladioli*.[47] Of course, additional trials will be required to verify the safety of inhaled GSH, especially in fragile patients who were excluded from the pilot study (culturing *B cepacia*, history of hemoptysis/pneumothorax, $FEV_1 < 30\%$ predicted, or severe asthma component.)

It should be noted that secondary GSH deficiency has been observed and perhaps plays an aggravating role in several other respiratory diseases, including COPD,[48] ARDS,[49] idiopathic pulmonary fibrosis of nonsmokers,[50] AIDS-related respiratory disease,[51] idiopathic interstitial pneumonia,[52] idiopathic respiratory distress syndrome,[53] and diffuse fibrosing alveolitis.[54] Therefore, this therapeutic approach might be useful in diseases other than CF.

In conclusion, while there is emerging evidence that primary GSH deficiency is an important element of the CF pathology, it must be understood that CF disease is certainly multifactorial in origin. Short of a genetic cure, however, effective treatment of CF should address the GSH deficiency. The findings from this pilot study are consistent with the hypothesis that augmentation of ELF GSH does significantly improve clinical indicators in patients with CF. Specifically, we observed a spectrum of fairly ameliorative results, most noteworthy of which

was significant improvement in small airway function. We believe it is clear that further investigation is required to determine the extent of the effectiveness of GSH on CF symptoms; therefore, higher powered studies of longer duration should be conducted that examine a larger array of clinical indicators, including inflammatory mediators and exhaled breath characteristics.

ACKNOWLEDGMENT: We thank Travis Dastrup, MS, Columbia University School of Medicine; Melissa Redding, Brigham Young University; Colleen Saxey, RN, Brigham Young University; and Nicholas Bishop, Brigham Young University, for assisting in conducting the trial, and for data input and analysis.

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March 16, 2018

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LT USPHS
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CDER/OC/OPRO
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RE: Docket FDA-2015-N-3534

Dear Ms. Hallman,

McGuff Compounding Pharmacy Services, Inc. (MCPS) is responding to the FDA's questions to the nomination of Glutathione's inclusion on the 503A bulk drug substances list due by Mar. 16, 2018.

Responses:

Q. To the extent possible, we request that you submit information regarding the historical use of glutathione in compounded products, such as the approximate number of prescriptions per year for compounded glutathione and the uses associated with those prescriptions for compounded glutathione.

A. For one compounding pharmacy, an average of 9,152 prescriptions were dispensed annually for various compounded preparations of glutathione as follows:

Formulation Strength & Size	Annual Prescriptions
Glutathione 100 mg/mL 10mL Injection	125
Glutathione 100 mg/mL 30mL Injection	920
Glutathione 200 mg/mL 5mL Injection	154
Glutathione 200 mg/mL 30mL Injection	7,490
Glutathione 100 mg/mL 30mL Inhalation	30
Glutathione 200 mg/mL 30mL Inhalation	433

Since the year 2000, over 436,000 units of compounded glutathione for injection and inhalation have been formulated. No serious adverse events have been reported to the compounding pharmacy over an 18 year period, substantiating the use of compounded glutathione preparations to be extremely safe.

Pharmacies aren't required to document the associated use with each prescription. However, some associated uses of compounded glutathione include Parkinson's Disease, peripheral obstructive arterial disease, pulmonary & respiratory conditions (e.g. COPD, asthma, etc.), enhanced therapeutic effect of some chemotherapeutic

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agents and alleviation of some adverse chemotherapy effects, immune system protection/enhancement, and management of oxidative stress.

Echoing similar sentiments in our initial response, we'd like to once again note the FDA's own guidance definition that an API or bulk drug substance is, "any substance that is intended for incorporation into a finished drug product and is intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body…." The value of compounded glutathione formulations can indeed be connected to glutathione deficiency in the body and not be tied to a specific disease state.

Individual Patient's Testimonial (Name Removed To Protect Identity):

This is to explain the benefits that my sister has obtained, through glutathione.

My sister has been fighting Parkinson's disease for 14 years. After several years of taking L-Dopa, this is no longer showing any effect. Since L-Dopa has not taken any effect in her organism, she can't move, and she depends 100% on other people to perform daily life activities. In addition, other symptoms are tight muscles and joint ache all day long. Her Dr. prescribed glutathione and after taking it, L-Dopa is showing effect in her body once again. Another glutathione's benefits has been, eliminating the side effects of L-dopa.

As you can see, it's vital to continue with Glutathione treatment in order to get a good quality life, and I really need and hope that you will be able to continue Glutathione Production.

Sincerely

In 1996 I was sprayed with Monitor, an organophosphate (OP) insecticide while surveying a farm. This began my nightmare of being sucked down into the pit. No help, vague answers, worsening symptoms.

The MDs I consulted ignored my statements that my symptoms began with the pesticide spraying. They diagnosed me according to where my presenting symptoms intersected with their specialties: asthma, fibromyalgia, etc. They prescribed synthetic chemicals/pharmaceuticals. In addition to my already disabling symptoms I got many of the listed side effects. They harmed me more than helped.

I need to state that previous to the pesticide spraying I had been vibrantly healthy. If I hadn't been, I wouldn't have survived being doused by an ag plane with Monitor when he unloaded a full tank right on top of the field I was working in.

Since I couldn't tolerate the prescribed drugs, I worked hard to detox and was slowly getting better. Then, my health began to decline dramatically in 2003. By the spring of 2004 I was diagnosed with "chemical hypersensitivity" and sent home to die. I was given 2 months to 2 years, maximum, to live. I

was told, "There is no treatment. There is no cure. It will only get worse with every exposure. Go home and avoid all (exposures)." My asthma specialist then told me he was kicking me out. He said he would not treat me; He would not see me; He would not help me; I wasn't to even ask. He went on to tell me that if I told anyone that he'd diagnosed me with this, he'd deny it. When I asked him if there was anyone who could possibly help me, he suggested I try an Environmental Illness specialist (EI). I did. The first thing that specialist did was measure my glutathione level. The test came back the lowest he'd ever seen. He described it as "in the sub-basement." I was diagnosed with "Severe glutathione deficiency".

It took me until 2007 to figure out why my health suddenly took a dramatic nose dive in '03-'04. A melon farming operation moved in within 500-600 ft of my house and began growing watermelons in February or March of 2003. Watermelons are grown using a number of toxic pesticides. The primary chemical they use is a highly volatile fungicide called Chlorothalonil. It kills all cells from fungal to mammalian by uptaking cellular glutathione.

They begin ground drilling (fumigating) of the soil with this fungicide as soon as the temperatures are high enough the soil is no longer frozen. They apply chlorothalonil as a fumigant again at planting, and apply it weekly with fertilizer until the end of growing season, by spray, fog or chemigation. FYI chlorothalonil is also the primary fungicide used in consumer products were there is moisture or spills, like upholstery, carpet, shower curtains, sink stoppers, mats, rainwear, anywhere mold may grow, and in many cleaning products. Its "everywhere."

I told that farmer's representative that I had a previous pesticide injury and needed to know before all pesticide applications, so I could protect myself. At that time he told me, "Its only fertilizer. Don't worry about it."

They had some kind of an accident with the chemical that year that was covered up. I was outside when it happened, smelt it and was horribly injured. It was not reported. I didn't yet know what it was. That farmer stopped growing in my neighborhood at the end of that season. A different melon grower took over and grew melons for the next 4 seasons in the fields adjacent to my property. He told the same lie, "Its only fertilizer. Don't you worry your little head about it." Occasionally, when caught, he'd name a chemical, Bravo or Daconil, but state it was completely harmless. It wasn't until in the winter of 2007 that I stumbled upon the answer while talking with an EPA inspector. Thats when I discovered the application protocol for chlorothalonil (aka Bravo, Daconil) was so intensive and weekly.

In the meantime my EI and I had tried every oral and topical application of glutathione remediation he could find. My system was too damaged. I couldn't make my own glutathione. Not enough to stay ahead. I couldn't absorb it or reconstruct it from the 3 components of the tri-peptide. I made progress in the off season when I could avoid all toxins and lost ground during pesticide season. My net health was declining each year as the off season was only 3-3.5 months. The damaging applications hadn't stopped when they quit growing melons. My spraying neighbors retaliated, because I had asked them to warn me in advance. They had no scientific knowledge or understanding of my condition. They'd never heard of it. They mocked me and dismissed my disability despite the letter from my specialist, who is an MD. I was fighting for my life every day. My life was ebbing rapidly away. I clung desperately to it and I suffered horrors and tortures that you cannot

imagine. By the grace of God I found some research on IV glutathione. I brought it to my EI and he carried the ball from there.

Over the years my heartless, retaliatory pesticide applying neighbors have nearly killed me more than two hundred times. I stopped counting in 2008. I've had life threatening episodes from accidental exposures hundreds of times more. If it were not for IV glutathione I would have died.

At first, following an exposure I would have to be rushed to my EI. Hospitals don't stock it. Their environmental chemicals are too toxic. Many times I'd get the exposure on a weekend or after hours and barely survive, in brutal agony, until we could get in. My positive response to IV glutathione is dramatic!

I still have PTSD from the horrors of the pesticide assaults. Pesticides were, after all, discovered as a by-product while the government was developing OP nerve agents as chemical weapons back in the time of the World Wars. Because the pesticide applications increased in my neighborhood with the increase in GMO Crop experimental plots, my emergencies increased. I had to have IV glutathione 3 times a week, at least, at one point. Due to the constant and unpredictable threats of chemical assaults and my desperate need I undertook learning how to self administer. This returned a small measure of control over my own life. It also enabled me to travel more safely, freeing me somewhat from being held imprisoned by chemicals in my own home, waiting for the next onslaught. Being able to inject glutathione when I needed it, and not suffer through the accumulative damage that occurred as the toxins rampaged through my unprotected body while I waited to get in to the doctor's, helped me to begin to gain ground. Time from exposure to remediation is critical.
My life and my family have been devastated. The damage done to my system from the heartless actions of a few unlawful pesticide applicators created my condition. My glutathione depletion is the result of the damage by those pesticides. IV Glutathione is the key to my recovery. My EI stated that my not having it "...would be catastrophic."

Of glutathione's hundreds of known biochemical operations, detox and RNA repair are two of the primary functions that I require the injected glutathione for, to manage my condition and to heal. Its as necessary to me as insulin injection is to a diabetic. Its as lifesaving as my old asthma inhalers were considered to be. Asthma inhalers I can't take because of their toxicity. All (synthetic chemical) pharmaceuticals have side effects. All (synthetic chemical) pharmaceuticals are toxic. I can't breakdown toxic chemicals and move them out of my body, so I can't do synthetic chemical pharmaceuticals, any more than I can endure pesticides. I need access to compounded IV glutathione and other nutraceuticals to survive and to repair the systems the pesticide exposures damaged.

Please, do not prevent my acquisition of this specially compounded pharmaceutical. Without it, I will die.

Dear FDA:

I have two *genetic* conditions for which liposomal glutathione is the only remedy. Inhaled glutathione, which requires a prescription, is superior

to oral liposomal glutathione. I have had genetic testing and my condition and the remedy has been confirmed my four internists and one specialist. Inhaled glutathione costs me $125 per month or $1,500 per year.

There is no prescription drug for my genetic conditions. Without glutathione, both myself and Medicare will incur the costs of numerous doctor's visits and hospitalizations when I get pneumonia. Without glutathione, I must have an Epi Pen which Medicare pays for at a cost of $600 between one and three times per year, or as much as $1,800 per year.

I implore you to keep glutathione available.

My genetic conditions are as follows:

(1) **Absent GSTM1 gene** weakens my immune system and without glutathione, I get every flu that comes around. During the bad flu season of 2009, I had the flu three times plus I had pneumonia. I have had no illnesses, not even a cold, since 2013 when I began treatment with glutathione.
(2) **Exceptionally low pancreatic enzymes**. The absent GSTM1 gene exacerbates my second genetic condition, which is exceptionally low pancreatic enzymes. This condition causes me to have a broad spectrum of low grade food allergies and intolerances. Without treatment with glutathione, after two days, I begin coughing up green phlegm, after four days I am sneezing and coughing, and left untreated, I get late night/early morning anaphylaxis. The build-up of histamine from the food allergies overflows when my immune system is at its lowest, i.e. between 1am and 3am, causing me to wake with anaphylaxis. I had to sleep with an Epi Pen next to my bed, and restrict myself so I could only sleep on my side so I wouldn't choke to death when I got an attack.

Untreated food allergies and intolerances weaken my immune system. I literally get sick from food. Glutathione strengthens my immune system so this doesn't happen.

Respectfully submitted,

Dear Friends at McGuff Compounding Pharmacy and to the reviewing committee of the FDA's PCAC,

Since beginning nebulized Glutathione, compounded by your laboratory, I have experienced exponential gains in the health of my lungs! The chronic inflammation I have suffered for years due to Asthma and a fungal infection following removal of a metastatic tumor in my right lung had left me with severe Bronchiectasis.

On the advice of my M.D., Dr. SS, I began nebulizing Glutathione twice daily and in less than a month I have enjoyed dramatic lessening of the coughing and mucus production that had plagued my waking hours and impacted my ability to sleep for more than two hours at a time without waking to an extreme cough and mucus production. I am already able to walk a longer

distance without severe dyspnea, talk and sing whole sentences without choking on a severe throat tickle and mucus production.

I had tried other products such as albuterol nebulized, and Mucomist but not experienced any reduction in the inflammation in my lungs. As the mucus plugging has been reduced by the Glutathione, I have measured an increase in my %SpO2 as tested by Pulse Oximeter. I have seen a welcome increase in my FEV6 or total end volume using a Spirometer to measure my lung capacity or TLC.

Glutathione MUST stay available for use by patients like myself. It would be a cruel and inhumane act to disallow this vital, non-injurious, non-side-effect, non-drug-addicting, natural substance to be taken off the market in the form it is currently provided, ie; compounded for use in a nebulizer or delivery via IV.

I entreat you to continue allowing the compounding of Glutathione by McGuff and other pharmaceutical laboratories, so that suffering patients such as myself may benefit from its use.

Hi,
Please don't deny my access to the medicinal use of Glutathione by discontinuing it's formulation by compounding pharmacies.

I use Glutathione 2-3 times per week to help support my body so that I am able to go about my daily activities to support myself, physically and monetarily. Glutathione helps me by increasing my energy as well as decreasing the achy-ness that I get through out my body when I don't use it regularly. Imagine going about your day feeling like you have a bad case of the flu coming on. That's what I feel when I have gone without my weekly shots of Glutathione on several occasions and I really, really don't want to have to do that again any time soon!

I am also a health care practitioner. I have heard from a number of my clients how helpful their shots of Glutathione can be in helping them to function in today's world.

Please know that there many individuals that would be effected by a negative decision regarding the formulation of Glutathione.

Warm Regards,

To Whom It May Concern,

I am using glutathione with great relief to my upper respiratory condition due to black mold.

I need to have continued access since it’s been a Godsend for me.

To whom it may concern:

It has recently come to my attention that the FDA May take away my access to glutathione. You see, I have a chronic illness that causes inflammation throughout my body- mainly in my brain and lungs. I feel it mostly in my lungs. The inflammation wreaks havoc in my body and remodels my lung tissue; causing them to be brittle and stiff. It is difficult for me to get air and

makes life challenging at times. I use glutathione twice a day in a nebulizer at home. It helps me so much. Hopefully, I won't be sick forever but while I am, I'd like to be able to keep up with my six year old. We like to walk, hike and do things together. Glutathione helps me do these things.

My husband XXXXXX has suffered from industrial asthma, and COPD ever since June of 1997. He is allergic to most of the prescription drugs and inhalers to treat it. The only thing he could use that he didn't have an allergic reaction to is albuterol. Around 2005 I read about the great benefits of using inhaled glutathione in a nebulizer. After doing some research we copied the information and took it to his doctor and asked him to prescribe it for my husband thru a compounding pharmacy. He started using the inhaled glutathione shortly after that and has continued to use it ever since. It has been amazingly life changing. The benefits of the glutathione (which is one of the body's major antioxidants) has kept my husband extremely healthy even with those serious lung conditions. It has allowed him to be much more active than most people with COPD and emphysema and asthma. Plus it has kept his immune system very strong, which helps him fight off colds and flu that could kill a person with those health issues. After he started using the inhaled glutathione his eye doctor was totally amazed at how his eye sight had improved and asked us what he had been doing to get such a major improvement in his eye sight. The only thing he had been taking was the glutathione. We absolutely believe his quality of life and his continuing good health, in spite of having such serious lung conditions, would never be as wonderful as it is if he had never started using the very beneficial inhaled form of glutathione. PLEASE, PLEASE, PLEASE continue to allow patients who need this antioxidant to allow them to remain as healthy as they possibly can. It is a LIFE SAVER !

Sincerely,

My husband has Parkinson's disease. He has been taking Glutathione in its absorbable form for several years. It really makes a difference. He has Parkinson's on one side of his body, his right side. I can tell visually when he needs to take more. His mouth on the right side of his face droops. Once he takes Glutathione, that fixes the droop. If I can see that much difference, I know it is working. He has maintained his Parkinson's symptoms, and feels Glutathione is keeping additional symptoms at bay. If he doesn't have access to Glutathione, will his Parkinson's worsen?

To whom it may concern,
Glutathione has greatly benefited me by reducing my chronic pain. Pain reduction prevents the need for narcotic pain medications that are highly addictive. Non-narcotic pain reduction improves my quality of life.

I am a graduate student at ASU. Glutathione helps decrease my brain fog, which produces better educational success.
This medication has helped me significantly, without it I would be in chronic pain and have brain fog.

Please keep glutathione on the market for patients like myself.

Sincerely,

Hello,

I am disheartened to hear that Glutathione may no longer be available. I depend on this medication to keep my immune system able to function properly. I have a debilitating disease for which no other treatments help. Glutathione is one of them that does help tremendously. Without it, I would not be able to function or get out of bed. Before starting treatment, I was bed bound and had to use a bedside urinal. I was running out of options until my doctor recommended trying glutathione.

Though I am medically disabled, glutathione has allowed me to work part time from home in my pajamas to help make ends meet. I have never asked the government for assistance of any kind. Despite my limitations, I have strived to work through my health challenges and provide a way for myself. Should I no longer have access to glutathione, I will likely become severely disabled again. If I miss even a dose, I am in bed the entire week. And this will prevent me from being able to provide for myself or lead any sort of life.

Please help keep these medications and treatments available to those of us who truly depend on them and need them. We have no other options and truly need them to live and function as a member of society. My body is so broken that it cannot properly function as it should. It cannot repair itself, or create its own cellular reactions and nutrients as most people's bodies do.

My very life depends on receiving glutathione. Please do not take it away or you will be taking away my life.

To the FDA:

I am a patient who has suffered with Multiple Chemical Sensitivity since 2011. When I am exposed to chemicals such as formaldehyde, VOC's, surfactants, perfumes and other chemicals from sources in everyday consumer products too numerous to list, I become ill. It starts with a headache, then progresses to severe brain fog, then to nausea, and if the exposure is toxic enough and I cannot get away from the exposure in time, I even lose consciousness.

Three years ago this condition had worsened to the point that I was regularly losing consciousness upon exposure to toxins. Living with MCS was crippling as far as living a normal life: I was not able to go into stores to shop, to public meeting rooms to attend meetings, to church to worship, to hair salons to get a haircut, to the dentist to have my teeth cleaned, to homes to visit friends, to hotel rooms to go on a vacation...etc. etc. The only place I was safe was confined to my home, where it took years of work to create a non-toxic living space.

For several years I took an oral liposomal form of Glutathione, without much perceived benefit because the glutathione is so easily broken down by digestive acids. Finally I found a doctor who prescribed for me compounded Glutathione which I breathed through a nebulizer once a day. Not only did a 1/2 cc daily dosing of nebulized Glutathione bring me back from the brink of passing out, but over time my level of sensitivity to exposures also decreased. Now I can go into these settings, where I was once too sick to go.

I had genetic testing performed which shows that I have numerous mutations in the genes which control the complex, multi-step biochemical process of synthesizing Glutathione from a whole host of nutrients and other

biochemicals. Because my body's ability to produce Glutathione is functioning at less than 30% of normal capacity, my body cannot detoxify itself when exposed to the multitude of chemicals which surround us all in daily life...and thus I developed Multiple Chemical Sensitivity.

Nebulizing Glutathione daily has restored my ability to participate in the normal activities of daily life for the first time in years. I have never experienced any side effects or anything negative from using Glutathione either orally or nebulized.
You cannot take this product away from people who desperately need it!!! The number of people with Chemical Sensitivities has exploded in this country in the last two decades; I am one of probably tens of thousands who suffers from this disabling condition. There are no drugs which can treat this condition. There are very few physicians who even know how to approach the treatment of this condition.

Just because it cannot be patented for pharmaceutical company profit, you should not ban its production through compounding. That is evil to prohibit a safe, affordable, accessible, effective form of treatment just because it cannot turn a profit for Big Pharma!!! Do not succumb to this evil!! And yes, your conscience must play a part in your decision-making, because your decisions will either provide a true means of healing to thousands upon thousands of human beings, or result in untold suffering and disability if you choose for wrong.

Your agency was created with the responsibility to help and protect the health of the American people...not the bottom line of Big Medicine and Big Pharma!!! I call on you personally to fulfill that mission.

To whom it may concern,

My name is XX, I use glutathione in a nebulizer daily. I have been treated for Lyme disease, and in the process found out that mold is something that if I come into contact with I can detox it from my body with the use glutathione. It has worked wonders for me, and it pains me to think that this simple, yet very important antioxidant could be taken away.

PCAC Reviewers,

Please note that I am a Glutathione user for the last four years. I first came aware of this medicine during a stay at the Lung Institute for stem cell therapy. While I noticed no 'noticeable' effect of the stem cell treatment (However, let me state that any therapy for COPD is not always readily noticeable) I did notice a immediate benefit from nebulizing with Glutathione--300mg/ml. My wife also remarked that my breathing at night was also much less strained and relaxed. Deeper and more regular. I decided that I would continue the Glutathione and went to Naturopathic Medical Clinic, Dr KP, at Grants Pass, Oregon. Dr. P prescribed Glutathione at 200 mg/ml, one ml. daily by nebulizer. I have used it since then. I find that it gives me much needed relief and that my daily life is significantly improved with its use. Also I would like to point out that my latest CT scan of my chest (11/14/2017) noted that I had "severe emphysema". Also please note that my last Spirometry exam of 12/23/2016 it was shown that my FEV1 indicated "end-stage emphysema". Yet, I am not on any oxygen therapy, walk 2 miles daily, exercise every other morning (one hour) and maintain my house and lawn. I

use 2 'emergency inhalers' every year and also use prescribed Stiloto Respimat. No steroids. Therefore, I would urge you strongly to leave the Glutathione for nebulizing on the ACCEPTABLE list for compounded medicines. I am receiving life giving therapy and would doubtless lose ground without it at a much faster rate. I have lost a Grandfather and an older brother to COPD and it is obvious that I am in much better shape than either of them because of this medicine. Please continue to allow me to help myself to a continued rewarding life.

Thank you for your consideration.

Hi , I am XX. I depend on glutathione to live a semi normal existence. I am missing the GSTM1 gene for my liver and kidney so I cannot produce any for those organs and i am homozygous for the mthfr gene and have only 10 percent function of the enzyme for methylation. There are many people like me . Taking away glutathione would be a death sentence. I made it to 43 without knowing why i was constantly sickened and felt poisoned and toxic just from normal environmental toxins , heavy metals, pesticides , perfume, chlorine, food additives etc. Constant migraines and pain and severe mercury toxicity were the first signs. Please don't take this valuable life saving natural substance away. if our bodies can not make enough we should be able to supplement it in a way that it gets deeply into our bodies and oral supplementation will not work. the damage to the gut from the toxicity has made it impossible to absorb any other way but intravenously. thank you for your understanding,

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My experience with Glutathione IV is very simple. Mayo Clinic in Jacksonville said there was nothing more they could do for me as they have tried all their protocols and nothing worked for me. I was given the nearest hospice address and phone number. I decided to contact another doctor before giving up on life.

Dr. BB introduced me to Glutathione IV's in his office, every week. I went from being unable to walk, experiencing pure hell due to pain and itching skin with blood ulcers, redness, and total weakness. Diagnosed with severe Dermatomyositis, I had been told 90 days was the most I would live.
My Glutathione test came as being off the chart low. Once the Glutathione infusions started it made the difference between life and death for me. What else can I say?

My neurologist Dr. H could not believe my recovery as he was certain I was not going to make it. He asked to see the bottle of Glutathione IV's to recommend to his patients.

It is a matter of life and death for me and I am certain for many others like me.

My daughter relies on nebulized Glutathione to help her breathe and swallow. Mainstream prescriptions have not successfully addressed her esophageal constriction. She is on oxygen 24/7, but still needs the Glutathione. It would be dangerous to her health and well-being if she no longer had access to Glutathione.

As a retired Federal Law Enforcement Officer, Supervisory Special Agent, with over 30 years of experience, I have seen what some prescribed “legal

drugs" are doing to our citizens of this country, many of these "legal" drugs are addictive and are killers.

What glutathione provides my body is the ability to work to it maximum efficiency with a clear mind. I am a cancer survivor with a compromised immune system and due to many surgeries and a bowel resection, my body it not 100 %. With these health issues, my body is aided by my use of Glutathione by the clearing of free radicals and other toxins that my body cannot clear with efficiency. Glutathione is not an addictive drug.

Glutathione has kept me healthy, active, alert and living. I find it interesting and very sad, that with all the drugs that cause death and destruction, the FDA is wanting to limit Glutathione.

My hope would be that the FDA recognizes the value of Glutathione and does not limit or eliminate its availability.

I have a son who is 16 years old, has Cystic Fibrosis ("CF") and has been inhaling Glutathione ("GSH") for many years. I cannot say enough how much GSH has benefited him. The majority of CF patients are in and out of the hospital yearly and even multiple times a year for what is referred to as a CF Tune-Up. My son has not been hospitalized for the past 15 years and I contribute this to GSH and many other homeopathic/natural medicines. If GSH is no longer available to us through compounding pharmacies, I am extremely worried the impact this will have on my son's health.
It is sad when it comes down to government greed over the lives of our children!

To whom it may concern,

Glutathione has played a very important role in my continuous recovery efforts from Early Onset Alzheimers Disease. My positive APOE4 blood test which is the genetic marker for early onset of this disease, my family history and unbeknownst to me my objective symptoms are proof that I have had this disease for years.

My Neurologist insists that a regiment of a specific diet and added Glutathione supplements will continue causing a reversal of this disease. I am thinking clearer and remembering specifics that I could not a short time ago.

Please do not remove Glutathione from my list of supplements that are very effective in reversing my symptoms of Alzheimers Disease.

I was diagnosed with COPD in 2008. Treatment was eight doses of different steroids daily. Every three weeks would spend a week in the hospital, getting steroid injections. Thus my life expectancy was not very long, doctors said all they could do was try to make me comfortable for my duration.

I was told of Integrated Heath in Scottsdale Az., that had success with Glutathione via a nebulizer. Started this treatment, and am still here.

The treatment was 100g twice a day. This day I am still using Glutathione with great success. Have not taken any steroids for approx 4 years, except for emergencies.

My quality of life is not good as pre COPD, but is a lot better than the steroid medications.

Q. Please provide any additional information that FDA should consider in its evaluation that may help to clarify the role of the nominated glutathione bulk substance in compounded drugs products in current clinical practice, such as statements or guidelines from professional medical societies.

A. Professionals' Statements:

To Whom it May Concern,

There is no alternative to reduced glutathione, either by injection or nebulization, for patients who require effective physiological antioxidant activity for treating COPD, asthma and many other conditions including those related to unintended consequences of radiation therapy and certain types of chemo-therapy.

W Bruce Milliman, ND
Naturopathic Academy of Naturopathic Physicians
(NAPCP-Founding President)

Dear FDA,

I've been working with glutathione for the past 8 years. I've never had a negative reaction and it has helped my patients suffering from chronic fatigue and other forms of chronic oxidative stress to be more functional. It has also helped patients with chronic neurodegenerative conditions who have not been responsive to conventional therapies.

Glutathione is a natural substance produced in the body. It is not a patentable drug. It does not fall under the jurisdiction of the FDA just like all other natural products. If the FDA attempts to regulate this substance it will trigger backlash in the community that is already starting to become more aware of the conflicts of interest that exist in a governmental body that is full of pharmaceutical industry insiders and unelected officials.

Physicians and patients will not be blocked from access to life changing natural substances and there will be push back. There is no justification to start regulating this substance after many years of safe use. There is no danger in its use and the only conceivable incentive for the FDA is to appease the pharmaceutical companies (who have bought out the FDA and in fact make up many of its officials) that are challenged by more root cause oriented approaches to health and healing.

If the FDA were to look at How Glutathione is used and then look at other alternatives available it will become obvious that the current conventional approach to chronic fatigue is not working. Glutathione is not only safe but indispensable for its role in the larger approach to treating a syndrome caused by chronic immune stimulation and thus oxidative stress.

Sincerely,
Jake Wardwell, D.O.

Patient KSS, female, age 25, had interstitial cystitis for 4 years, was only able to hold 3 oz of urine in bladder and was in constant bladder pain. She took weekly bladder instillations at her doctors office for 1 ½ years, a combination of heparin and elmiron, with only mild improvement of pain. Her other diagnoses included fibromyalgia, anxiety, moderate depression. Unable to work, and in acute distress and desperation, she was recommended by her physician to locate a practitioner to give her IV Glutathione. She started receiving IV Glutathione 1200 mg along with a modified Myers infusion in Feb 2016. Shortly thereafter she was able to start a part time job. She stopped using her cane for walking! Despite being surgically diagnosed with endometriosis and told she would likely never conceive, after trying for 4 years she and her husband did conceive, 2 months after starting the weekly Glutathione and Myers' infusions. She was able to maintained the pregnancy, and she gave birth to a healthy baby boy. Glutathione was administered throughout her pregnancy. She stopped infusions in Dec 2016, and was given instructions to return if her symptoms returned, but she is doing quite well and IV therapy seems to be no longer needed.

Pt M.B, is a 34 year old Iraq War veteran, with c/o chronic eruptive pruritic non-contagious skin condition he and several other veterans had contracted while in Iraq. He'd been suffering with this for the past 10 years, without relief despite trying many medications. Predominantly an itchy, painful blistering rash in patches all over his body, and notable for worsening and flaring up more severely during times of stress. The Veteran Administration in Syracuse NY were not able to successfully treat this over the years, nor explain or pinpoint the etiology. He received IV Glutathione 1000mg once a week for 12 weeks. He has found now that most all affected areas are healed up, just some scarring remaining… and even during the stressful times when the rash would ordinarily flare up, a couple of blisters might form but they would quickly heal up. He completed the Glutathione 1000mg and is quite happy with the results.

Elizabeth G. Salon, R.N.C., M.S., F.N.P.
Family Nurse Practitioner
Integrative Health

I use I.V. glutathione frequently for my patients with cardiovascular, cerebrovascular, and neurodegenerative diseases, e.g., atherosclerosis, cardiomyopathy, post-myocardial infarction, stroke, Alzheimer's disease, ALS, MS, Parkinsonism, as well as for those receiving stem cell therapy. It is a vitally important agent for all of these conditions, and provides significant therapeutic benefits for my patients. I must continue to have ready access to I.V. glutathione.

Sincerely,
Mitchell A. Fleisher, M.D., D.Ht., D.A.B.F.M., Dc.A.B.C.T.
Center for Integrative & Regenerative Medicine

Hello,
I'm a naturopathic physician at Sojourns Community Health Clinic. I am writing to help ensure compounded preparations remain available for my patients.

In my practice, I have used Reduced Glutathione 2000mg IV pushes for many patients. Patients have had relief from a range of symptoms including: headaches, fatigue, cognitive impairment, and chronic pain. It has been especially useful in my patient population with chronic pain and chronic fatigue syndrome. Patients have reported being able to better function in their lives for days following treatment, many reporting dramatic improvement. Some patients receive IV Glutathione weekly, others monthly. It is an excellent treatment that has allowed many of my patients feel better and be able to participate in life again at work, school and home.

It would be a terrible loss not to be able to offer IV Glutathione to my patients in the future. Many would experience a decrease in quality of life without it. Please keep compounded glutathione available. Thank you for your work on the topic.

Yours,
Anne Van Couvering, ND

The FDA should review the use of Glutathione for liver diseases such as hepatitis and others, neurodegenerative conditions like dementia, various lung diseases including COPD and conditions characterized by high levels of toxic metals like mercury. Glutathione administered via nebulization is especially useful for pulmonary conditions, in dose ranges of 120mg twice daily. Dosage ranges for the intravenous use of Glutathione are typically from 800mg to 2,400mg for liver conditions, dementias and for the removal of toxic heavy metals. Glutathione for intravenous use is supplied in the concentration of 100mg or 200mg/ml. The links below reveal just a small portion of the relevant clinical data supporting the use of glutathione for the conditions mentioned.

https://www.naturalmedicinejournal.com/journal/2011-02/health-dividend-glutathione
http://erj.ersjournals.com/content/16/3/534.long
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2249747/
https://www.ncbi.nlm.nih.gov/pubmed/27603810
https://bmcgastroenterol.biomedcentral.com/articles/10.1186/s12876-017-0652-3
https://www.sciencedirect.com/science/article/pii/S0009898103002006
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2596047/

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http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.556.7222&rep=rep1&type=pdf
http://www.iaea.org/inis/collection/NCLCollectionStore/_Public/41/131/41131299.pdf

Dr. Ayo Bankole
Inland Naturopathic Medical Center

In 1994, the Journal of Nutritional Biochemistry stated, "Disease states due to glutathione deficiency are not common."

Well, 25 years and 90 thousand journal articles later we have found this statement to be false on all levels. Glutathione is recognized as an extremely important intracellular antioxidant that also plays a central role in the detoxification and elimination of potential carcinogens and toxins. Studies have found that glutathione synthesis and tissue glutathione levels become significantly lower with age, leading to decreased ability to respond to oxidative stress or toxin exposure.

The higher the glutathione peroxidase in the plasma or red blood cells, the more your body is running through and out of glutathione. Now consider the follow…

Total glutathione peroxidase activity was elevated in females 65 years of age or older. Cigarette smoking significantly elevated glutathione peroxidase. Alcohol elevated glutathione peroxidase, with the highest levels seen in drinkers who also smoked. Increased glutathione peroxidase was also seen in vigorous exercise, especially triathletes and marathoners.

According to the National Cancer Institute, dairy products, cereals and breads are low in glutathione. Fruit and vegetables have moderate to high amounts of glutathione. Frozen versus fresh foods had similar amounts of glutathione. Processing and preservation resulted in considerable loss of glutathione.

A 27% reduction in glutathione has been reported in the cerebrospinal fluid of schizophrenic patients.

Studies have shown that dietary glutathione enhances the metabolic clearance and reduces net absorption of dietary peroxidized lipids, which cause intense cellular damage.

- High altitude exposure reduces glutathione levels.
- Glutathione functions as an antioxidant and can maintain vitamin C in its reduced and functional form.
- Chronically low glutathione levels are seen in premature infants, alcoholic cirrhotics and individuals with HIV.

Glutathione increases sperm motility patterns and sperm morphology. In a double-blind, placebo-controlled crossover trial of infertile patients, patients were randomly and blindly assigned to treatment with one injection every other day of either glutathione at 600 mg or an equal volume of placebo. All

the glutathione selected patients showed an increase in sperm concentration and a highly statistically significant improvement in sperm motility, sperm kinetic parameters and sperm morphology. Want to get pregnant? Make sure your husband has optimal levels of glutathione.

From the journal of Digestion:Glutathione is extremely important in normal functioning of the pancreas, being needed for normal folding of the proteins that will ultimately form key digestive enzymes when the pancreas is stimulated after a meal. In patients with chronic pancreatitis, it has been found that glutathione is often significantly depleted, suggesting that lack of glutathione has a role in the generation and/or maintenance of the disease. In addition, many patients suffering from chronic pancreatitis appear to be under xenobiotic or oxidant stress, creating an even greater need for glutathione. Since the pancreas is under relative glutathione "stress" during the normal process of packing and secreting digestive enzymes, it is easy to see how the lack of glutathione could have a role in chronic pancreatitis.

From the Journal of Brain Research Reviews: Glutathione depletion can enhance oxidative stress and may increase levels of excitotoxic (toxins that excite neurons to the point of death) molecules, which may initiate cell death in specific nerve cell populations. Evidence of oxidative stress and reduced glutathione status is found in Lou Gehrig's disease, Parkinson's disease and Alzheimer's disease.

From the Annals of Pharmacotherapy: Glutathione is important in DNA synthesis and repair, protein and prostaglandin synthesis, amino acid transport, metabolism of toxins and carcinogens, enhancement of immune function, prevention of oxidative cell damage and enzyme activation.

From the Journal Acta Dermato-Venereologica: Low levels of blood glutathione were found in patients with pemphigoid, acne conglobata, polymyositis, rheumatoid arthritis, scleroderma, systemic lupus erythematosus, atopic dermatitis, eczema and psoriasis.

From the Journal of the Federation of American Societies for Experimental Biology: Intracellular glutathione enhances the immunologic function of lymphocytes (perhaps the most important immune cell line in preventing infection and cancer). Low levels of glutathione limit the optimal functioning of T cells. Cytotoxic T cell (necessary to eliminate cancer) responses and interleukin-II-dependent functions are inhibited even by a partial depletion of the intracellular glutathione pool.

From the journal of Ocular Pharmacological Therapy: Susceptibility of the lens nucleus to oxidative damage and loss of transparency has been shown in experimental animal models, including exposure to hyperbaric oxygen, x-ray and UVA light. Depletion of glutathione allows the levels of oxidant to damage lens tissue and structure. From the Journal of Laboratory and Clinical Science: An increased incidence of low glutathione levels in apparently healthy subjects suggests a decreased capacity to maintain metabolic and detoxification reactions that are stimulated by glutathione. The authors stated that glutathione status, physical health, and longevity are closely related.

From the Lancet: The plasma glutathione in young, healthy adults was 0.54 umol/L; in healthy elderly it was 0.29 umol/L; in elderly outpatients it was 0.24 umol/L; and in elderly inpatients it was 0.17 umol/L. Aging results in a decrease in plasma glutathione and an increase in oxidative damage in apparently healthy individuals.

Simply put, if you want young cells and the ability to overcome disease you need to work on getting your glutathione levels up!

Matt Angove, ND, NMD
American College For Advancement In Medicine (ACAM)
ACAM Integrative Medicine Blog

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Please let us know if you are in need of any further information.

Sincerely,

Ronald M. McGuff, President/CEO
McGuff Compounding Pharmacy Services, Inc.
2921 W. MacArthur Blvd., STE 142
Santa Ana, CA 92704

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Alcohol 33 (2004) 191–197

ALCOHOL

Chronic ethanol ingestion and the risk of acute lung injury: a role for glutathione availability?

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Received 9 March 2004; received in revised form 30 July 2004; accepted 5 August 2004

Abstract

Although pulmonary function is not altered, a history of alcohol abuse is an independent outcome variable in the development of acute respiratory distress syndrome. In the absence of cirrhosis, alcohol abuse decreased glutathione, the key antioxidant lining the alveolar space, by 80% and is associated with alveolar barrier leak. Neither the glutathione pool nor barrier leak was corrected by abstinence for 1 week. This aberrant glutathione homeostasis may contribute to enhanced alveolar permeability, thereby increasing susceptibility to the development of acute respiratory distress syndrome. In a rat model, chronic ingestion of ethanol decreased pulmonary glutathione concentration, increased alveolar barrier permeability, and increased the risk of acute lung injury. In alveolar type II cells, chronic ingestion of ethanol altered cellular functions such as decreased surfactant processing, decreased barrier integrity, and increased sensitivity to cytotoxin-induced apoptosis in vitro and in vivo. In alveolar macrophages, chronic ingestion of ethanol decreased phagocytosis of microorganisms and decreased cell viability, events that would increase the risk of pneumonia. A central role for glutathione availability was demonstrated by the normalization of cellular function and viability of type II cells and macrophages as well as decreased sensitivity to endotoxemia-induced acute lung injury when glutathione precursors were added to the ethanol diet. These results support the suggestion that chronic ingestion of ethanol increased the risk of acute lung injury not through ethanol per se but through the chronic oxidative stress that resulted from ethanol-induced glutathione depletion. Because chronic oxidative stress alters cellular functions and viability, the lung becomes more susceptible when a second hit such as sepsis occurs. © 2004 Elsevier Inc. All rights reserved.

Keywords: Acute respiratory distress syndrome; Acute lung injury; Alcohol; Type II cell; Alveolar macrophage; Glutathione

1. Alcohol abuse increases the incidence of acute respiratory distress syndrome

Morphologically, acute respiratory distress syndrome (ARDS) is characterized by extensive alveolar epithelial cell destruction and flooding of the alveolar airspace with proteinaceous fluid (Ware & Matthay, 2000). Acute respiratory distress syndrome remains a common and severe form of acute lung injury, which frequently complicates pneumonia. Findings of studies have demonstrated that a history of alcohol abuse is an important independent variable in the development of ARDS. Patients with a history of alcohol abuse chronically have both a greater incidence and severity of ARDS (Moss et al., 1996). The link between alcohol abuse and acute lung injury is of enormous importance, particularly because results of two large epidemiologic studies have indicated that approximately 50% of patients with sepsis-associated ARDS have a significant history of alcohol abuse (Moss et al., 1996, 2003). Moreover, patients with ARDS, who often require prolonged ventilatory support, have a greater incidence of secondary ventilator-associated pneumonia (Chastre et al., 1998). Ventilator-associated pneumonia is independently associated with increased hospital mortality, organ dysfunction, and time on mechanical ventilation (Bercault & Boulain, 2001; Kollef et al., 1997), with approximately 20% of the patients with a history of alcohol abuse (Bercault & Boulain, 2001).

2. Chronic alcohol ingestion results in oxidative stress and alters glutathione homeostasis

Only the liver and the gastric mucosa have the high affinity isoform of alcohol dehydrogenase, but alcohol can also be metabolized by means of cytochrome P450 if concentrations remain elevated (Lieber, 1993). Although the lung does not have alcohol dehydrogenase, alcohol metabolism through the cytochrome p450 system in the lung is significant (Manautou et al., 1992). In both p450 and alcohol dehydrogenase metabolism of alcohol, the major by-product is acetaldehyde, which causes oxygen radical generation and

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Editor: T.R. Jerrells

0741-8329/04/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi: 10.1016/j.alcohol.2004.08.002

lipid peroxidation (Lieber, 1993). Thus, metabolism of alcohol to acetaldehyde causes oxidative stress by means of lipid peroxidation and reactive oxygen species production, regardless of the mechanism involved in its metabolism. With acetaldehyde generation, there is antioxidant utilization and depletion.

Glutathione (GSH) is a thiol antioxidant that is present in high intracellular concentrations as well as in many extracellular fluids. Glutathione is predominantly synthesized in the liver, and it is involved in many biologic pathways, including the synthesis of proteins and DNA, transport of amino acids, and enzyme activity. It is also critical for cellular protection, such as the detoxification of reactive oxygen species, the conjugation and excretion of toxic molecules, and the control of the inflammatory cytokine cascade.

With chronic alcohol abuse, hepatic GSH synthesis and the enzymes that use GSH for peroxide detoxification, GSH peroxidase and GSH *S*-transferase, are decreased (Lieber, 1993). Even without cirrhosis (Jewell et al., 1986), hepatocyte GSH depletion precedes development of the histologic changes of alcohol-mediated hepatotoxicity, which supports the suggestion of a central role of GSH availability in alcohol-mediated hepatoxicity (Lieber, 1993).

3. Alcohol alters glutathione homeostasis within the lung

In the fluid lining the alveolar space, GSH is essential for the detoxification of endogenous and exogenous oxidant radicals and protection of cells residing in the airway and alveolus from inhaled oxidants. Oxidant stress within the alveolar space such as occurs during pneumonia or ARDS necessitates protection by means of antioxidants such as GSH to circumvent severe lung injury. Under normal conditions, the alveolar type II cell maintains a GSH concentration of greater than 400 μM in the fluid lining the alveolar space, several hundred times higher than the concentration in plasma and one of the highest concentrations identified for any extracellular fluid (Morris & Bernard, 1994). In otherwise healthy alcohol-dependent individuals (age <45 years, normal nutritional indices, normal lung function, and no evidence of liver disease), the GSH concentrations in the alveolar epithelial lining fluid were decreased by approximately 80% compared with findings for subjects without a history of alcohol abuse (Moss et al., 2000). Such dramatic decreases in the reduced/oxidized glutathione ratio (GSH/GSSG) concentrations of the fluid lining the alveolar space support the idea of oxidative stress and would place the alveolar epithelium as well as the lung at greater risk of oxidative injury during sepsis or ventilatory oxygen therapy. This decrease in GSH/GSSG concentrations was associated with a twofold increase in protein concentrations in the fluid lining the alveolar space of individuals who chronically abused alcohol compared with findings for control subjects (Burnham et al., 2003). Thus, a history of alcohol abuse resulted in a subclinical defect in alveolar epithelial barrier permeability. Even with 1 week of abstinence, the concomitant GSH deficiency and elevated protein concentration in the lining fluid persisted (Burnham et al., 2003). These results supported the suggestion that aberrant GSH homeostasis in alcohol-dependent individuals may contribute to enhanced alveolar permeability, thereby increasing susceptibility to development of ARDS.

In a rat model of chronic ingestion of ethanol, pulmonary GSH homeostasis was similarly impaired, resulting in chronic oxidative stress in the epithelial lining fluid, as evidenced by decreased GSH and increased GSSG concentrations (Holguin et al., 1998). Gluthathione precursors during ethanol ingestion improved the GSH/GSSG concentrations, as well as improved net vectorial fluid transport and bidirectional protein permeability in vivo in the ethanol-fed rat model (Guidot et al., 2000). Thus, dysfunction of the alveolar epithelial barrier occurred as a result of ethanol-induced GSH depletion. An important role for GSH availability as a predisposing factor to the development of acute lung injury was also demonstrated by the ability of GSH precursors to decrease activation of matrix metalloproteinases and collagen degradation during acute endotoxemia in the ethanol-fed rat (Lois et al., 1999). Also, addition of GSH precursors to the ethanol diet attenuated endotoxemia-induced acute lung injury, as assessed by protein leak into the alveolar space, hypoxemia, and functional surfactant phospholipids (Brown et al., 2001a; Holguin et al., 1998; Velasquez et al., 2002). Thus, many of the intrinsic defects associated with chronic ingestion of ethanol are associated with decreased GSH availability, which supports the suggestion that GSH precursors could potentially decrease the risk of ARDS in those patients with a history of alcohol dependence.

4. Alcohol alters glutathione homeostasis and cellular functions of alveolar type II cells

Alveolar type II cells have diverse functions in maintaining the unique microenvironment of the alveoli. These functions include ion/water transport to maintain “dry” alveoli, surfactant secretion to reduce surface tension during exhalation, and GSH transport to maintain antioxidant homeostasis at the tissue/air interface. When the epithelium is denuded, as in acute lung injury, type II cell proliferation and differentiation are critical for repair of the alveoli and restoration of gas exchange. In alveolar type II cells, chronic ingestion of ethanol particularly decreased mitochondrial GSH in a manner dependent on the extent of ethanol ingestion (Brown et al., 2001b; Guidot & Brown, 2000). This depletion of mitochondrial GSH resulted in sensitization of the type II cell to inflammatory mediator–induced apoptosis and necrosis in vitro and in vivo (Brown et al., 2001b). When repair of the denuded epithelium by type II cells is delayed, injury is exacerbated through continued alveolar flooding, continued migration of activated neutrophils and fibroblasts into the airspace, increased oxidant injury, fibrotic lesions, and morbidity (Fig. 1).

Type II cell functions other than viability were also altered by chronic ingestion of ethanol. Monolayers derived from

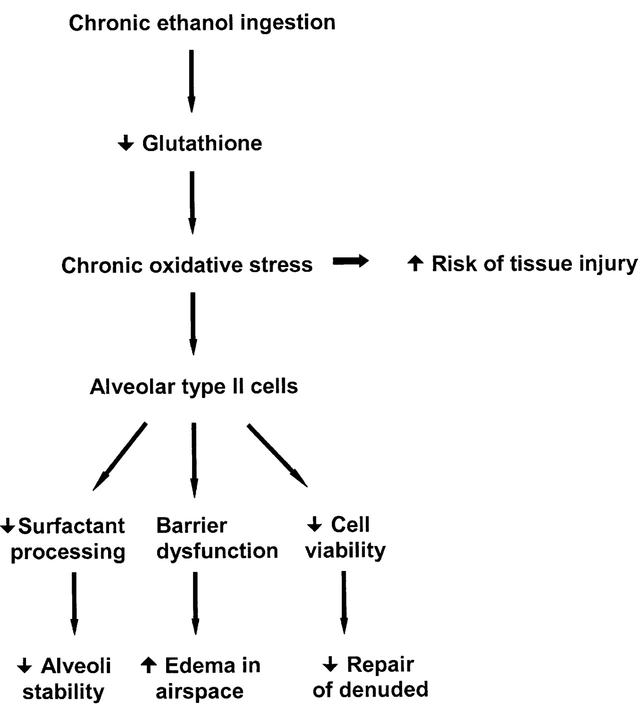

Fig. 1. Chronic ingestion of ethanol results in alveolar type II cell dysfunction. In a rat model, chronic ingestion of ethanol results in glutathione depletion, and the subsequent chronic oxidant stress increases the risk of tissue injury. As chronic oxidative stress is extended, alveolar type II cell functions become compromised. With decreased surfactant synthesis, secretion, and reutilization, the surface tension increases and the alveoli become unstable. With increased permeability of the alveolar epithelium, alveolar flooding also contributes to alveoli instability. With increased sensitivity to cytotoxin-induced apoptosis, the capacity of the type II cell to proliferate and repopulate the denuded alveolar epithelium becomes compromised, and reestablishment of gas-exchanging alveoli becomes similarly compromised. The capacity of glutathione precursors to maintain these cellular functions supports the suggestion that glutathione depletion and the subsequent chronic oxidant stress are the key events in ethanol-induced injury to type II cells.

type II cells isolated from ethanol-fed rats had greater macromolecule leak than observed for monolayers of cells from pair-fed control rats (Guidot et al., 2000). Results of these in vitro studies correlated with findings of in vivo studies and supported the suggestion that chronic ingestion of ethanol significantly altered epithelial barrier integrity and may explain the increased protein concentrations in the fluid lining the alveolar space of subjects with a history of alcohol abuse. Similarly, chronic ingestion of ethanol depressed type II cell synthesis and secretion of surfactant phospholipids (Guidot & Brown, 2000; Holguin et al., 1998). Chronic ingestion of ethanol was also associated with a switch from surfactant phospholipids in an active form to an inactive form during sepsis (Velasquez et al., 2002). This conversion from a functional to a nonfunctional phospholipid form is initiated by shear stress and oxidative damage, and the phospholipids must be recycled and resynthesized into functional forms by type II cells. This decreased capacity of type II cells to maintain surfactant in its functional form may not be physiologically relevant except during stress such as sepsis.

A key role for GSH in this sensitization process was demonstrated by the ability of GSH precursors to maintain the mitochondrial GSH pool during ethanol ingestion and attenuate the increased sensitivity to cytotoxin-induced apoptosis (Brown et al., 2001a, 2001b). Therefore, alveolar type II cells are similar to hepatocytes in that chronic ingestion of ethanol selectively decreases the mitochondrial GSH pool and that mitochondrial function and cell viability are modulated by ethanol-induced decreases in mitochondrial GSH availability. Similarly, preservation of mitochondrial GSH during ethanol ingestion was also associated with the capacity of the type II cell to maintain barrier integrity (Guidot et al., 2000) and surfactant in its functional form (Velasquez et al., 2002). In other clinically relevant studies, oral administration of GSH precursors after weaning from the ethanol restored type II cell functions such as surfactant synthesis and secretion (Guidot & Brown, 2000). Thus, alveolar type II cell dysfunction could be rescued with GSH precursors after cessation of ethanol intake.

5. Chronic alcohol abuse impairs alveolar macrophage function

The respiratory tract is continuously exposed to airborne microorganisms that can cause disease. Whereas most particles are removed by the upper airways, the inhaled particles that do reach the alveoli are cleared primarily by the resident alveolar macrophages through phagocytosis and the production of reactive oxygen species. Both chronic and acute alcohol use result in defects in innate and adaptive immunity, which increases susceptibility to infections (Nelson & Kolls, 2002). This impaired local antimicrobial defense is partially due to decreased ability of alveolar macrophages to phagocytize and clear encapsulated bacteria from the airways (Baughman & Roselle, 1987; Greenberg et al., 1999). Equally important is impaired release of proinflammatory cytokines, chemokines, and oxidant radicals required for microbial killing (Omidvari et al., 1998).

6. Alcohol abuse increases the incidence and severity of pulmonary infections

According to the National Vital Statistics Report for 2001, pneumonia is the seventh leading cause of death in the United States (Anderson & Smith, 2003). In subjects with alcohol dependence, the prototypic infection is pneumonia, with 25% to 50% of all patients admitted to the general hospital with pneumonia having a concomitant diagnosis of alcoholism (Torres et al., 1991). Among all elderly patients admitted to hospitals, 20% to 40% will have alcohol-related disorders, and such disorders are as common in the elderly as are those due to myocardial infarctions (Adams et al., 1993). Patients with a history of alcohol dependence are also at an increased risk for the development of both community-acquired and nosocomial pneumonia, with higher hospitalization-associated charges, higher intensive care unit use, and longer inpatient stays (Saitz et al., 1997), as well as

mortality (Fine et al., 1996). In the intensive care unit, the rate of morbidity and mortality in alcohol-dependent patients is two to four times greater than in subjects without a history of alcohol dependence (Spies et al., 1996).

Under stressed conditions such as hyperoxia, alveolar macrophages rely on the GSH pool of the alveolar epithelial lining fluid to provide GSH for uptake (Forman & Skelton, 1990) to protect themselves from oxidant injury (Loeb et al., 1988) and maintain membrane integrity during their respiratory burst (Pietarinen et al., 1995). A role for extracellular GSH in the maintenance and protection of alveolar macrophages is supported by the need for GSH availability during phagocytosis and the respiratory burst in neutrophils (Rajkovic & Williams, 1985) and monocytes (Seres et al., 2000). When GSH availability in the alveolar macrophage is limited, the generation of high-energy nucleotides is impaired (Pietarinen et al., 1995), and cellular functions, such as phagocytosis and microbial clearance, are compromised (Fig. 2). Thus, the decreased alveolar macrophage function associated with chronic alcohol abuse may be a result of alcohol-induced decreases in GSH availability in the alveolar epithelial lining fluid and the subsequent chronic oxidative stress in the macrophage. Consequently, GSH deficiency in the alveolar epithelial lining fluid could represent one factor predisposing alcohol-dependent subjects to the development of more severe and fatal pneumonias and represent a potential therapeutic target site.

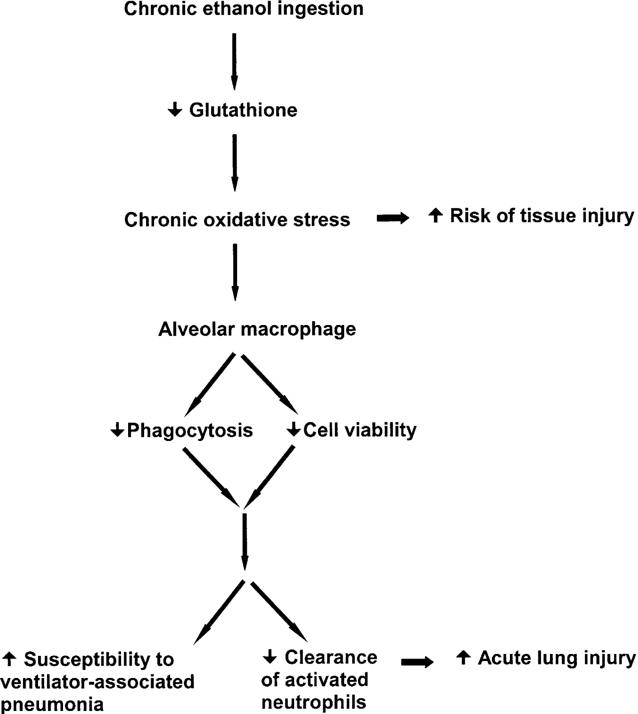

Fig. 2. Chronic ingestion of ethanol results in alveolar macrophage dysfunction. Findings of both clinical and animal studies have demonstrated that acute and chronic ingestion of ethanol results in compromised alveolar macrophage phagocytosis. In the rat model, chronic ingestion of ethanol decreased not only phagocytosis but also macrophage viability. With decreased numbers of viable macrophages to participate in phagocytosis, coupled with decreased phagocytosis by the viable macrophages, the capacity to clear microorganisms is severely compromised. This results in increased susceptibility to ventilator-associated pneumonia and subsequent increased acute lung injury. Compromised phagocytosis also means that activated neutrophils will not be cleared, which will potentiate acute lung injury. The capacity of glutathione precursors to maintain the fluid lining the alveolar space as well as macrophage viability and phagocytosis supports the suggestion that chronic oxidant stress is a key event in ethanol-induced injury to alveolar macrophages.

7. Ethanol-induced alveolar macrophage dysfunction depends on glutathione availability

With the use of the rat model of chronic ingestion of ethanol (Lieber–DeCarli diet; 36% of total calories), alveolar macrophages were isolated. After 24-h culture in Dulbecco's modified Eagle's medium (DMEM)-F12 medium, 56% ± 3% of the macrophages obtained from pair-fed control rats were apoptotic as assessed by caspase 3 activation (Fig. 3). In the group that ingested ethanol, macrophage apoptosis was increased to 85% ± 4%. Addition of the GSH precursor procysteine (L-2-oxothiaxolidine-4-carboxylate; final concentration, 0.35%) to the ethanol diet maintained apoptosis at control values despite the chronic presence of ethanol. When treated with fluorescein isothiocyanate–labeled, inactivated *Staphylococcus aureus*, only 41% ± 5% of the macrophages obtained from ethanol-fed rats were positive for phagocytosis compared with 81% ± 3% for control macrophages (Fig. 4). When the GSH precursor procysteine was

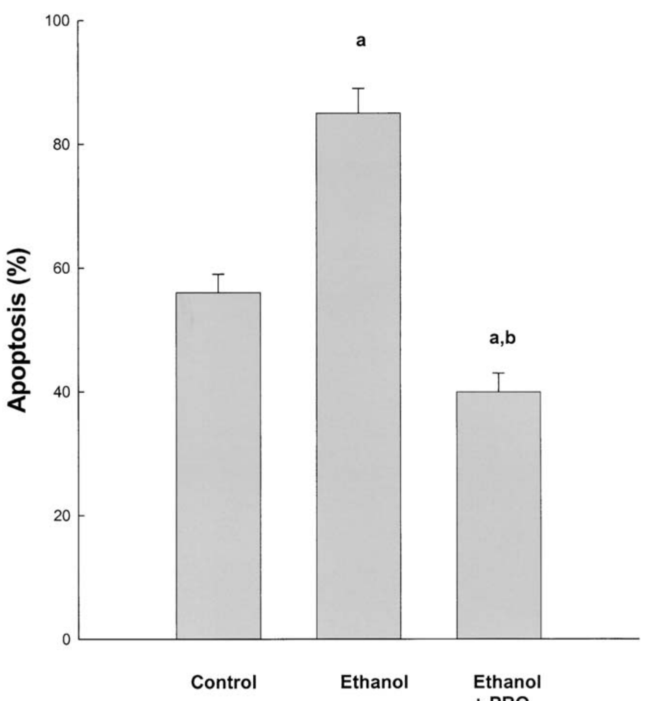

Fig. 3. Basal macrophage caspase-3 activation. After 24 h in culture, macrophages were treated with a cell-permeable fluorescent substrate for caspase-3 (PhiPhiLux-G1D2) for 1 h. Fluorescence was determined by quantitative digital analysis. Bar heights represent mean percentage of cells positive for caspase 3 activity ± standard error of the mean for five or more rats. PRO = Procysteine. a = $P < .05$ compared with values for control group; b = $P < .05$ compared with values for ethanol ingestion group.

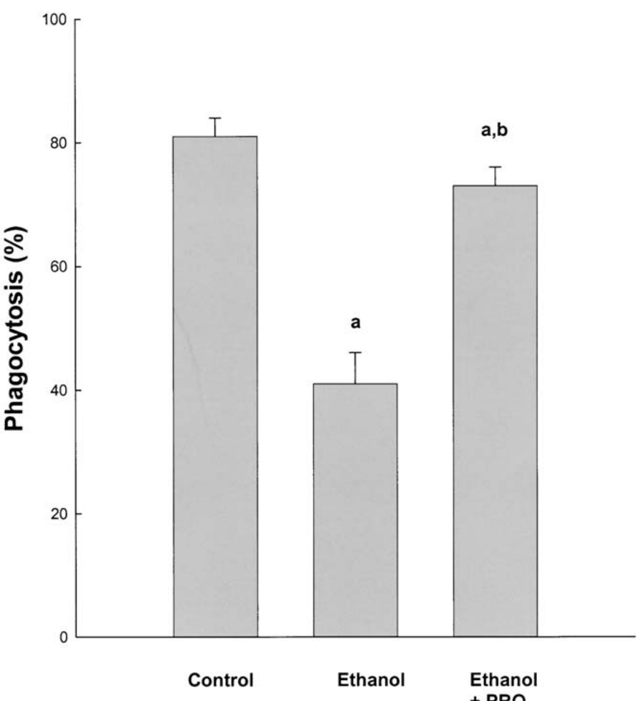

Fig. 4. Alveolar macrophage phagocytosis. Rats were fed the Lieber–DeCarli diet with or without ethanol for 6 weeks, and macrophages were subsequently obtained and isolated. Phagocytosis of fluorescein isothiocyanate–labeled inactivated *Staphylococcus aureus* (1:1 ratio; macrophage:bacteria) was determined after a 2-h incubation. Any macrophage containing green fluorescence was considered a cell with active phagocytosis (10 experimental fields per set). Bar heights represent the percentage of macrophages with active phagocytosis ± standard error of the mean for six rats. PRO = Procysteine. a = $P < .05$ compared with values for control group; b = $P < .05$ compared with values for ethanol ingestion group.

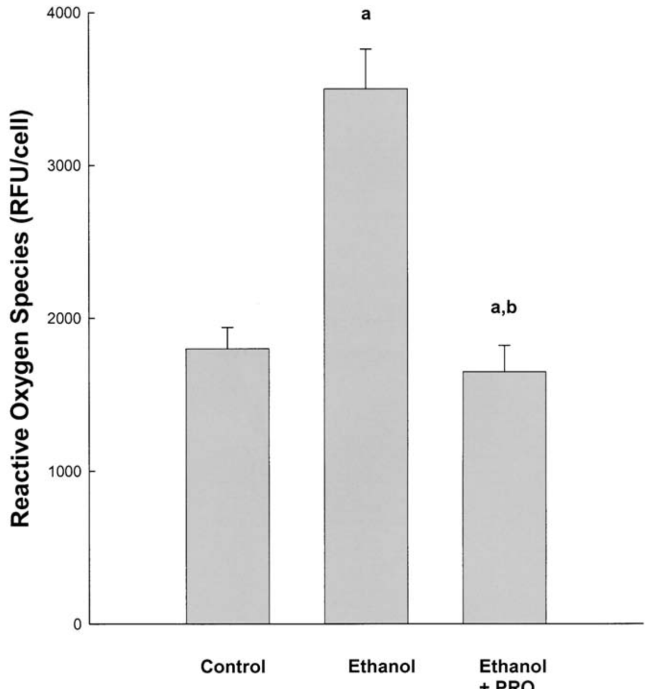

Fig. 5. Basal reactive oxygen species generation in macrophages. Rats were fed the Lieber–DeCarli diet with or without ethanol for 6 weeks, and macrophages were subsequently obtained and isolated. Isolates were cultured for 24 h and treated with MitoTracker Red CM-H2XRos (2 μM). Fluorescence was determined by quantitative digital analysis (Q imaging). Bar heights represent mean relative fluorescent units per cell (RFU/cell ± standard error of the mean) for seven rats. PRO = Procysteine. a = $P < .05$ compared with values for control group; b = $P < .05$ compared with values for ethanol ingestion group.

added to the ethanol diet, macrophage phagocytosis was maintained at control values. With the use of the redox-sensitive fluorophore MitoTracker Red CM-H2XRos, chronic ingestion of ethanol resulted in a twofold increase in mitochondrial reactive oxygen species (Fig. 5). In contrast, procysteine supplements normalized the mitochondrial oxidant stress (Fig. 5). The capacity of the GSH precursor procysteine to maintain macrophage cell viability and phagocytosis paralleled the capacity to maintain the GSH concentration in the alveolar epithelial lining fluid (Fig. 6). These results supported the suggestion that chronic ingestion of ethanol depletes the alveolar epithelial lining fluid of GSH and GSH availability to the alveolar macrophage. Glutathione depletion subsequently results in chronic oxidative stress in the

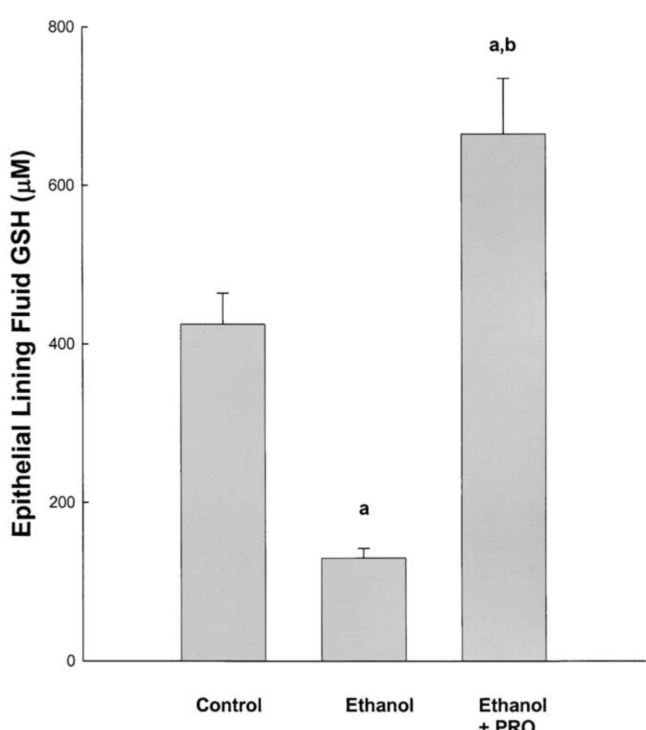

Fig. 6. Glutathione (GSH) in epithelial lining fluid. Rats were fed the Lieber–DeCarli diet with or without ethanol for 6 weeks. The epithelial lining fluid was collected by bronchoalveolar lavage, and GSH concentration was determined by high-performance liquid chromatographic analysis. Urea was used to correct for the dilution resulting from the lavage procedure. Bar heights represent mean values ± standard error of the mean for eight rats. PRO = Procysteine. a = $P < .05$ compared with values for control group; b = $P < .05$ compared with values for ethanol ingestion group.

alveolar macrophage and subsequent loss of cellular functions, such as viability and phagocytosis. Macrophage phagocytosis is important for clearance of not only microorganisms but also activated neutrophils that migrate into the airspace (Ware & Matthay, 2000). Thus, ethanol-induced decreases in alveolar macrophage phagocytosis may also exacerbate injury during ARDS because of the decreased capacity to clear activated neutrophils in the airspace.

Findings of our studies in ethanol-mediated lung dysfunction, coupled with the large body of work on ethanol-mediated liver dysfunction, support the idea that GSH deficiency may play a clinically significant role in the development of ARDS through impaired viability and function of alveolar type II cells and alveolar macrophages. These results may have important consequences for not only the understanding of how alcohol abuse predisposes to respiratory infections and ARDS but also the ability to design and test effective strategies to decrease lung injury in this particular patient population.

Acknowledgments

This work was supported by National Institute on Alcohol Abuse and Alcoholism grants R01 AA12197, R01 AAHL11660-03, and P50 AA135757 (Emory Alcohol and Lung Biology Center), by National Heart, Lung, and Blood Institute grant R01 HL67399-01, and by the Alcoholic Beverage Medical Research Foundation. Dr. Theresa W. Gauthier was supported by the National Institute on Alcohol Abuse and Alcoholism grant R01 AA13979 and the Alcoholic Beverage Medical Research Foundation.

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Biochim Biophys Acta. Author manuscript; available in PMC 2013 May 1.

Published in final edited form as:
Biochim Biophys Acta. 2012 May ; 1822(5): 625–630. doi:10.1016/j.bbadis.2011.10.003.

Elevation of Glutathione as a Therapeutic Strategy in Alzheimer Disease

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Abstract

Oxidative stress has been associated with the onset and progression of mild cognitive impairment (MCI) and Alzheimer disease (AD). AD and MCI brain and plasma display extensive oxidative stress as indexed by protein oxidation, lipid peroxidation, free radical formation, DNA oxidation, and decreased antioxidants. The most abundant endogenous antioxidant, glutathione, plays a significant role in combating oxidative stress. The ratio of oxidized to reduced glutathione is utilized as a measure of intensity of oxidative stress. Antioxidants have long been considered as an approach to slow down AD progression. In this review, we focus on the elevation on glutathione through N-acytl-cysteine (NAC) and γ-glutamylcysteine ethyl ester (GCEE) as a potential therapeutic approach for Alzheimer disease.

Keywords

Alzheimer disease (AD); Mild Cognitive Impairment (MCI); Amyloid β-peptide; Glutathione (GSH); N-acetylcysteine (NAC); γ-Glutamylcysteine ethyl ester

1. Introduction

Alzheimer disease (AD) is a largely sporadic, age-related neurodegenerative disorder pathologically characterized by the accumulation of abnormal protein deposits, including extracellular amyloid plaques, intracellular neurofibrillary tangles (NFT), and loss of synaptic connections within selective brain regions [1]. One of the main components of amyloid plaques is the amyloid β-peptide (Aβ), generated by the proteolytic cleavage of the amyloid precursor protein (APP) by β- and γ-secretases. Aβ exists in many forms, such as soluble, aggregated, oligomeric, protofibrillar, and fibrillar forms [2; 3], and a number of studies have demonstrate that the oligomeric form of Aβ is highly toxic and associated with oxidative stress [4; 5; 6].

Aβ(1–42)-associated free radicals can abstract an allylic hydrogen-atom from the unsaturated acyl chains of lipid molecules within the lipid bilayer, thereby leading to the

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initiation of lipid peroxidation processes [7; 8]. The process of lipid peroxidation generates highly reactive products, such as 4-hydroxy-2-nonenal (HNE) and acrolein, that can further react with proteins and enzymes, effectively amplifying the effects of Aβ(1–42)-induced free radical processes [8; 9].

Under normal conditions, oxidative stress and damage are combated by endogenous antioxidant compounds and enzymes within the cell. However, the brain is particularly vulnerable to oxidative damage due to the high levels of unsaturated lipids, oxygen, redox metal ions, and relatively poor antioxidant systems. As previously reported by our laboratory and others, both AD and mild cognitive impairment (MCI) brains have significantly decreased levels of antioxidant enzymes, making the brain more vulnerable to Aβ(1–42)-induced toxic effects [10]. Oxidative stress is also evident in AD brain by marked levels of protein, lipid, DNA, and RNA oxidation, neuronal dysfunction and death [11; 12]. Consequently, one way of boosting defenses in the brain is by assisting the antioxidant defense system particularly endogenous glutathione (GSH) and glutathione-related enzymes.

2. Glutathione (GSH)

The most prevalent antioxidant in the brain, glutathione, is found in millimolar concentrations in most cells. A thiol-containing molecule, GSH is capable of reacting with reactive oxygen species (ROS) and nucleophilic compounds such as HNE and acrolein, lipid peroxidation products that react with thiols in proteins. Reduced GSH reacts with free radicals to form oxidized glutathione (GSSG), which can be catalyzed by the enzyme glutathione peroxidase (GPx) or occur independently. GSSG is recycled back to two GSH molecules by GSH reductase (GR) utilizing the reducing equivalents of NADPH (Figure 1). Glutathione S-transferases (GST) are a group of enzymes that catalyze the reaction between GSH and nucleophilic compounds such as HNE and acrolein. The resulting glutathione-*S*-conjugates are effluxed from the cell by the multidrug resistance protein-1 (MRP-1) [13; 14]. In AD hippocampus, GST and MRP-1 are covalently bound by the lipid peroxidation product HNE, rendering them inactive [13; 15]. Thus, glutathione-*S*-conjugates are not readily formed or exported, possibly increasing HNE levels in the cell [16].

Post-translational modification of proteins by glutathionylation is reversible by glutaredoxin, a thiol transferase [17]. Redox sensitive proteins could be protected from oxidative stress by glutathionylation. Indeed, several proteins in AD inferior parietal lobule (IPL), including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-enolase, and p53, were identified as glutathionylated [18; 19]. GAPDH and α-enolase also have decreased activity in AD brain, and were previously reported to be oxidatively modified [20; 21; 22]. GAPDH and α-enolase are enzymes in the energy producing glycolytic pathway; oxidative modification and decreased activity may contribute to the alteration in glucose metabolism noted in AD [23]. Moreover, both enzymes have pro-survival functions in addition to roles in glycolysis. Oxidative dysfunction of these enzymes is deleterious to neurons [24; 25].

GSH levels are decreased in diseases with oxidative stress - including AD - and with age [26]. In AD peripheral lymphocytes, GSH levels are decreased and GSSG levels are increased, consistent with increased oxidative stress [27]. The ratio of GSSG to GSH is used as a marker of redox thiol status and oxidative stress. Indeed, with increasing progression of AD, GSSG and GSSG/GSH levels are found to increase. Lloret and colleagues found a linear correlation between increased GSSG levels and decreased cognitive status of AD patients using the Mini Mental Status examination (MMSE) [28].

Mild cognitive impairment (MCI) is often referred to as a transitional period between normal cognitive aging and mild dementia or probable AD. Many individuals with amnestic MCI develop AD, suggesting MCI is the earliest stage of AD [29; 30]. Several studies have

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demonstrated oxidative stress in MCI brain. In MCI hippocampus, a brain region highly affected in AD, superoxide dismutase (SOD) and GST activity is decreased, although protein expression was increased. The ratio of GSH/GSSG was decreased consistent with oxidative stress conditions. No significant difference in GPx or GR enzyme activity was noted [31]. Many enzymes are redox sensitive and easily oxidized, rendering them inactive even though protein expression level is high. Lipid and protein oxidative stress products were also elevated in the superior and middle temporal gyri of MCI brain [9; 32; 33]. Recent reports demonstrated peripheral serum levels of MCI and AD patients had significantly decreased GPx and SOD activity compared to age-matched controls, but did not differ from each other [34]. These researchers also showed increased levels of lipid peroxidation product malondialdehyde (MDA) compared to controls, with a significant increase from MCI to AD. Several previous studies also reported an increase in peripheral lipid and protein oxidation in AD and MCI patients [35; 36; 37; 38]. Decreased SOD and GPx antioxidant activity over time, leads to an accumulation of H_2O_2 and lipid peroxidation, possibly leading to the pathological alterations characteristic of AD. The above studies all concluded that oxidative stress conditions in early AD are already present in MCI, and the decreased antioxidant activity, particularly glutathione, may initiate the progression to AD [37]. A recent study demonstrated that MCI patients that progressed to AD displayed an increased distribution of the ApoE ε4 allele, a risk factor for sporadic AD, and displayed a significant decrease in the ratio of oxidized to reduced glutathione and vitamin E levels compared to MCI patients that remained at MCI status over time [39]. Oxidative stress indices increased over time in both MCI and MCI patients that progressed to AD, with no difference between the two groups. This study confirms that a decrease of antioxidants, particularly reduced glutathione, over time is a major contributor to the progression of MCI to AD. Increased peripheral oxidative stress indices, such as MDA, TBARS, or protein carbonyls, could potentially be used as a biomarker for diagnosing the onset of MCI, while a steady decrease of reduced glutathione may be a biomarker for progression to AD. An early diagnosis would allow early intervention utilizing appropriate antioxidants and other therapies.

Glutathione is comprised of the amino acids glutamate, cysteine, and glycine. Glutamate and glycine are found in millimolar concentrations, whereas free cysteine is limited with most non-protein cysteine being stored within GSH. Two enzymes are involved in synthesis of GSH: γ-glutamylcysteine ligase (also called γ-glutamylcysteine synthetase) and gluthathione synthase (Fig. 2). Because the physiological amount of brain-resident cysteine limits the formation of GSH, most current research has focused on increasing cysteine levels in the brain as an indirect way to increase the levels of GSH. In particular, N-acetyl-L-cysteine (NAC) is known to directly increase brain cysteine levels, allowing for increased biosynthesis of GSH in the brain and periphery [40]. Additionally, γ-Glutamylcysteine ethyl ester (GCEE) introduces the precursor for the last step in GSH synthesis, guiding cysteine directly towards GSH synthesis in the brain and periphery and avoiding the feedback inhibition of γ-glutaminecysteine ligase.

3. N-Acetyl-L-Cysteine (NAC)

NAC (Figure 3) has been shown to be an effective precursor to GSH production and crosses the blood brain barrier (BBB) [41; 42]. NAC provides cysteine, the rate limiting substrate in glutathione synthesis. NAC acts as an antioxidant by increasing GSH levels and by directly interacting with free radicals. Intraperitoneal (i.p.) injection of NAC to rodents increased GSH in brain and synaptosomes and offered protection against peroxynitrite, hydroxyl radicals, acrolein, and oxidative stress induced by 3-nitro-propionic acid [40; 43; 44; 45]. NAC also improved neuronal survival in the hippocampus after ischemic-reperfusion [46].

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Pretreatment with NAC in mice receiving intracerebroventricular (i.c.v.) injections of Aβ had improved learning and memory compared to vehicle-treated animals [47]. NAC also increased GSH levels, protected against Aβ-induced protein and lipid peroxidation, and decreased acetylcholine levels and choline acetyltransferase (ChAT) activity [47]. SAMP8 (Senescence Accelerated Mouse) mice overexpress APP resulting in elevated levels of Aβ in the brain. SAMP8 mice administered NAC had improved cognition in the T-maze footshock avoidance paradigm and the lever press appetitive task [42]. Recently, AD-relevant APP/PS-1 mice were orally administered NAC in drinking water for 5 months, before deposition of Aβ occurred in the brain. The antioxidant administered before Aβ induced oxidation occurred decreased protein and lipid oxidation, nitration of proteins, and increased glutathione peroxidase and reductase activity compared to age matched controls [48]. Such treatment clearly decreased oxidative stress *in vivo* in mice brain.

In AD brain and neuronal cultures exposed to Aβ, dying cells display characteristics of apoptosis [49]. A shift in redox status due to NAC changes the signaling pathways involved in the apoptosis signaling cascade [50; 51]. NAC protection against Aβ involves several signaling pathways involved in apoptosis including: activation of the Ras/ERK pathway, stimulating p35/Cdk5 activity, and reduced phosphorylation/deactivation of MLK3-MKK7-JNK3 signaling cascade [50; 51; 52]. NAC also acts as a transcription factor activating the RAS-ERK pathway, rescuing neurons from apoptotic cell death [52]. Therefore, in addition to antioxidant properties, and increasing GSH levels, NAC protects against Aβ toxicity through activation of anti-apoptotic signaling pathways.

NAC may play a role in amyloid precursor protein (APP) processing and Aβ formation. Aβ results from two proteases cleaving APP: β-secretase and γ-secretase. NAC down-regulates APP gene transcription, resulting in undetectable levels of APP mRNA in neuroblastoma cells. This activity may be related to decreased binding activity of transcription factor NF-κB, which is increased by oxidative stress and Aβ [53]. Another group demonstrated that NAC significantly decreased soluble levels of Aβ(1–40) and Aβ(1–42) and modestly reduced insoluble Aβ(1–40) in TgCRND8 transgenic mice that overexpress the APP gene [54]. Olivieri *et al.* (2001) showed NAC affected APP processing and increased levels of Aβ(1–40) by itself, suggesting the influence of β-secretase and γ-secretase cleavage of APP in neuroblastoma cells [55].

The role of Pin1 has been investigated in APP processing. Pin1 catalyzes the structural formation of phosphorylated Ser/Thr-Pro for dephosphorylation of APP. In AD models and AD brain, this motif remains phosphorylated resulting in increased Aβ production [56; 57]. Our laboratory demonstrated oxidation and decreased levels of Pin1 in MCI and AD brain [9; 58; 59]. Utilizing proteomics, we identify elevated levels of Pin1 in preclinical AD (PCAD) brain [60], consistent with the notion that PCAD subjects, characterized by normal scores on tests of cognition but having AD-like pathology in brain, respond to elevated Aβ by increasing expression of Pin1. Our laboratory also demonstrated, NAC treatment slightly elevated Pin1 in APP/PS1 mice over a 5 month period, possibly decreasing Aβ induced oxidative stress [48]. Results concerning NAC's effect on Aβ formation requires further study.

NAC capped quantum dots were utilized to block fibril formation of Aβ by blocking the active site of fibrils, nuclear fibrils, or protofibrils, possibly through hydrogen bonding [61]. Free NAC was unable to block Aβ fibril formation. Future antifibrilogenesis may involve quantum dot technology.

Neprilysin is a principal degrading peptidase of Aβ. In AD affected brain regions, neprilysin is oxidatively modified by HNE and has decreased levels and activity [62; 63].

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Preincubation with NAC was able to prevent HNE and Aβ-induced HNE addition to neprilysin and thus maintain neprilysin activity [64]. We suggest that NAC may be protective through modulation of Aβ formation and degradation via influence on APP transcription, processing, signaling pathways, and preventing oxidative stress.

Alzheimer disease presents a prominent neuroinflammation component. Astrocytes are the main supplier of GSH to microglia and neurons. During chronic inflammation and oxidative stress, astrocytes release toxic inflammatory mediators and free radicals, accelerating activation of microglia and neurodegeneration [65]. Recently, decreased intracellular glutathione was correlated with the release of pro-inflammatory factors TNF-α, IL-6, and nitrite ions and activation of the inflammatory pathways, P38 MAP-kinase, Jun-N-terminal kinase, NF-κB, in human microglia and astrocytes [66]. Extracellular GSH attenuated the BSO-reduction of intracellular levels of GSH in the above microglia and astrocytes, suggesting involvement of a membrane channel or transporter. NAC directly inhibited inflammatory factor NF-κB and blocked production of nitric oxide from inducible nitric oxide synthase and inflammatory cytokines [67]. Increasing glutathione levels with NAC in glial cells and astrocyes may confer protection against the neuro-inflammation component of AD.

Given the multi-faceted way NAC is capable of modulating AD (see Figure 4), patient supplementation with NAC has been addressed. In a previous study by Adair *et al.* (2001), late-stage AD patients supplemented with NAC over a six month period not only tolerated the treatment well, but also demonstrated significantly improved performance on the Letter Fluency Task and the Wechsler Memory Scale Immediate Number Recall [68], although, measures of oxidative stress in peripheral blood did not differ significantly [68]. More recently, AD patients were given a vitamin/nutriceutical supplement that included folate, vitamin B12, α-tocopherol, S-adenosyl methionine, NAC, and acetyl-L-carnitine [69]. All cognitive endpoints were found to favor the multi-supplement. Several antioxidant clinical trials had no effects or marginal positive effects on MCI progression to AD or AD [70; 71; 72]. They did not include a multi-supplement approach or a glutathione enhancing drug. The failures in many antioxidant clinical trials likely arise from starting the therapies in the late stages of AD, not monitoring drug levels and markers for the *in vivo* therapeutic effect of the drug, not utilizing a multi-antioxidant approach that covers both lipophilic and hydrophilic areas of the cell or recycle the oxidized antioxidants back to the reduced state, and not taking into account the basal redox status of the subjects in the trials [10; 73; 74]. These limitations must be taken into consideration when determining if an antioxidant therapy would be beneficial in slow or preventing the progression of MCI and AD.

4. γ-Glutamylcysteine Ethyl Ester (GCEE)

Another effective means for increasing biosynthesis of GSH is GCEE (Figure 5) [75]. γ-Glutamylcysteine formation is the rate-limiting step for the biosynthesis of GSH. Providing γ-glutamylcysteine bypasses the feed-back inhibition by GSH on γ-glutamylcysteine synthetase (GCS), the enzyme that catalyzes production of γ-glutamylcysteine. Attachment of an ethyl ester moiety allows γ-glutamylcysteine to more easily cross the cell membrane and blood-brain barrier (BBB). Protection against myocardial ischemic-reprefusion and mydocardial dysfunction in Se-deficient rats was afforded by GCEE [76; 77]. GCEE is able to increase brain and mitochondrial GSH levels and protect synaptosomes, neuronal cells, and mitochondria against peroxynitrite damage [78; 79]. Neuronal cells were also protected against Aβ(1–42)-induced protein oxidation, loss of mitochondrial function, and DNA fragmentaion by GCEE up-regulation of GSH. GCEE did not, however, disrupt Aβ(1–42) fibril formation [80; 81]. Aβ(1–42) is known to deplete GSH cellular levels which can lead to neuronal death. However, 24 hours after Aβ(1–42) addition, GSH and GCS levels

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increase intracellularly, offering protection against Aβ(1–42)-induced apoptosis in cortical neurons [82; 83; 84]. Recently, *i.p.* injections of GCEE protected against kainic acid induced ROS and downregulated c-fos mRNA in the cortex and hippocampus of rats [85]. GCEE may react directly with ROS due to the cysteine residue and/or increase GSH, which can protect against ROS and nucleophilic compounds.

5. Conclusions

Oxidative stress is a known characteristic of MCI and AD. Up regulation of endogenous antioxidants is vital in combating oxidative stress and thus helping to slow the advancement of MCI and Alzheimer disease. Glutathione is the most abundant and versatile endogenous antioxidant with many enzyme systems to enhance its function. NAC (FDA approved) and GCEE are known to increase glutathione in the brain and periphery and protect against ROS-producing substances *in vivo*. More research needs to be invested in GCEE, since it has no known harmful effects and by-passes the feedback inhibition cycle of glutathione. Increasing glutathione remains a promising therapeutic strategy to slow or prevent MCI and Alzheimer disease.

Acknowledgments

This work was supported in part by NIH grants to D.A.B. [AG-05119].

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Highlights

- Glutathione (GSH) is the most abundant endogenous antioxidant in brain
- Oxidative stress is a prominent feature of Alzheimer disease and MCI brain
- Elevation of GSH in vivo protects brain against AD-relevant Abeta(1–42)
- Elevation of GSH in brain induces several protective pathways

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Figure 1.
Recycling of glutathione (GSH) and oxidized glutathione (GSSG).

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Figure 2.
Synthesis of Glutathione

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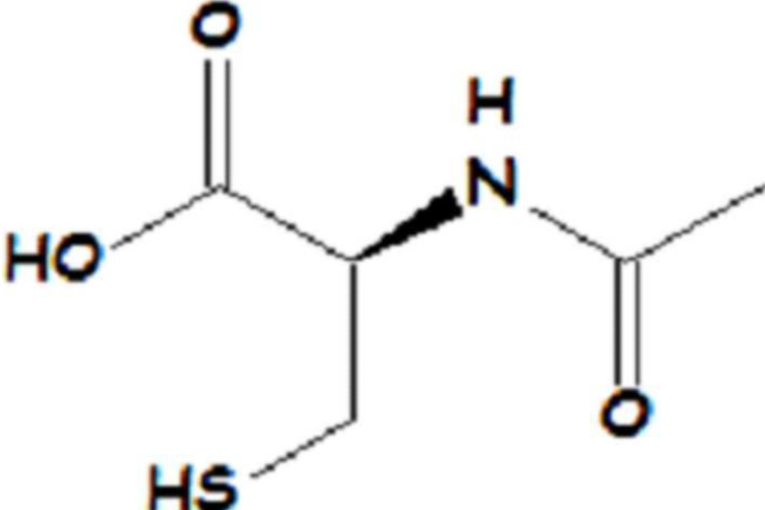

Figure 3.
Structure of N-acetyl-L-cysteine (NAC).

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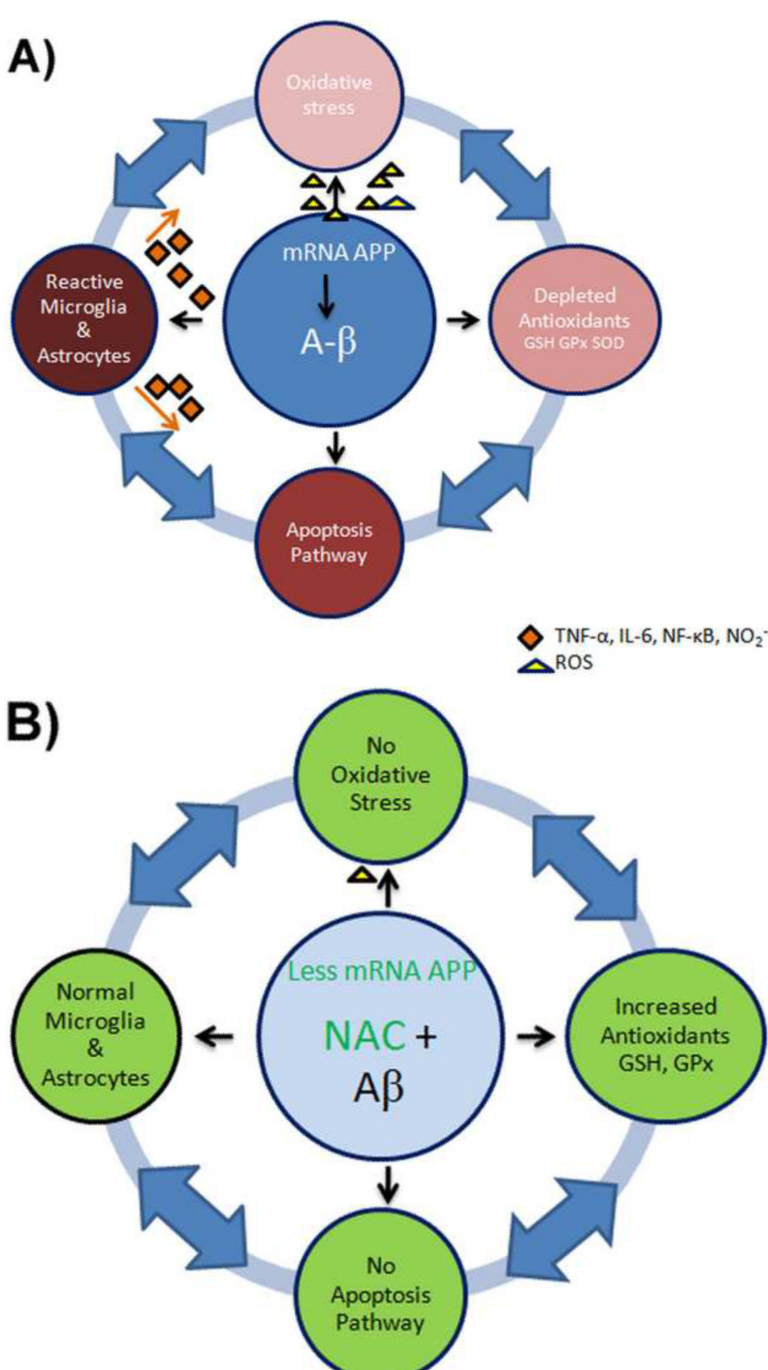

Figure 4.

A) Aβ produces ROS that eventually leads to the depletion of antioxidants and oxidative stress in Alzheimer disease. The increased oxidation induces apoptotic signaling pathways and inflammation in astrocytes. Astrocytes release toxic inflammatory mediators and free radicals, accelerating activation of microglia and neurodegeneration, connecting the cycle of negative events perpetuating AD.

B) NAC down-regulates APP gene transcription, resulting in undetectable levels of APP mRNA. Thus, since less Aβ is transcribed, fewer free radicals are produced by Aβ. NAC increases antioxidant levels of glutathione and reacts with ROS preventing oxidative stress.

The decreased oxidation in the cells induces anti-apoptotic signaling pathways and prevents inflammation of the cell. NAC directly inhibits inflammatory factor NF-κB and blocks production of nitric oxide from inducible nitric oxide synthase and inflammatory cytokines.

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Figure 5.
Structure of γ-glutymylcysteine ethyl ester (GCEE).

Biochimica et Biophysica Acta 1810 (2011) 1045–1051

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Review

Glutathione biochemistry in asthma☆

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ARTICLE INFO

Article history:
Received 17 September 2010
Accepted 21 January 2011
Available online 31 January 2011

Keywords:
Glutathione
Asthma
Oxidative stress
Redox balance
Glutathione peroxidase
Glutathione reductase
Glutathione-S-transferase
Glutaredoxin

ABSTRACT

Background: Oxidative stress in an important hallmark of asthma and much research has therefore focused on the predominant antioxidant in the lungs, namely the tripeptide glutathione.

Major conclusions: In lung samples of patients with asthma increased levels of glutathione are typically observed, which appear to relate to the level of pulmonary inflammation and are therefore regarded as an adaptive response to the associated oxidative stress. Also in blood samples increased total GSH levels have been reported, representing the systemic inflammatory component of the disease. In addition, a number of the antioxidant enzymes involved in the maintenance of the GSH/GSSG ratio as well as enzymes that utilize GSH have been found to be altered in the lungs and blood of asthmatics and will be summarized in this review. Very few studies have however linked enzymatic alterations to GSH levels or found that either of these correlated with disease severity. Some animal studies have started to investigate the pathophysiological role of GSH biochemistry in asthma and have yielded surprising results. Important in this respect is the physiological role of the GSH redox equilibrium in determining the outcome of immune responses, which could be deregulated in asthmatics and contribute to the disease.

Scope of review: Clinical data as well as animal and cell culture studies regarding these aspects of GSH in the context of asthma will be summarized and discussed in this review. This article is part of a Special Issue entitled: Biochemistry of Asthma.

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1. Introduction

The tripeptide glutathione (L-γ-glutamyl-L-cysteinyl-glycine) is the key antioxidant that protects the lungs from free radical mediated injury. Because a large portion of pulmonary oxidative stress occurs on the extracellular surface of the lung epithelium, glutathione is highly abundant in the epithelial lining fluid of the respiratory tract with concentration around 400 μM [1], thereby exceeding plasma levels by 100-fold. Intracellular levels range from 1 to 10 mM and under physiological conditions, 95% of glutathione is in the reduced form [2,3].

Glutathione first of all is a non-enzymatic defense molecule by acting as a sacrificial target for reactive oxygen species (ROS), whereby it becomes oxidized to the dimeric form GSSG. The enzymatic machinery involving glutathione is part of the enzymatic antioxidant defense mechanism. In the glutathione peroxidase (GPx)-catalyzed reduction of H_2O_2 and organic hydroperoxides GSH acts as a co-substrate and is itself oxidized to GSSG. GSH is recycled back from GSSG at the expense of NADPH by glutathione reductase (GR). Alternatively, GSSG can be excreted by cells. Extracellular glutathione is metabolized by the enzyme γ-glutamyl transferase (GGT), providing L-cysteine, which is taken up by the cells by the L-cys amino acid transport system for internal rebuilding of glutathione [4]. Upon oxidative stress, recycling is not sufficient to maintain the redox state and as an adaptive response de novo synthesis of GSH, in which L-cysteine is first coupled to glutamate by glutamate cysteinyl ligase (GCL) and next to glycine by GSH synthase can be initiated (Fig. 1).

In addition, GSH can form adducts with xenobiotics and proteins, reactions which are catalyzed by glutathione-S-transferase (GST) enzymes. The adduct formation with xenobiotics aids in their elimination, whereas the binding to protein thiols is a posttranslational modification that is termed *S*-glutathionylation and protects proteins against irreversible oxidations and can modulate their function. The biochemistry of nitric oxide interaction with glutathione, leading to the formation of *S*-nitrosoglutathione and protein *S*-nitrosylation, plays an important role in lung physiology and asthma as well and is addressed elsewhere in this issue.

Asthma is a chronic inflammatory disorder caused by a heterogeneous group of factors, including allergens and chemical irritant exposures in susceptible subjects. Moreover, there are different phenotypes with regard to the age of onset as well as inflammatory profile and response to therapy. Regardless, oxidative stress is considered a hallmark of all subtypes of asthma and is believed to play a pathophysiological role in the disease by causing damage to airway epithelial cells, leading to bronchial hyperresponsiveness and airway obstruction. Oxidative stress and altered redox chemistry that arise from irritant exposure and inflammation are not only linked to damage but importantly have now also been shown to modulate immune responses. Therefore, given the pivotal role of glutathione in

☆ This article is part of a Special Issue entitled: Biochemistry of Asthma.

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doi:10.1016/j.bbagen.2011.01.010

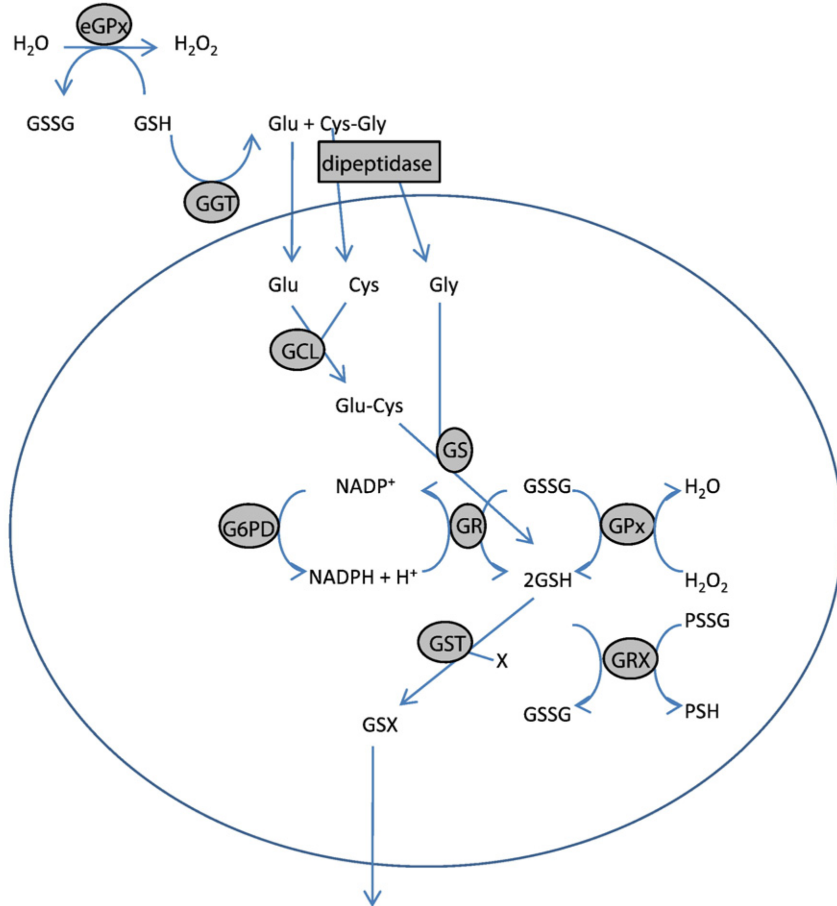

Fig. 1. Overview of glutathione-related biochemistry. eGPx: extracellular glutathione peroxidase, GGT: γ-glutamyltranspeptidase, GCL: glutamate cysteinyl ligase, GS: glutathione synthase, G6PD: glucose 6 phosphate dehydrogenase, GR: glutathione reductase, GPx: glutathione peroxidase, GST: glutathione-S-transferase, GRX: glutaredoxin, X: xenobiotic, PSH: reduced protein, PSSG: *S*-glutathionylated protein.

providing protection against oxidative insults, it is considered an important factor in all phenotypes of asthma. This review will however mostly focus on the current knowledge of glutathione biochemistry in "classical atopic" asthma in adults and children, as well as in the ovalbumine (OVA) model of allergic airway disease in mice.

2. GSH/GSSG

2.1. Local

A number of studies have reported on pulmonary glutathione in patients with asthma as compared to healthy controls as an assessment of oxidative stress. When comparing these studies it is important to keep in mind the type of sample used (lavage fluid vs. sputum vs. exhaled breath condensate), the type of glutathione measured (GSH vs. GSH/GSSG ratio vs. total glutathione), the type of patients (adult vs. children, chronic vs. mild, atopic vs. non-atopic), pharmacotherapy, and the assays and corrections used as these factors can help to explain the variations in results and concentration ranges observed.

In induced sputum supernatant total GSH in healthy controls is in the low micromolar range and is generally increased in asthmatics (Table 1) [5–8]. It has been found to positively correlate with sputum cell counts, which include both inflammatory cells as well as sloughed off epithelial cells [6,9]. Most studies did either not report on GSSG concentrations separately or specifically stated that the levels were below the detection limits of the assay. Reports on the ratio are therefore also lacking. One study however reported that both reduced and oxidized glutathione were increased in asthmatics but found the ratio not to be significantly altered in patients with asthma [9], whereas another found GSSG not to be significantly altered in asthmatics [6]. The elevated levels of GSSG can be considered a marker of oxidative stress, whereas increased total or reduced GSH levels can be regarded as an adaptive response to the increased oxidative burden in the lungs. In contrast to the correlation with inflammation, these studies have however yielded very few indications of a relation between total glutathione and disease severity. On a technical note, as demonstrated by Beier et al., the typical use of DTT should be avoided when measuring glutathione levels in induced sputum samples as concentrations above 0.001% raise the glutathione concentrations and can cause loss of GSSG. Moreover, she demonstrates that increased concentrations are observed when the induction time is extended [7], warranting standardization of induction and processing of sputa. Interestingly, in mild atopic asthmatics allergen challenge resulted in an increase in GSH levels and a reduction in GSSG levels after 24 h only in early responders, possibly providing protection against the late-phase allergic reactions. In dual early and late responders there was no effect of allergen challenge on glutathione, but the proportion of GSSG was found to correlate with sputum eosinophil numbers post challenge in all patients [10]. On the other hand, patients experiencing an acute exacerbation were found to have GSH levels lower then stable asthmatics, but still significantly higher than healthy controls and GSH levels during exacerbations were found to correlate with sputum neutrophil counts [5].

Bronchoalveolar lavage (BAL) is an invasive technique to obtain a diluted sample of epithelial lining fluid through bronchoscopy. Measurements of glutathione in BAL fluid (BALF) are therefore

Table 1
Glutathione in induced sputum samples. *Significant difference compared to control or baseline. EAR: early phase allergen responders, LAR: late phase allergen responders.

Result	Method	Reference
Total GSH	DTNB	Beier [6]
Control 4 μM		
Mild asthma 9.2 μM*		
Persistent asthma 8.7 μM*		
GSSG		
No significant difference		
Total GSH	DTNB	Beier [7]
Control 12.1 μM		
Asthma 20.2 μM*		
Total GSH	DTNB	Beier [10]
EAR		
Baseline 3.3 μM		
Allergen challenge 5.9 μM*		
LAR		
Baseline 3.5 μM		
Allergen challenge 2.8 μM		
Total GSH	DTNB	Dauletbaev [8]
Control 3.9 μM		
Asthma 6.4 μM		
Total GSH	DTNB	Deveci [5]
Control 0.34 μM		
Stable asthma 8.53 μM*		
Acute asthma 2.85 μM		
GSH	DTNB	Wood [9]
Control 1.2 μM		
Asthma 4.1 μM*		
GSSG		
Control 2.6 μM		
Asthma 5.9 μM*		
Total GSH		
Control 7 μM		
Asthma 15.3 μM*		

Table 2
Glutathione in BALF samples. Significant difference compared to control* or baseline**.

Result	Method	Reference
Total GSH	DTNB	Comhair [13]
Control 268 μM		
Asthma		
Baseline 282 μM		
Allergen challenge 165 μM**		
GSSG		
No significant difference		
Total GSH	Dansylchloride HPLC	Fitzpatrick [16] children
Control 260 μM		
Non-obstructed asthma 134 μM*		
Obstructed asthma 129 μM*		
GSH		
Control 231 μM		
Non-obstructed asthma 94 μM*		
Obstructed asthma 57 μM*		
GSSG		
Control 29 μM		
Non-obstructed asthma 39 μM*		
Obstructed asthma 72 μM*		
GSH	DTNB	Kelly [12]
Control 0.41 μM		
Asthma 0.3 μM		
GSSG		
Control 0.08 μM		
Asthma 0.28 μM*		
Bronchial total GSH	DTNB	Smith [11]
Control 13 μM		
Asthma 23.9 μM*		
Alveolar total GSH		
Control 23.3 μM		
Asthma 36.5 μM*		

more scares and are performed only in patients with mild disease. Concentrations of glutathione in BALF of healthy controls are higher than reported values in sputum and demonstrate a greater range as well. But as in sputum, BALF of patients with asthma is found to contain higher levels of both total and oxidized glutathione [11,12], albeit not consistently (Table 2). Moreover, higher concentrations can be found in alveolar washes when compared to bronchial washes and differences between asthmatics and healthy controls can be observed in both compartments even though the alveolar space is thought not to be affected by the disease [11]. Lastly, allergen challenge in atopic patients acutely decreased reduced glutathione levels without significantly affecting GSSG levels [13]. This study sampled BALF as early as 10 min post-challenge with could explain the discrepancy with the increased levels of GSH in sputum 24 h after challenge as was discussed above [10]. In order to investigate the role of alterations in glutathione redox chemistry in allergen-induced bronchoconstriction studies need to be designed that sample at multiple time points.

A more recently employed and non-invasive technique to obtain lung lining fluid samples is exhaled breath condensate (EBC). This involves cooling of normally exhaled air and thereby condensing molecules and volatile particles arising from the lungs. Using this technique, glutathione levels have been studied in children with asthma and in contrast with the adult specimen in the studies described above, reduced glutathione levels are found to be lower in children with asthma compared to age-matched healthy controls (Table 3) [14,15]. This contrasting observation between adults and children was also made in sputum samples of children with asthma, where in addition increases in GSSG as well as the ratio were observed [16]. The study by Corradi et al. furthermore demonstrates that glucocorticoid (GC) treatment of an acute exacerbation increased GSH levels [15]. Again though, no significant correlations with disease severity could be observed. Importantly, the levels of glutathione measured in EBC are in the low nM range and HPLC-based assays are therefore needed to detect GSH.

2.2. Systemic

Oxidative stress is not only present in the lungs of patients with asthma but can also be observed in the circulation. Alterations in glutathione levels and redox state have therefore been reported in blood of asthmatics as well. Importantly, in sputum samples most of the glutathione is found in the cell free supernatant, indicating its principal role in protecting the lung from extracellular, whereas most glutathione in the circulation is found inside the red blood cell in order to preserve its role in oxygen physiology. Furthermore, the percentage of oxidized glutathione is typically lower in blood when compared to lung lining fluid suggesting either more effective recycling to GSH or lower levels of free radicals in the circulation.

Most studies found total glutathione levels in whole blood or erythrocytes of adult patients with asthma to be increased compared to healthy controls (Table 4) [17–20], which is the same trend as observed in the lungs. As reported in lung specimen, in children in contrast lower levels of plasma GSH were observed in conjunction with decreased levels of the precursor amino acids L-glycine and

Table 3
Glutathione in EBC samples. Significant difference compared to control* or compared to untreated**.

Result	Method	Reference
GSH	Phtalaldehyde HPLC	Corradi [15] children
Control 14 nM		
Exacerbated asthma 5.96 nM*		
GC-treated exacerbation 8.44 nM**		
GSH	Phtalaldehyde HPLC	Dut [14] children
Control +/− 16 nM		
Mild asthma +/− 7 nM*		
Moderate asthma +/− 7.3 nM*		

Table 4
Glutathione in blood samples. Significant difference compared to control or untreated* and compared to untreated**. GC: glucocorticoid, Hb: hemoglobin.

Result	Method and sample	Reference
Total GSH	DTNB whole blood	Hasbal [23] children
Control 2.52 μmol/g Hb		
Asthma baseline 2.79 μmol/g Hb		
Asthma GC-treated 2.4 μmol/g Hb**		
GSSG	DTNB RBC	Mak [20]
Control 44.98 μM		
Asthma smoker 167.4 μM*		
Asthma non-smoker 174 μM*		
Total GSH	DTNB whole blood	Nadeem [17]
Control 0.61 mM		
Asthma 0.83 mM*		
Total GSH	DTNB whole blood	Nadeem [18]
Stable asthma 0.698 μM		
Acute asthma 0.705 μM		
Total GSH	DTNB RBC	Pennings [22]
Exacerbated asthma 7 μmol/g Hb		
GC-treated exacerbation 6.4 μmol/g Hb*		
GSH	DTNB plasma	Sackesen [21] children
Control +/− 5.92 μM		
Asthma +/− 4.65 μM*		
GSH	DTNB RBC	Vural [19]
Control 0.49 μmol/g Hb		
Asthma 0.59 μmol/g Hb*		
GSH	DTNB whole blood	Wood [9]
Control 836 μM		
Asthma 832 μM		
GSSG		
Control 24.8 μM		
Asthma 5.7 μM		
Total GSH		
Control 908 μM		
Asthma 834 μM		

L-glutamine [21]. The variability in glutathione values reported for blood is much greater than for lung samples, which could partially be explained by the even greater need for avoiding post sampling artificial loss of GSH due to the complex redox chemistry of blood. Moreover, the different ways of expressing the concentrations and correction factors used make comparisons between studies more difficult. The single study reporting GSSG levels found no alterations in erythrocytes of patients with asthma but also failed to observe increased GSH levels [9]. In patients experiencing an acute disease exacerbation blood glutathione levels were not different when compared to stable disease state [18]. In two studies inhaled GCs were found to attenuate total glutathione levels which was related to a reduction in circulating numbers of eosinophils [22,23]. The reduction in GSH levels could either be the result of the anti-inflammatory effects of GCs or a direct inhibitory effect of GCs on de novo synthesis of glutathione [24]. These finding are however in contrast to the observed induction of total GSH levels by GCs after an exacerbation in the lungs [15], as well as increased hepatic GSH synthesis after systemic delivery of GCs [25], although inhaled GCs might not have systemic effects.

3. Enzymes

3.1. Nrf2

A number of GSH regulatory enzymes are induced by the redox-sensitive transcription factor nuclear erythroid 2 p45-related factor 2 (Nrf2). Under the influence of oxidative stress Nrf2 will detach from its cytosolic inhibitor Keap1, translocate to the nucleus and bind antioxidant response elements (ARE) which are located in the promoter region of many antioxidant genes, including GCL catalytic subunit (GCLC), GCL modifier subunit (GCLM) and several GSTs [26]. Nrf2 levels or its activation have not been investigated thus far in lungs of patients with asthma and data on the individual antioxidant genes under Nrf2 control, some of which will be discussed below, do not indicate an unambiguous role for the transcription factor.

3.2. GSH synthesis

Although in COPD the enzymes involved in GSH synthesis have been investigated [27], they have not received much attention in asthma. No data are currently available on the expression of these enzymes in asthmatics and only one genetic study has been performed so far which observed a protective association of a genotype in GCLM for allergic asthma, but at the same time was found to be associated with an increased risk for non-allergic asthma [28]. One of the allelic variants in this genotype is known to suppress the oxidant-induced upregulation of GCLM gene expression and is associated with lower plasma GSH levels [29]. But the study did however not investigate enzyme expression or GSH levels. Clearly, a more integral approach in genetic studies is needed to help understand the involvement of the enzymes and genetic variations in asthma.

3.3. GPx

The GPx contain a selenocysteine within their active site and use low molecular weight thiols such as GSH to reduce H_2O_2 and lipid hydroperoxides. Four GPx enzymes have been described, encoded by four different genes. GPx1 is the classical ubiquitous cellular GPx, GPx2 is the gastrointestinal specific form, GPx3 or eGPx is a secreted variant, and GPx4 is the membranous phospholipid GPx [30–33]. Given again the importance of the lung lining fluid in protecting the lung tissue and the high levels of GSH present in it, eGPx can be considered the most important GPx in lungs. However, it is estimated that only approximately 50% of pulmonary GPx activity can be attributed to eGPx [32].

Comhair et al. demonstrated increased protein levels and activity of eGPx in BALF from patients with asthma compared to healthy controls, in conjunction with higher mRNA expression in bronchial epithelial cells obtained from patients compared to controls. In vitro experiments furthermore demonstrated that ROS and associated changes in the glutathione redox state are drivers of this enhanced expression of eGPx [34]. Intracellular forms of GPx and total GPx activity have not found to be altered in lung samples of patients with asthma [13,35], despite the clear presence of intracellular oxidative stress. Data on GPx in blood of asthmatics are conflicting; some studies have found no statistical difference in blood of asthmatics compared to controls [36–38], whereas others found attenuated GPx activity in serum [39] or platelets [40–42]. Also in children Bibi [43] and Sackesen [21] reported decreased GPx levels in RBC and plasma of asthmatics, respectively, the latter together with decreased plasma GSH. During exacerbations the data are more consistently pointing towards decreased GPx in both adults [44] and children [43].

3.4. GR

There is only one GR in mammalian cells and it requires NADPH for the reduction of GSSG to GSH. Studies measuring GR in asthma are more scant. In stable adults GR activity in BALF cells was not different between asthmatics and controls [11] and also in children no differences in BALF GR were observed [16]. Systemically, specifically in male subjects with asthma it was found that erythrocyte GR activity was associated with disease as in subjects with current symptoms of bronchial hyperreactivity a 5.7% higher enzyme activity was observed compared to healthy controls. On the other hand, this study found that in general GR activity was higher in women than men [45]. Furthermore, during an asthma exacerbation erythrocyte GR was found to decrease [36].

3.5. GSTs

Members of the glutathione-*S*-transferase (GST) superfamily have been under investigation in asthma since they are critical in the protection of cells against toxic products of ROS-mediated reactions. GST-P1 is the predominant cytosolic GST enzyme in human lung and it was shown that polymorphisms are strongly associated with asthma [46]. GST-M, GST-A and GST-T are also expressed in lung epithelium but to a lesser extent. GST-M1 and T1 single-nucleotide polymorphisms have also been associated with asthma (for review, see ref. [47]). These findings were confirmed by a recent meta-analysis [48] which also highlighted the complex gene–gene and gene–environment interactions that should be taken into account when studying these enzymes. However, not all polymorphisms associated with asthma have been shown to functionally affect GST activity and GST activity in BALF of children with severe asthma was not found to be altered compared to healthy controls [16]. There is thus clear genetic evidence for the association of GSTs with asthma but their mechanistic involvement remains to be elucidated.

3.6. GRX and PSSG

As part of the antioxidant properties of glutathione, it can be conjugated to protein thiols through its proper cysteine thiol group, providing direct protection against irreversible oxidations. The formation of these protein-glutathione mixed disulfides is a post-translational modification termed *S*-glutathionylation that has been demonstrated to occur in a number of proteins and affect their activity [49]. *S*-glutathionylation occurs under physiological conditions and is enhanced after oxidative stress. GST-P1 has been shown to be involved in the conjugation of glutathione to proteins [50], whereas glutaredoxins (GRX) can reverse the modification [51]. GRX and protein *S*-glutathionylation have not been investigated in patients with asthma. We have demonstrated in the OVA mouse model of allergic airway disease that the expression and activity of GRX1 are increased, predominantly in bronchial epithelium [52]. It remains to be further investigated whether this is associated with enhanced release of glutathione from proteins to increase free GSH but also potentially leaving them more vulnerable to oxidative damage. On the other hand, altered activity of target proteins through alterations in their *S*-glutathionylation status could play a role in the disease as well.

4. Animal studies investigating the pathophysiological role of altered GSH in asthma

4.1. Nrf2

The possible importance of Nrf2-driven antioxidant gene expression in asthma has been demonstrated by enhanced susceptibility of Nrf2 k.o. mice to the OVA model of allergic airway disease [53]. This was evidenced by more pronounced mucus cell hyperplasia and infiltration of eosinophils into the lungs of k.o. animals compared to wild-type littermates. The genetic disruption of the Nrf2 pathway also resulted in a lowered antioxidant status of the lungs caused by lower basal expression, as well as marked attenuation of the transcriptional induction of multiple antioxidant genes, including GCLM, GCLC, GST-A3, GST-P2 and GR. Moreover, in separate studies Nrf2 has been shown to play a central role in controlling the redox equilibrium of dendritic cells, thereby influencing antigen presentation activity [54]. Together these studies warrant investigation of Nrf2 activity in patients with asthma which could next provide a new therapeutic target.

4.2. GPx

In contrast to clinical asthma samples, GPx1 is increased in the OVA model. Furthermore, in the OVA model of allergic airway disease GPx1 k.o. mice were found to be protected, with attenuation of eosinophil infiltration, goblet cell hyperplasia, collagen deposition and airway hyperresponsiveness (AHR). In vitro studies demonstrated that CD4+ T helper (Th) cells derived from these GPx1 k.o. mice produced more ROS and interleukin (IL)-2 after stimulation as well as enhanced proliferation. In differentiation assays moreover, they were found to be biased to Th1 and Th17. These data indicate that the modulating effects of GPx1 on immune cell proliferation and differentiation predominate over the protection against oxidative damage [55].

GPx2 expression has also been found to be elevated in airway epithelium in the OVA model of allergic airway disease, but in contrast to the GPx1 k.o. GPx2-deficient mice showed enhanced airway inflammation and reactivity in this model [56]. Discrete localization and functions of different GPx forms could explain protective vs. enhancing role. There are indications that GPx2 might function less as a GSH peroxidase and could be more involved in inhibiting prostaglandin synthesis and enhance other anti-inflammatory mechanisms [56]. Remarkably, eGPx genetically manipulated mice have not been tested in asthma models of asthma.

4.3. GGT

Mice deficient in GGT are unable to metabolize extracellular GSH and develop intracellular GSH deficiency, but have augmented levels of GSH in the ELF. When an IL-13 intratracheal instillation model of asthma was applied to these mice they were found to be protected against AHR, mucus hyperplasia and oxidative stress, without attenuation of airway inflammation, but in the presence of a 10-fold increase in ELF GSH concentration. Similar data were obtained using a pharmacological inhibitor of GGT, indicating that lung lining GSH provides protection against AHR and mucus hypersecretion [57], again highlighting the extracellular importance of GSH in the lungs.

4.4. Immune function modulation

Not only does GSH play an important role in the protection against oxidative stress associated with asthma, but a number of studies have recently demonstrated that the GSH redox state of immune cells is a key factor in the balance of Th1/Th2 immune responses and antigen presentation. For instance, lowering intracellular GSH levels was shown to negatively impact antigen processing in antigen-presenting cells, especially of antigens containing disulfide bonds [58] and also Nrf2 has been shown to influence antigen presentation activity by controlling the redox equilibrium of dendritic cells [54]. With respect to the Th1/Th2 imbalance as mentioned above, T cells derived from GPx1 k.o. mice are biased towards Th1 and Th17 and could explain the protective phenotype of these mice to asthma [55]. In antigen-presenting cells, it was furthermore demonstrated that a more reducing intracellular content with respect to glutathione favors Th1 development through the production of IL-12 [59]. Conversely, exposure of macrophages to the Th1 cytokine IFNγ increased the GSH/GSSG ratio whereas exposure to the Th2 cytokine IL-4 decreased this ratio [60]. Administration of γ-glutamylcysteinylethyl ester (GCE) as a precursor for GSH to monocytes in vitro as well as in the OVA model furthermore increased IL-12 production and suppressed typical Th2 cytokines IL-4 and IL-5 and eosinophil chemotaxis [61].

In general these data emphasize that GSH and associated antioxidant enzymes do not only play an important role in asthma through protect against oxidative stress and damage, but that GSH/GSSG imbalances and deregulated expression of associated enzymes can in addition disturb discrete receptor-induced ROS-mediated signaling events that are involved in pathways important in immune responses and asthma.

5. Treatments targeting glutathione

5.1. Boosting GSH

Nebulization of GSH itself has been tested clinically, but it was found that the administered GSH had a poor half-life and resulted in limited cellular uptake. Surprisingly moreover, it was found that in patients with mild asthma it caused airway hyperreactivity, cough and breathlessness, which was hypothesized by the authors to result from sulfite formation [62]. To circumvent some of these issues, *N*-acetylcysteine (NAC) has been tried as a source of L-cysteine in order to increase pulmonary GSH levels with variable rates of success in animal models of asthma. This could be due to the fact that NAC has the disadvantage of being acidic in solution and high doses are still needed to achieve pulmonary levels that have been shown to protect against ROS. Therefore the molecule *N*-acetylcysteine amide (AD4) that is not negatively charged at the carbonyl group has been developed. Moreover, AD4 can cross membranes better and has shown protective effects on airway inflammation and hyperresponsiveness in mouse models of allergic airway disease [63]. Similar positive effects have been achieved in mice with glutathione ethyl ester which can be taken up by cells as well [61,64]. Clinical trials in patients with asthma are yet to be performed with these and other compounds to test their therapeutic efficacy.

5.2. Selenium and enzyme mimetics

A number of studies have found plasma levels of the trace element selenium to be significantly lower in patients with asthma [65–67]. Selenium deficiency is associated with lower levels of GPx, and it has furthermore been shown that selenium can interact with adhesion molecule expression and have anti-inflammatory effects in patients with asthma [68]. In vitro and in an animal model of asthma, selenite was also shown to improve GPx activity and inhibit the activation of the transcription factor NF-κB and consequent inflammation [69]. The only randomized control trial to date on selenium supplementation in asthmatics, which was conducted in patients with chronic non-atopic asthma, showed increased serum selenium levels and increased platelet GPx in conjunction with clinical improvement. However, no measurable changes in objective parameters of lung function and airway hyperresponsiveness were observed [70]. Proper and larger studies clearly need to be conducted before selenium supplementation can be recommended for patients with asthma.

Some of the GSH-related enzymes could also be restored by administration of mimetic compounds. For instance, the selenium based organic complex Ebselen is a GPx mimetic that has been shown to be a very powerful antioxidant ex vitro. Studies in asthma have however not been reported.

6. Conclusion

In recent years, the research into glutathione in asthma has moved away from being merely a marker of oxidative stress and by proxy inflammation to trying to understand the full scale of glutathione related biochemical events and their relation to the disease. Clearly however in order to fully reach this goal, more comprehensive clinical studies will need to be conducted which no doubt will be complicated by the recent push to sub-phenotype this condition. In addition, the use of various transgenic mouse strains either deficient or over-expressing glutathione associated antioxidant enzymes can further delineate their pathophysiological role. Here it might be necessary to further tease apart their contribution in the various cell types involved in asthma, such as for instance airway epithelium, smooth muscle or immune cells. The end result hopefully will be better therapeutic strategies aimed at restoring physiological glutathione levels.

Acknowledgements

NLR is supported by a VENI grant from the Netherlands Organization for Scientific Research NWO/ZonMW (016.086.090) and the Netherlands Asthma Foundation (3.2.09.049).

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Glutathione and Adaptive Immune Responses against *Mycobacterium tuberculosis* Infection in Healthy and HIV Infected Individuals

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Abstract

Glutathione (GSH), a tripeptide antioxidant, is essential for cellular homeostasis and plays a vital role in diverse cellular functions. Individuals who are infected with Human immuno deficiency virus (HIV) are known to be susceptible to *Mycobacterium tuberculosis* (*M. tb)* infection. We report that by enhancing GSH levels, T-cells are able to inhibit the growth of *M. tb* inside macrophages. In addition, those GSH-replenished T cell cultures produced increased levels of Interleukin-2 (IL-2), Interleukin-12 (IL-12), and Interferon-gamma (IFN-γ), cytokines, which are known to be crucial for the control of intracellular pathogens. Our study reveals that T lymphocytes that are derived from HIV infected individuals are deficient in GSH, and that this deficiency correlates with decreased levels of Th1 cytokines and enhanced growth of *M. tb* inside human macrophages.

Citation: Guerra C, Morris D, Sipin A, Kung S, Franklin M, et al. (2011) Glutathione and Adaptive Immune Responses against *Mycobacterium tuberculosis* Infection in Healthy and HIV Infected Individuals. PLoS ONE 6(12): e28378. doi:10.1371/journal.pone.0028378

Editor: Shabaana Khader, University of Pittsburgh, United States of America

Received June 28, 2011; **Accepted** November 7, 2011; **Published** December 2, 2011

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Funding: The study was supported by start-up funds from Western University of Health Sciences and Potts Memorial Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Although, one of the co-authors of this manuscript is a Managing Partner for Your Energy Systems (a small biotech company), this does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

HIV is the cause of acquired immunodeficiency syndrome (AIDS). Both HIV-1 and HIV-2 cause AIDS, but HIV-1 is found worldwide, whereas HIV-2 is found primarily in West Africa [1,2,3]. Blood monocytes, CD4 T lymphocytes and resident macrophages are important *in vivo* cell targets for HIV infection and their role in AIDS pathogenesis are well documented [1,2,3-5]. HIV preferentially infects and kills CD4+ T lymphocytes, resulting in the loss of cell-mediated immunity and a high probability that the host will develop opportunistic infections including tuberculosis (TB).

M. tb is endemic in every part of the world. This organism accounts for nearly 1.7 million deaths annually, making it the leading bacterial cause of death worldwide [1]. Once thought to be controlled, the incidence of TB is rising in many areas caused in part by the emergence of drug-resistant strains and the HIV epidemic. Furthermore, nearly one-third of the world is latently infected with *M. tb*, making eradication of the organism difficult [1].

Although TB remains the leading cause of morbidity and mortality due to any one infectious agent worldwide [6] our understanding of its immunopathogenesis is still incomplete.

M. tb infection begins as primary TB with the deposition of bacilli in the alveoli, which are phagocytosed by alveolar macrophages [7]. Active TB is characterized by a profound and prolonged suppression of *M. tb*-specific T cell responses, as evidenced by decreased production of the cytokines IL-2 and IFN-γ [8-11,12]. Overproduction of immunosuppressive cytokines [IL-10 and transforming growth factor (TGF)- β] by mononuclear phagocytes has been implicated in decreased T cell function during TB [8,13-15].

GSH plays a major role in the maintenance of the cellular redox state. GSH scavenges peroxide species which can be harmful to the cells. GSH also plays a role in the normal function of the immune system [3]. Of particular interest, is the ability of GSH to enhance the activation of lymphocytes which play a major role in the pathology of HIV infection. GSH has been demonstrated to be depleted in HIV positive individuals [3]. Low GSH levels have been shown to result in the activation of nuclear factor κB (NFκB), which is necessary for active transcription of the HIV provirus [4,5,16]. Depleted GSH levels have also been shown to play a role in the apoptosis of $CD4^+$ T cells. As depletion of these T cells is the major pathology of the HIV virus, replenishment of GSH represents an exciting prospect for treatment as a supplement to highly active antiretroviral therapy (HAART) [17].

Our earlier studies indicated that GSH facilitates the control of growth of intracellular *M. tb* in both murine and human macrophages and has direct antimycobacterial activity [18-20]. Furthermore, our recent studies indicate that GSH in combination with IL-2 and IL-12 augments natural killer (NK) cell functions to control *M. tb* infection [21].

The ability of GSH to augment the activity of NK cells in the control of *M. tb* infection as indicated by our previous studies [18-20] led us to hypothesize that GSH will also augment the activity of T-cells resulting in the control of *M. tb* infection inside macrophages. Further, we believe that the decreased intracellular growth of *M. tb* will be accompanied by increased production of Th1 cytokines that are essential for the control of intracellular pathogens. We tested our hypothesis by performing extensive *in vitro* studies using monocytes and T cells that are isolated from blood of healthy subjects and individuals with HIV infection. Our results indicate that individuals with HIV infection have significantly lower levels of GSH in their T cells in comparison to healthy subjects. The decrease in the GSH levels correlated with increased growth of *M. tb* inside human macrophages and reduced levels of Th1 cytokines in both plasma and cell free supernatants derived from monocyte-T cell co-cultures. Our results signify the importance of GSH in augmenting the functions of T lymphocytes to limit the growth of *M. tb* inside monocytes and macrophages.

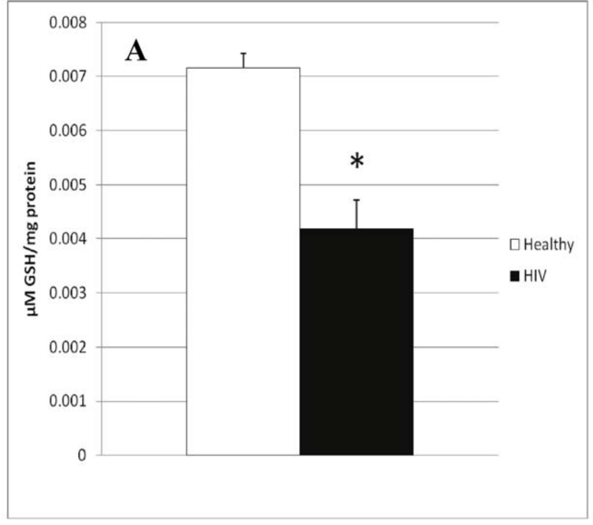
Results

Assay of GSH levels in T cells from healthy and HIV-infected subjects

GSH levels were determined in freshly isolated T cells from thirteen healthy subjects and thirteen individuals with HIV infection. We observed that GSH concentrations were significantly lower in T cells isolated from individuals with HIV infection compared to T cells from healthy subjects (Figure 1a). The decreased levels of intracellular GSH in T cells from HIV-infected individuals correlated with increased levels of TNF-α and free radicals. Decreased GSH levels in T cells of HIV positive individuals are likely to impair the adaptive immune responses against *M. tb* infection. NAC-treatment of T cells from individuals with HIV infection resulted in significant increase in the levels of GSH (Figure 1b). Treatment of T cells from HIV positive individuals with BSO resulted in significant decrease in the levels of GSH (Figure 1b).

Assay of free radicals and TNF-α in plasma samples

MDA is a naturally formed byproduct of the interaction between free radicals and lipid molecules which occurs in the body. The interaction between free radicals and lipid molecules produces lipid peroxides which are unstable and decay to MDA and other products. Concentrations of MDA are indicative of the level of cellular damage due to free radicals and therefore are also indicative of levels of free radicals. Measurement of MDA concentrations in the plasma of thirteen healthy and thirteen HIV infected individuals revealed a significant increase in the amounts of MDA present in the plasma of HIV infected individuals over those found in healthy individuals (Figure 2a). We observed an eight-fold increase in the levels of TNF-α in the plasma samples of HIV infected individuals compared to healthy subjects (Figure 2b). The difference in plasma levels of TNF-α did not reach statistical significance due to a high variance in the TNF-α concentrations measured in HIV infected individuals.



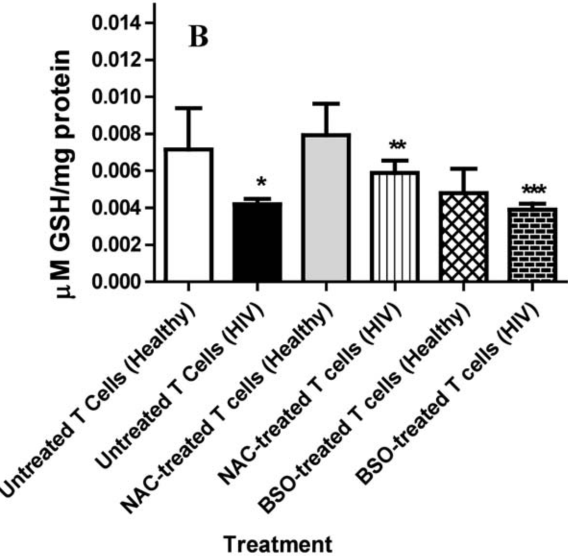


Figure 1. Assay of GSH levels in T cells isolated from healthy and HIV positive individuals. Intracellular levels of GSH in freshly isolated T cells from healthy volunteers and HIV-infected individuals was determined by spectrophotometry, using an assay kit from Calbiochem (Figure 1a). T cells (2×10^5/well) purified from PBMCs using nylon wool columns were pelleted by centrifugation and an equal volume of ice cold 5% MPA was added to the pellet. Supernatants were collected after centrifugation and analyzed for total GSH using an assay kit from Calbiochem, as per manufacturer's instructions. Total GSH in the samples were normalized with protein. Proteins in the samples were estimated by Bradford's method using Bio-Rad reagent. We also tested the effects of NAC and BSO in increasing and decreasing the intracellular levels of GSH, respectively in T cells isolated from both healthy subjects and individuals with HIV infection (Figure 1b). T cells (2×10^5/well) isolated from healthy and HIV positive subjects were treated as follows: mock treatment, treatment with NAC (10 mM) and treatment with BSO (500 μM). Following overnight incubation, T cells were pelleted and used for GSH measurement as per manufacturer's instructions. Results shown in Figure 1b are averages from experiments performed using T cells isolated from three healthy individuals and eight individuals with HIV infection. * represent significant difference in the levels of GSH between untreated T cells derived from healthy subjects versus untreated T cells derived from individuals with HIV infection. **denotes significant difference in the levels of GSH between untreated T cells versus NAC-treated T cells from HIV positive subjects. *** denotes statistically significant difference in the levels of GSH between NAC-treated T cells and BSO-treated T cells from individuals with HIV infection.
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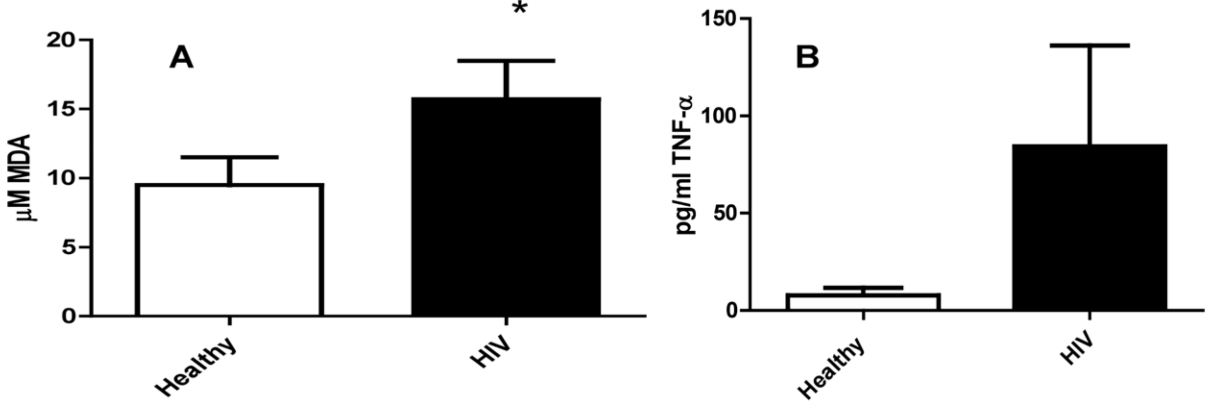

Figure 2. Assay of free radicals and TNF-α in plasma samples from healthy and HIV positive individuals. Plasma samples separated from blood of healthy volunteers and HIV-infected individuals were used for measurement of free radicals (Figure 2a) and TNF-α (Figure 2b). Free radical levels in plasma samples derived from healthy subjects and individuals with HIV infection was determined by measuring the levels of MDA using a colorimetric assay kit from Cayman. Levels of TNF-α in the plasma samples were determined by ELISA using assay kits procured from eBioscience. Results in Figure 2 are averages of data collected from thirteen healthy subjects and thirteen individuals with HIV infection.
doi:10.1371/journal.pone.0028378.g002

Determination of intracellular viability of *M. tb* in monocyte-T cell co-cultures from healthy and HIV positive individuals

We then tested the intracellular survival of *M. tb* in co-cultures of monocytes and T cells that were isolated from healthy subjects and individuals with HIV infection. Results from the six healthy subjects revealed a two-fold increase in the growth of *M. tb* inside monocytes cultured both in the presence and absence of T cells (Figure 3a). Incubation with NAC (10 mM)-treated T cells resulted in stasis in the growth of *M. tb* inside monocytes (Figure 3a). Incubation with BSO-treated T cells resulted in abrogation in the growth inhibition and a six-fold growth of *M. tb* inside monocytes confirming that GSH enhances the functions of T lymphocytes to control *M. tb* infection inside monocytes (Figure 3a). Whereas in HIV positive individuals, we observed a six-fold increase in the growth of *M. tb* inside monocytes cultured in the absence of T cells (Figure 3b) and five-fold increase in the growth of *M. tb* in monocytes cultured in the presence of mock-treated T cells (Figure 3b). These results indicate that monocytes and T cells from HIV positive individuals are unable to restrict the growth of *M. tb* to the same extent as healthy individuals. Treatment of T cells from HIV positive subjects with 10 mM NAC and co-incubation with infected monocytes resulted in reduction in the fold increase in growth of *M. tb* inside monocytes compared to other treatment

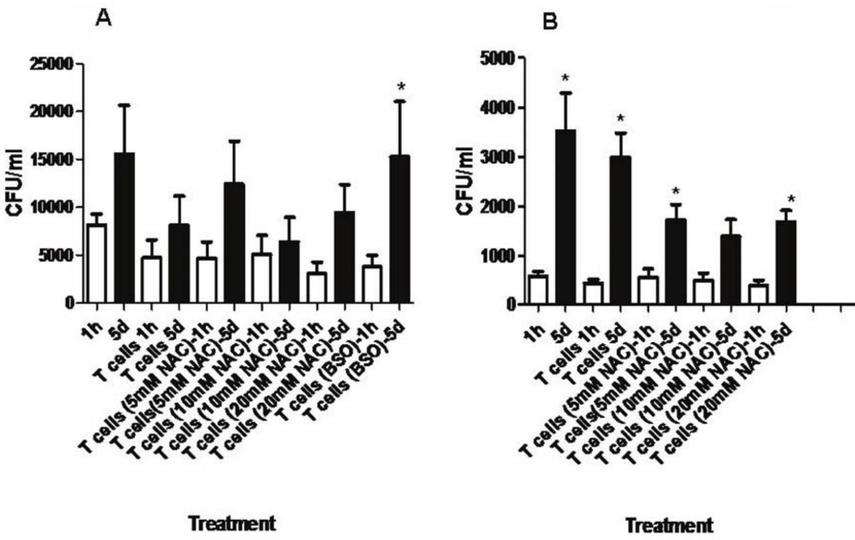

Figure 3. Intracellular survival of H37Rv inside T cell-monocyte co-cultures. We determined the intracellular survival of H37Rv inside T cell-monocyte co-cultures from healthy subjects (Figure 3a) and individuals with HIV infection (Figure 3b). Human monocytes were infected with the processed virulent laboratory strain of *M. tb*, H37Rv at a multiplicity of infection of 10:1. T cells were purified using nylon wool column. T cells were treated as follows: a) no additives b) NAC (5 mM) c) NAC (10 mM) d) NAC (20 mM) and e) BSO (500 µM) for 24 h. T cells were washed, resuspended in fresh media and then added to the infected monocytes. Infected monocytes-T cell co-cultures were terminated at 1 hour and 5 days post-infection to determine the intracellular survival of H37Rv inside human monocytes. Monocyte lysates were plated on 7H11 medium enriched with ADC to estimate the growth or killing of H37Rv. Results shown in Figure 3a are averages from five different experiments performed in triplicate. Results shown in Figure 3b are averages from seven different experiments performed in triplicate.
doi:10.1371/journal.pone.0028378.g003

conditions. However, NAC (10 mM)-treatment of T cells did not result in complete stasis in the growth of *M. tb* as seen in healthy subjects (Figure 3b). We also observed a poor uptake of *M. tb* by monocytes derived from individuals with HIV infection compared to healthy individuals as evidence by lower colony counts recovered from the co-cultures of monocytes and T cells (Figure 3b). This reduced uptake of *M. tb* could possibly be due to impaired phagocytic capacity of monocytes that are derived from HIV positive individuals.

Assay of IL-12, IL-2 and IFN-γ in supernatants derived from T cell-monocyte co-cultures

To determine whether the growth inhibition of *M. tb* in monocyte-T cell co-cultures is accompanied by increased production of IL-12, IL-2 and IFN-γ, we quantified the levels of these cytokines in the cell free-supernatants from co-cultures of *M. tb*-infected monocytes and T cells (from healthy subjects and individuals with HIV infection) by ELISA (Figure 4). We observed an increase in the production of IL-12, IL-2 and IFN-γ in monocyte+NAC-treated T cell co-cultures derived from healthy subjects (Figure 4a, b and c). These results indicate that increasing GSH in T cells favors the synthesis of Th-1 cytokines leading to the control of *M tb* infection. We also quantified the levels of IL-12, IL-2 and IFN-γ in monocyte-T cell co-cultures from individuals with HIV infection (Figure 4d and e). We were unable to detect IL-12 in the culture supernatants. IL-2 and IFN-γ were detected in monocyte-T cell co-cultures however the levels of these cytokines were almost one log lower compared to the healthy individuals (Figure 4d and e). This signifies the consequences of HIV infection on T cell functions. Since mycobacteria are not internalized by T cells the downregulation in the production of IFN-γ and IL-2 by T cells is an HIV induced effect.

Assay of IL-12, IL-2, IFN-γ and IL-10 levels in plasma samples from healthy and HIV-infected subjects

Measurement of Th-1 cytokines such as IL-12, IL-2 and IFN-γ in plasma samples from thirteen healthy and thirteen HIV infected individuals revealed a significant reduction in the levels of these

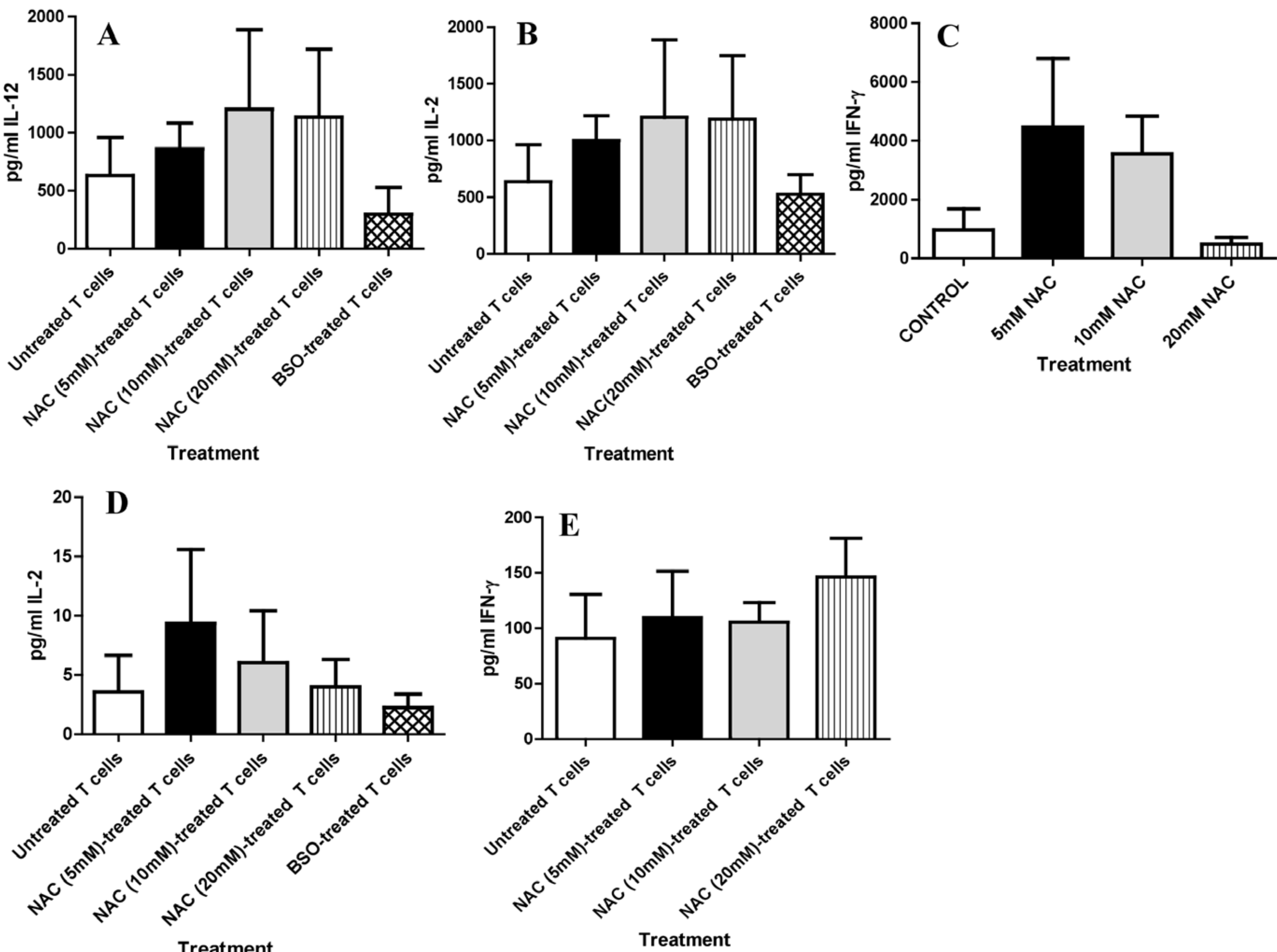

Figure 4. Assay of IL-12, IL-2 and IFN-γ in supernatants derived from co-cultures of T cells-*M. tb*-infected monocytes. Levels of IL-12, IL-2 and IFN-γ were assayed in supernatants derived from co-cultures of T cells-*M. tb*-infected monocytes from healthy individuals (Figure 4a, b and c) and individuals with HIV infection (Figure 4d and e) T cells were purified using nylon wool column. T cells were treated as follows: 1) no additives 2) NAC (5 mM) 3) NAC (10 mM) 4) NAC (20 mM) and 5) BSO (500 μM) for 24 h. Following incubation with stimulants, T cells were washed, re-suspended in fresh RPMI containing human serum without any stimulants and then added to the infected monocytes. Supernatants were collected from co-cultures of H37Rv-infected monocytes-T cells at 5 days post-infection were filtered and assayed for the levels of IL-12, IL-2 and IFN-γ using assay kits from eBioscience. Data in Figures 4a, b and c represent means±SE from four different healthy individuals. Data in Figures 4d and e represent means±SE from six different individuals with HIV infection.
doi:10.1371/journal.pone.0028378.g004

cytokines in HIV infected individuals when compared to healthy subjects. We observed a statistically significant (i.e., 50%) decrease in the levels of IL-12 in plasma samples from individuals with HIV infection compared to healthy subjects (Figure 5a). Decreased levels of IL-12 will interfere with the differentiation of Th0 to Th1 subset [22]. We also observed a 20% decrease in the levels of IL-2 in plasma samples derived from individuals with HIV infection compared to healthy subjects (Figure 5b).Decreased levels of IL-2 will interfere with the amplification of T cell responses [9]. Furthermore, we observed a significant decrease in the levels of IFN-γ in plasma samples from individuals with HIV infection compared to healthy subjects (Figure 5c). Low levels of IFN-γ will result in enhanced susceptibility to intracellular infections [10]. Importantly, we observed a five-fold increase in the levels of IL-10 in the plasma samples derived from individuals with HIV infection compared to healthy subjects (Figure 5d). However this increase is not statistically significant. Increased IL-10 may promote viral replication by inhibiting effector immune response from both arms of the innate and adaptive immunity [14].

Discussion

Blood monocytes and resident macrophages are important *in vivo* cell targets for HIV infection and their role in AIDS pathogenesis is well documented [2]. These cells of innate immune defenses usually survive HIV infection, serve as a major virus reservoir, and function as immunoregulatory cells through secretion of several pro-inflammatory cytokines and chemokines in response to HIV infection, thereby recruiting and activating CD4+ T cells which serve as new target cells for the virus [3-5].

GSH is the primary antioxidant compound employed by human cells in the maintenance of the cellular redox balance. GSH is a tripeptide made of glutamine, cysteine, and glycine and has been shown to be important for normal function of the immune system. In particular, we have previously demonstrated that GSH is important for the control of intracellular *M. tb* infection by the cells of the immune system [18-21]. Conditions of depleted GSH have been demonstrated to activate transcription factors that are necessary for active replication of the HIV within lymphocytes [3-5, 16 &17].

In this study, we observed that GSH levels are compromised in T lymphocytes derived from individuals with HIV infection (Figure 1) and this decrease correlated with increased levels of TNF-α and free radicals in the plasma compared to healthy subjects (Figure 2).

TNF-α, a pro-inflammatory cytokine is considered to play a critical role in the origin and progression of HIV infection [23]. The immuno-regulatory response of the host influences the pathogenesis of HIV-1 infection, triggering monocytes, macrophages, and natural killer cells to produce TNF-α [24]. As a result, there is a positive correlation between HIV-1 viremia and TNF-α levels in serum of HIV-1 infected patients. This relationship suggests that reducing TNF-α levels may also reduce occurrence of HIV-1 viremia. In excess, TNF-α may cause severe inflammatory damage and toxicity, making control of its production and secretion highly important. Regulating its release serves as a potential means of therapy for HIV-1. TNF-α can also induce other pro-inflammatory cytokines such as IL-6 and IL-8, which aid in the upregulation of viral replication [25]. Studies have also shown the ability of TNF-α to stimulate production of anti-

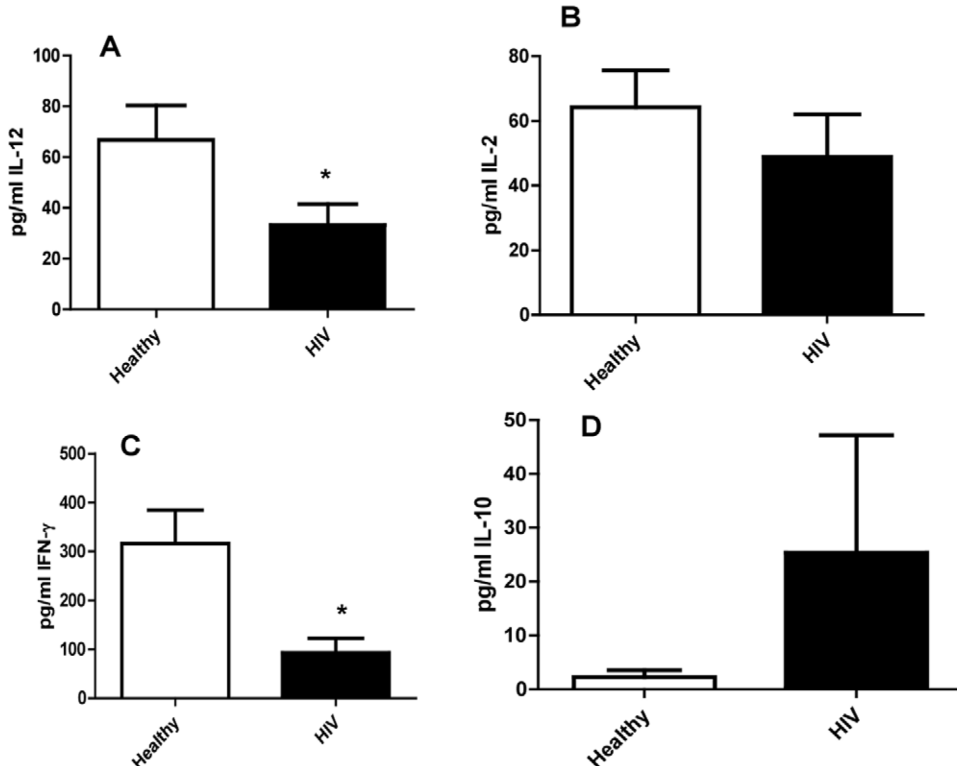

Figure 5. Assay of IL-12, IL-2, IFN-γ and IL-10 in plasma samples from healthy and HIV positive individuals. Plasma samples separated from blood of healthy volunteers and HIV-infected individuals were used for measurement of IL-12 (Figure 5a), IL-2 (Figure 5b), IFN-γ (Figure 5c) and IL-10 (Figure 5d) by ELISA using assay kits from eBioscience. Results in Figure 5 are averages from data collected from thirteen healthy subjects and thirteen individuals with HIV infection.
doi:10.1371/journal.pone.0028378.g005

inflammatory cytokine IL-10, preventing further inflammation by causing TNF-α inhibition [26]. TNF-α is secreted during the early phase of acute inflammatory diseases. Its pathogenic role in HIV-1 infection involves activation of NF-κB, stimulating apoptosis of T lymphocytes. Tissue and plasma samples of hosts express high levels of TNF-α, contributing to fever, anorexia, and other symptoms of HIV/AIDS.

It has been shown that HIV infection results in increased production of free radicals and TNF-α by macrophages [23,24]. TNF-α stimulates the production of free radicals. Moreover, enhanced levels of free radicals are likely to increase TNF-α in various cells.

Chronic oxidative stress is often associated with HIV infection and research indicates a benefit for increased antioxidant vitamins and supplements in reduction of DNA base damage, which in turn can slow progression of infection [27]. The progression of HIV is correlated with a decreased immunity. One way in which this decreased immunity progresses is by free radical overload of monocytes and granulocytes leads to deficiency of antioxidant mechanisms which, in turn, may lead to the loss of CD4 cells often seen in the progression of HIV [28]. The decreased immunity may also be related to the reactive oxygen species and free radical presence which is higher in HIV infected patients. With HIV infection progression there is an increased production of reactive oxygen species which leads to the theory of free radical mediated apoptosis of lymphocytes which reduces the ability for immune response to progressive HIV infections [28]. With regards to CD4 T cell counts the apoptosis of lymphocytes by free radicals leads to progression of immunodeficiency and makes for a quicker transition from HIV infection to AIDS [29]. Furthermore, there is a link to lipid peroxidation observed in patients with HIV or AIDS to a deficiency of antioxidants which leads to free radical proliferation [30].

Results of the co-culture studies using monocytes and T cells from healthy individuals indicate a complete inhibition in the growth of *M. tb* when infected monocytes were co-incubated with 10 mM NAC-treated T-cells (Figure 3a). Furthermore, co-incubation of infected monocytes with BSO-treated T cells demonstrated six-fold increase in the intracellular growth of *M. tb* (Figure 3a). In contrast to healthy subjects, we observed a several-fold increase in the growth of *M. tb* in co-cultures of monocytes and T cells derived from individuals with HIV infection (Figure 3b). Treatment of T cells derived from HIV positive individuals with 10 mM NAC resulted in reduction in the fold increase in growth of *M. tb* inside monocytes compared to other treatment conditions but did not result in complete stasis in the growth of *M. tb* as seen in healthy subjects (Figure 3b). This is the first study to demonstrate that treatment with a GSH-enhancing agent increases the functional activity of human T-cells resulting in the control of *M. tb* infection. Our results indicate that treatment of T cells from healthy subjects with NAC leads to the increased production of IL-12, IL-2 and IFN-γ (Figure 4a). These cytokines drive the Th1 response and are crucially important for the control of intracellular pathogens like *M. tb*.

In our study, we also observed decreased levels of IL-12, IL-2 and IFN-γ in plasma samples from individuals with HIV infection compared to healthy subjects (Figure 5a, 5b and 5c). Activated antigen presenting cells secrete IL-12 which causes Th cell differentiation into the Th1 subset of cells [22]. These Th1 cells then secrete a characteristic Th1 profile of cytokines consisting of IL-2 and IFN-γ. IL-2 induces proliferation of naïve Th cells (Th_0), amplifying the Th response. IFN-γ induces further IL-12 production in activated antigen presenting cells, amplifying the Th1 response, and suppressing any Th2 response [10]. IFN-γ also plays an important role in the activation of innate immune cells such as macrophages to control intracellular infections [10,31]. Our results are consistent with other published reports that in individuals infected with HIV, the normal Th1 response to viral infection is shifted to a Th2 response [31,32].

We also observed increased levels of IL-10 in plasma samples from individuals with HIV infection compared to healthy subjects (Figure 5d). IL-10 inhibits T cell proliferation and IFN-γ production. Elevated levels of IL-10 in serum during advanced HIV infection may enhance immune suppression, allowing for opportunistic infections.

Programmed death-1 (PD-1) and IL-10 are both upregulated during HIV infection. Blocking interactions between PD-1 and programmed death ligand-1 (PD-L1) and between IL-10 and IL-10 receptor (IL-10R) results in viral clearance and improves T cell function in animal models of chronic viral infections [33]. Additionally, blockade of the IL-10 pathway augmented *in vitro* proliferative capacity of HIV-specific CD4 and CD8 T cells in individuals with HIV. IL-10 blockade also increased cytokine secretion by HIV-specific CD4 T cells [34]. Our results therefore confirm the previous findings of other investigators that the levels of IL-12, IL-2 and IFN-γ are decreased in the plasma samples of individuals with HIV infection (who in turn have higher IL-10) [32-34].

Further, our results show that individuals infected with HIV have significantly lower levels of GSH within their T cells in comparison to healthy subjects and this decrease correlated with reduced production of Th1 cytokines and compromised control of *M. tb* infection (Figure 1, 3, 4 and 5).

To conclude, our results indicate that HIV enhances the synthesis of TNF-α and free radicals leading to decreased levels of GSH in T lymphocytes. The decreased GSH levels cause impaired production of Th1 cytokines resulting in enhanced growth of *M. tb* inside macrophages (Figure 6). Our findings suggest the possibility of efficacy for supplemental GSH therapy in HIV-*M. tb* co-infected individuals. In our future studies, we will further characterize the effects of decreased GSH in altering the functional activity of various T cell sub-populations.

Materials and Methods

Subjects

A total of 26 volunteers (13 healthy subjects and 13 individuals with HIV infection) were recruited for the study. Individuals with HIV infection were recruited from the Foothills AIDS project. Healthy subjects without HIV infection or a history of TB were recruited from the university faculty and staff. All HIV-infected volunteers had been diagnosed with HIV-1, were taking some form of anti-retroviral treatment (ART), and had CD4+ T-cell counts between 271 and 1415 cells per mm^3. Thirty five milli-liters of blood was drawn once from both healthy volunteers and individuals with HIV infection after obtaining a signed informed consent. All our studies were approved by both the Institutional Review Board and the Institutional Biosafety Committee of Western University of Health Sciences.

Separation of blood components

Blood collected from healthy and HIV-infected volunteers was subjected to density gradient centrifugation using histopaque (Sigma) to separate plasma, red blood cells (RBC), and peripheral blood mononuclear cells (PBMC). PBMCs were further processed to isolate monocytes and T cells. Plasma collected was used for measurement of cytokines (IL-12, IL-2, IFN-γ, IL-10 and TNF-α) and free radicals.

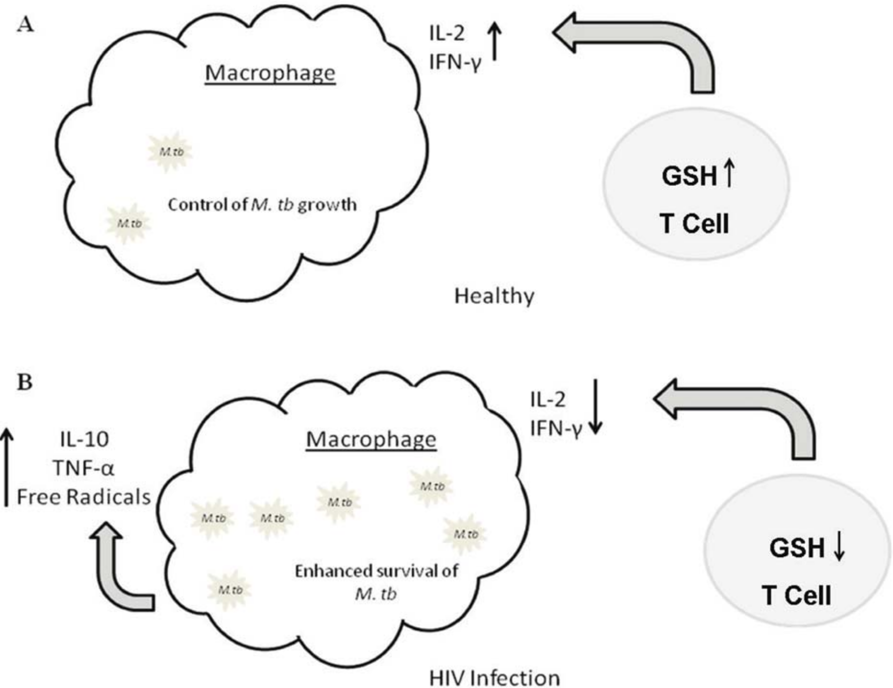

Figure 6. Model describing the mechanism by which GSH-enhanced T cells control *M. tb* infection in macrophages.
doi:10.1371/journal.pone.0028378.g006

Isolation of human monocytes

PBMCs isolated from the blood of healthy volunteers and individuals with HIV infection were distributed into Poly-L-Lysine (Sigma) coated 96-well plates (1×10^5/well) and incubated overnight at 37°C, 5% CO_2, to allow monocytes to adhere. Non-adherent cells were removed and used for isolation of T cells.

Purification of T lymphocytes

Autologous T lymphocytes were isolated from non-adherent cells (from both healthy volunteers and individuals with HIV infection) using nylon wool columns (Polysciences). T cells were subjected to various treatments such as treatment with a GSH enhancing agent, N-acetyl cysteine (NAC) and treatment with a GSH synthesis inhibitor, buthionine sulphoximine (BSO). Specifically, T cells were treated as follows: no additives, treatment with NAC (5 mM), NAC (10 mM), NAC (20 mM), and treatment with BSO (500 µM), for 24 h. T cells were then washed, resuspended in fresh media containing no additives and used for various assays such as measurement of GSH, determination of the intracellular viability of *M. tb* inside monocytes and cytokine production.

Assay of GSH levels in T cells from healthy and HIV-infected subjects

GSH levels were measured in freshly isolated T cells from healthy subjects and individuals with HIV infection. Intracellular levels of GSH in T cells were determined by spectrophotometry using an assay kit from Calbiochem. Briefly, T cells (2×10^5/well) were pelleted by centrifugation and an equal volume of ice cold 5% metaphosphoric acid (MPA) was added to the pellet. Supernatants collected after centrifugation were analyzed for total GSH as per manufacturer's instructions. Total GSH in the samples was normalized with protein. Proteins in the samples were estimated by Bradford's method using Thermo Scientific Coomassie Protein Assay Reagent.

Assay of GSH levels in NAC/BSO-treated T cells from healthy and HIV-infected subjects

We tested the effects of NAC and BSO in increasing and decreasing the intracellular levels of GSH respectively, in T cells isolated from both healthy subjects and individuals with HIV infection. T cells (2×10^5/well) isolated from healthy and HIV positive subjects were treated as follows: mock treatment, treatment with NAC (10 mM) and treatment with BSO (500 µM). Following overnight incubation, T cells were pelleted and used for GSH measurement as per manufacturer's instructions.

Assay of free radicals and TNF-α in plasma samples

Free radical levels in plasma samples derived from healthy subjects and individuals with HIV infection was determined by measuring the levels of malondialdehyde (MDA) using a colorimetric assay kit from Cayman. Levels of TNF-α in plasma samples derived from healthy subjects and individuals with HIV infection were determined by enzyme linked immuno-sorbent assay (ELISA) using assay kits from eBioscience.

Preparation of bacterial cells for monocyte infection

All infection studies were performed using the virulent laboratory strain of *M. tb*, H37Rv inside the biosafety level 3 (BSL-3) facility. *M. tb* was processed for infection as follows: static cultures of H37Rv at their peak logarithmic phase of growth (between 0.5 and 0.8 at A600) were used for infection of monocytes. The bacterial suspension was washed and re-suspended in RPMI (Sigma) containing AB serum (Sigma). Bacterial clumps were disaggregated by vortexing five times with 3 mm sterile glass beads. The bacterial suspension was passed through a 5 µm syringe filter (Millipore) to remove any further clumps. The total number of organisms in the suspension was ascertained by plating. Processed H37Rv was frozen as stocks at -

80°C. At the time of infection, frozen stocks of processed H37Rv were thawed and used for monocyte infection.

Co-incubation of *M. tb*-infected monocytes with autologous T cells

Adherent monocytes were infected with processed H37Rv at a multiplicity of infection of 10:1 and incubated for 2 hours for phagocytosis. Unphagocytosed mycobacteria were removed by washing the infected monocytes three times with sterile PBS. Infected monocytes were cultured in RPMI containing 5% AB serum in presence and absence of autologous T cells. Prior to co-incubation with infected-monocytes, autologous T cells were incubated overnight with stimulants (NAC/BSO), washed with PBS, re-suspended in fresh RPMI containing AB serum (without any stimulants) and then added to the infected monocytes (monocyte: T cell ratio was adjusted to 1:1). Infected monocyte-T cell co-cultures were terminated at 1 hour and 5 days post-infection to determine the intracellular survival of H37Rv. Infected monocytes cultured in the absence of T cells were used as negative controls.

Termination of infected monocytes-autologous T cell co-cultures

M. tb-infected monocytes cultured in the presence and absence of T lymphocytes were terminated at 1 hour and 5 days post-infection. During termination, supernatants were removed and adherent monocytes were lysed by addition of 200 µl sterile distilled water. 25 µl of 10-fold diluted lysates were plated on 7H11 medium (Hi Media) enriched with albumin dextrose complex (ADC), to estimate the extent of H37Rv growth or killing in co-cultures of monocytes and T cells. Cell-free supernatants were plated to determine extracellular H37Rv growth. Cell-free supernatants were also used for determining the levels of IL-12, IL-2 and IFN-γ.

Assay of cytokines in supernatants derived from T cell-monocyte co-cultures

Cell free-supernatants from co-cultures of *M. tb*-infected monocytes and T cells (from healthy controls and individuals with HIV infection) collected at 5 days post-infection were filtered using 0.2 micron syringe filters (Millipore) and assayed for the levels of IL-12, IL-2 and IFN-γ. Cytokines were assayed using ELISA kits from eBioscience.

Assay of IL-12, IL-2, IFN-γ and IL-10 in plasma samples

Levels of IL-12, IL-2, IFN-γ and IL-10 in plasma samples derived from healthy subjects and individuals with HIV infection were determined by ELISA using assay kits from eBioscience.

Statistical Analysis

Statistical analysis of the data was carried out using Prism and Statview® and the statistical significance was determined using an unpaired *t*-test. Differences were considered significant at a level of $P < 0.05$.

Acknowledgments

We thank the staff at the Western University medical center for their help with blood draw. We thank the volunteers for their participation in this study.

Author Contributions

Conceived and designed the experiments: VV. Performed the experiments: VV CG DM AS SK DG MT MF. Analyzed the data: VV CG DM. Contributed reagents/materials/analysis tools: FTK FG. Wrote the paper: VV.

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GLUTATHIONE

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PERSPECTIVES AND SUMMARY

This ubiquitous tripeptide (L-γ-glutamyl-L-cysteinylglycine), usually the most prevalent intracellular thiol, is now known to function directly or indirectly in many important biological phenomena, including the synthesis of proteins and DNA, transport, enzyme activity, metabolism, and protection of cells. The multifunctional properties of glutathione are reflected by the growing interest in this small molecule on the part of investigators of such diverse subjects as enzyme mechanisms, biosynthesis of macromolecules, intermediary metabolism, drug metabolism, radiation, cancer, oxygen toxicity, transport, immune phenomena, endocrinology, environmental toxins, and aging.

This chapter is concerned with current progress in unraveling the biochemical bases of the physiological roles of this important compound. Detailed information is now available about glutathione synthesis and its metabolism by the reactions of the γ-glutamyl cycle, and its function in reductive processes that are essential for the synthesis (and the degradation) of proteins, formation of the deoxyribonucleotide precursors of DNA, regulation of enzymes, and protection of the cell against reactive oxygen compounds and free radicals. In addition, glutathione is a coenzyme for several reactions; it conjugates with foreign compounds (e.g. drugs) and with compounds formed in metabolism (e.g. estrogens, prostaglandins, leukotrienes), and thus participates in their metabolism.

An important recent finding is that cellular turnover of glutathione is associated with its transport, in the form of GSH, out of cells. The functions of such GSH transport include formation by membrane-bound γ-glutamyl transpeptidase of γ-glutamyl amino acids, which can be transported into certain cells, and thus serve as one mechanism of amino acid transport. Transported GSH probably also functions in reductive reactions that may involve the cell membrane and the immediate environment of the cell. In the mammal, such transported GSH may enter the blood plasma and be transferred to other cells. Glutathione thus appears to be a storage form and a transport form of cysteine.

Much of the new information about glutathione has arisen through studies with selective inhibitors of the enzymes involved in its metabolism. Thus, inhibition in vivo of γ-glutamyl transpeptidase, γ-glutamyl cyclotransferase, 5-oxoprolinase, and glutathione synthesis has been achieved, and the effects observed have contributed importantly to the understanding of glutathione metabolism and function. Studies on the inhibition of γ-glutamyl transpeptidase have elucidated the transport of GSH and the formation and transport of γ-glutamyl amino acids. Inhibition of glutathione synthesis by sulfoximine compounds that inactivate γ-glutamylcysteine

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synthetase has also contributed to such knowledge, as well as to information about the roles of glutathione in protection against both free radicals and reactive oxygen compounds, and in metabolism. These enzyme inhibitors, and other compounds that increase in vivo glutathione synthesis have opened the way to selective modulation of glutathione metabolism; this has made several new therapeutic approaches possible.[1]

METABOLISM OF GLUTATHIONE—AN OVERVIEW[2]

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Glutathione is synthesized intracellularly (Figure 1) by the consecutive actions of γ-glutamylcysteine synthetase (Reaction 1) and GSH synthetase (Reaction 2). Reaction 1 is feedback inhibited by GSH. The breakdown of GSH (and also of GSSG and S-substituted GSH) is catalyzed by γ-glutamyl transpeptidase, which catalyzes transfer of the γ-glutamyl moiety to acceptors—amino acids, e.g. cystine, glutamine, and methionine, certain dipeptides, water, and GSH itself—(Reaction 3). GSH occurs mainly intracellularly and a major fraction of the transpeptidase is on the external surface of the cell membranes. GSH transported across cell membranes interacts with γ-glutamyl transpeptidase. γ-Glutamyl amino acids formed by γ-glutamyl transpeptidase are transported into cells; evidence for such formation and transport of γ-glutamyl amino acids is given below. Intracellular γ-glutamyl amino acids are substrates of γ-glutamyl cyclotransferase (Reaction 4), which converts these compounds into the corresponding amino acids and 5-oxo-L-proline. The ATP-dependent conversion of 5-oxo-L-proline to L-glutamate is catalyzed by the intracellular enzyme 5-oxo-prolinase (Reaction 5). The cysteinylglycine formed in the transpeptidase reaction is split by dipeptidase (Reaction 6). These six reactions constitute the γ-glutamyl cycle, which thus accounts for the synthesis and degradation of GSH. Two of the enzymes of the cycle also function in the

[1] More than 2000 current papers on glutathione have come to the authors' attention through a computer search and the kindness of many investigators, who have supplied us with manuscripts. Because of space limitations we cannot cite the full literature here; while we have included references to many recent developments, we have probably inadvertently overlooked some relevant papers. Readers should also consult the published proceedings of several recent meetings and earlier reviews (1–8).

[2] We follow current usage in abbreviating glutathione as GSH and glutathione disulfide as GSSG. In this field it is common to use the term glutathione (GSH) to include both GSH and GSSG, because the relative amounts of each form may not be known, and the analytical methods used may determine the sum of both forms. Although to some extent we follow this practice here, we indicate, where known, the predominant redox form. The term "total glutathione" has been used in the literature to indicate the sum of GSH and GSSG in GSH equivalents.

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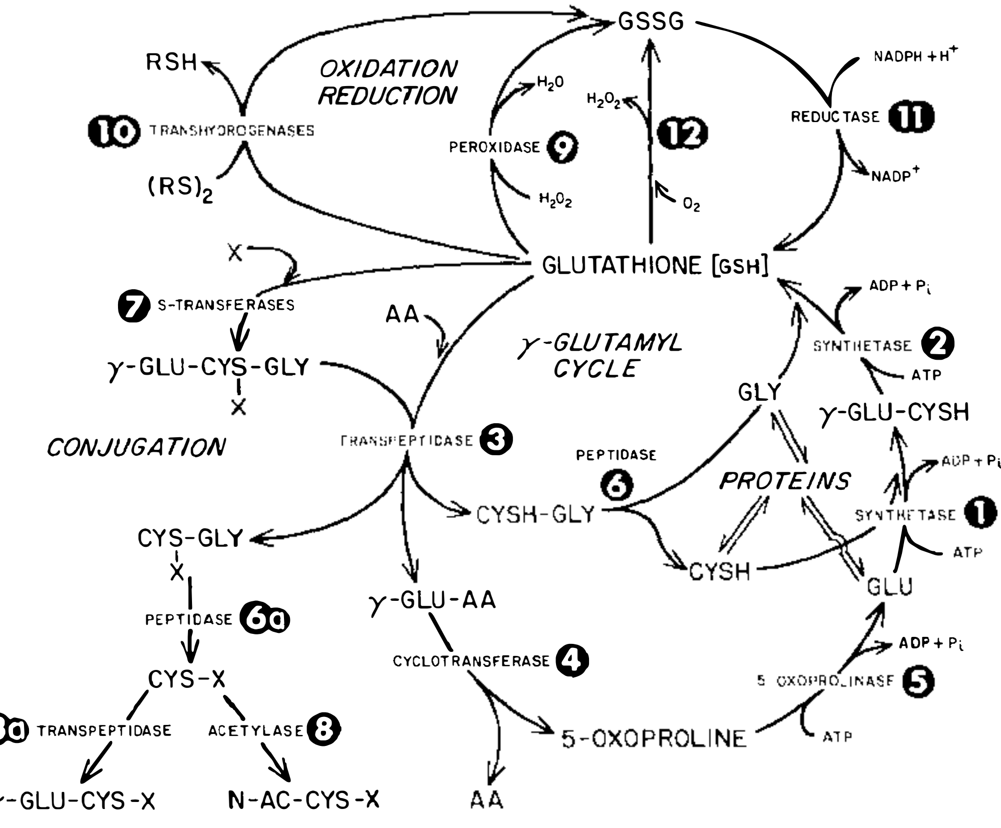

Figure 1 Overall summary of glutathione metabolism (see text): Reaction 1. γ-GLU-CYSH synthetase; Reaction 2. GSH synthetase; Reaction 3 and 3*a*. glutamyl transpeptidase; Reaction 4. γ-glutamyl cyclotransferase; Reaction 5. 5-oxoprolinase; Reactions 6 and 6*a*. dipeptidase; Reaction 7. GSH S-transferases; Reaction 8. *N*-acetylase; Reaction 9. GSH peroxidase; Reaction 10. transhydrogenases; Reaction 11. GSSG reductase; Reaction 12. oxidation of GSH by O_2; conversion of GSH to GSSG is also mediated by free radicals.

metabolism of S-substituted GSH derivatives, which may be formed nonenzymatically by reaction of GSH with certain electrophilic compounds or by GSH S-transferases (Reaction 7). The γ-glutamyl moiety of such conjugates is removed by the action of γ-glutamyl transpeptidase (Reaction 3), a reaction facilitated by γ-glutamyl amino acid formation. The resulting S-substituted cysteinylglycines are cleaved by dipeptidase (Reaction 6*a*) to yield the corresponding S-substituted cysteines, which may undergo N-acylation (Reaction 8) or an additional transpeptidation reaction to form the corresponding γ-glutamyl derivative (Reaction 3*a*); the latter reaction has been demonstrated with a substrate of exogenous origin (9), and with leukotriene E (10) (see p. 741).

Intracellular GSH is converted to GSSG by selenium-containing GSH peroxidase, which catalyzes the reduction of H_2O_2 and other peroxides (Reaction 9); there is evidence that certain GSH S-transferases can also catalyze such reactions. GSH is also converted to GSSG by transhydrogenation (Reaction 10); a number of reactions of this type are considered later. Reduction of GSSG to GSH is mediated by the widely distributed enzyme GSSG reductase which uses NADPH (Reaction 11). Extracellular conversion of GSH to GSSG has also been reported; the overall reaction requires O_2 and leads to formation of H_2O_2 (Reaction 12) (see p. 733). GSSG is also formed by reaction of GSH with free radicals.

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TRANSPORT OF GLUTATHIONE

The intracellular level of GSH in mammalian cells is in the millimolar range (0.5–10 mM), whereas micromolar concentrations are typically found in blood plasma. Several lines of evidence (2) indicate that γ-glutamyl transpeptidase is accessible to external substrate, and that the enzyme is largely bound to the outer surface of cell membranes. Nevertheless, many findings indicate that intracellular GSH is the major substrate of transpeptidase. The finding of an enzyme and its substrate on opposite sides of a membrane led to the postulate that intracellular GSH is transported to the membrane-bound transpeptidase (11). Studies on a patient with γ-glutamyl transpeptidase deficiency who has marked glutathionuria and glutathionemia (12) led to the suggestion that transport of intracellular GSH to the plasma and glomerular filtrate in this patient reflects an aspect of the normal process that provides substrate to the membrane-bound enzyme (11, 13). Thus, in the absence of significant transpeptidase activity, substantial amounts of GSH appear extracellularly. This interpretation is supported by studies in which marked glutathionuria and glutathionemia were found in mice and rats that had been treated with transpeptidase inhibitors (14, 15, 16). Animals given such inhibitors exhibit glutathionuria

during and for a short period after such treatment without microscopic evidence of tissue damage. With one inhibitor, urinary concentrations as high as 29 mM were observed (16). The kidney is a major source of such urinary GSH (14), but some undoubtedly arises also from the liver.

When the inhibitors of GSH synthesis (e.g. prothionine sulfoximine, buthionine sulfoximine) are given to mice and rats, plasma GSH levels decrease substantially (2, 14, 15). The rapid and marked increase in plasma GSH after inhibiting transpeptidase, and the considerable decrease seen soon after inhibition of GSH synthesis indicate active turnover of plasma GSH. The findings suggest that there is normally an appreciable flow of GSH from liver into plasma, and that cells that have high transpeptidase levels utilize plasma GSH; the products formed (including γ-glutamyl amino acids) enter the cells. The major organs involved in this inter-organ circulation of GSH are the liver and kidney, but undoubtedly other organs also participate. Studies on anephric animals treated with transpeptidase inhibitors show that about 67% of the plasma GSH is used by the kidney and the remainder by extrarenal transpeptidase [(15); see also (15a)]. These in vivo investigations indicate that GSH is normally translocated to membrane-bound transpeptidase as a discrete step in the γ-glutamyl cycle (14); in vitro studies support this. Thus, GSH export from cells to medium was found in human lymphoid cells (17, 18) and skin fibroblasts (19), and macrophages (20). Isolated perfused liver preparations show efflux of GSH to the perfusate (21).

Independent evidence for inter-organ GSH transport came from studies on the plasma levels of GSH in various blood vessels of the rat (22). Hepatic vein plasma has a much higher level than does arterial blood plasma or that obtained from the inferior vena cava. Renal vein plasma has about 20% of the level found in arterial plasma, indicating that kidney has a mechanism in addition to filtration for removal of plasma GSH (15a, 22, 23). Disappearance of plasma GSH on passage through the kidney decreased markedly when either of two types of transpeptidase inhibitors were given; this indicates that the nonfiltration mechanism that utilizes GSH involves the action of γ-glutamyl transpeptidase (22). Although it was previously believed that virtually all renal transpeptidase is localized on the brush border side of the cell, electron micrographic studies (24) provide evidence that there is transpeptidase on the basolateral side as well. Thus, transpeptidase acts on plasma GSH on both the brush border and the basolateral sides. About 80%–90% of renal tubular GSH arises from kidney cells; the remainder comes from the plasma (25, 26). The amount reaching the tubule from the plasma (estimated for a 30-g mouse from the glomerular filtration rate and the plasma GSH level) is about 0.8 μmole/hr. The

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turnover rate of renal GSH indicates a flow of about 4.1 μmole/hr from renal cells to tubule; most of this is utilized in the tubule. It has been tentatively estimated that about 2.4 μmole/hour of GSH is utilized on the basolateral side (26). Such basolateral utilization of GSH, described earlier as "extraction" (15a) and "uptake" (27), is mainly the result of extracellular conversion to other products, chiefly the amino acid constituents of GSH (28).

Liver GSH is transported in substantial amounts to hepatic vein plasma and to the bile; in the rat the GSH levels are, respectively, about 26 μM (22) and 1–6 mM (29–33). Rat bile also contains about 1 mM cyst(e)inylglycine and about 0.2 mM cyst(e)ine (32, 33). Studies on rat bile are often complicated by the high entrance of the pancreatic ducts into the bile duct, so that the bile collected contains pancreatic juice; this may lead to high GSSG values and low values of total glutathione. Proper cannulation yields "pure" bile, which has very low transpeptidase activity and 1–6 mM GSH; pancreatic juice, which has no detectable GSH or GSSG, has high levels of transpeptidase (33). The GSH level in bile decreases markedly after treatment of rats with buthionine sulfoximine, and it increases approaching intracellular levels after treatment with a transpeptidase inhibitor (33). The findings suggest that GSH transported from hepatocytes interacts with the transpeptidase of ductule cells, and that there is substantial reabsorption of metabolites by ductule epithelium. These findings on intact rats indicate a greater extent of GSH export from liver cells into bile than has been deduced by analysis of the excreted bile. In the intact rat, about 12 and 4 nmoles/g/min of GSH appear in the hepatic vein and bile, respectively.

Studies on isolated perfused rat liver indicate transport of GSH and GSSG into the total perfusate of 12 and 1 nmole/g/minute, respectively (21, 33a). It was concluded (33b, 33c), that GSSG and GSH-conjugates are released from the liver preferentially into bile by a process in which these compounds mutually compete for secretion. Other data [see Figures 1*B* and 3 in (34); see also (34a)] show, however, that substantial amounts of GSSG and GSH-conjugates enter the venous outflow. The quantitative aspects of the suggested competition indicate that the system has a much greater affinity for GSH-conjugates than for GSSG (33). The data on transport of GSH and GSSG from liver to bile obtained with the isolated perfused liver system differ substantially from those obtained on anesthetized intact rats. A major difference is that, in contrast to the intact rat, little or no GSH is found in the bile in the perfusion system.

Intracellular glutathione is normally over 99% GSH; GSH is the major transport form. Analyses of mouse blood plasma (35) and rat blood plasma, i.e. arterial, hepatic vein, renal vein, and vena cava (22, 36), and rat bile (31,

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29) show that about 90% is in the GSH form. About 70% of mouse urinary glutathione was found as GSH after potent inhibition of transpeptidase (16). Human lymphoid cells export glutathione that is at least 90% GSH (17). These findings contrast markedly with earlier reports that rat blood plasma (15a, 21, 23, 37) and bile (34) contain glutathione that is predominantly GSSG (see p. 748). In earlier studies that suggested that GSH and GSSG are transported across cell membranes, the possibility that the GSH and GSSG found extracellularly was related to oxidative or other types of cell damage could not be excluded.

Under conditions of marked toxicity or oxidative stress, intracellular GSSG increases substantially (38), and there may be a mechanism for its export. It has been concluded that the export of glutathione from erythrocytes involves transport of GSSG (39–42); however, normal transport of GSH is difficult to exclude. GSSG transport was reported in erythrocytes whose GSSG level (normally, $<0.1\%$) was artificially raised by a GSH-oxidizing agent; such transport did not occur in ATP-depleted cells. Evidence for an active transport system for GSSG has been obtained in other studies on erythrocytes (40, 41) and lens (43); a similar pathway may exist in liver (38, 44). Beutler (39) has suggested that active transport of GSSG may be an emergency mechanism to protect cells from toxic effects of GSSG. The question as to how much GSSG transport occurs normally is difficult to answer. Intracellular GSSG levels are extremely low, and accurate measurement of these small amounts in the presence of very large amounts of GSH constitutes a formidable technical problem.

The rate of transport of GSH from lymphoid cells has been found to be proportional to the intracellular GSH level (18). Little if any intact GSH is taken up by these cells. Lymphoid cells that have been depleted of GSH by treatment with buthionine sulfoximine, and which are therefore more sensitive to the effects of irradiation (18), are not protected by suspension in GSH-containing media (45). Repletion of intracellular GSH by suspending cells in media containing GSH takes place by a process involving enzymatic degradation of the external GSH, uptake of the products, and intracellular resynthesis of GSH (45).

Transport of GSH out of cells is a property of many cells (2, 46). In the mammal, this seems to function in the transfer of cysteine sulfur between cells. Transport of GSH may protect the cell membrane against oxidative damage by maintaining essential SH-groups. It may provide a way of reducing compounds in the immediate environment of the cell; this might protect the cell or facilitate transport of certain compounds, e.g. disulfides. Transport of GSH to membrane-bound transpeptidase leads to γ-glutamyl amino acid formation, a process that is part of a transport system and which serves also in the recovery of the amino acid constituents of GSH.

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TRANSPORT OF γ-GLUTAMYL AMINO ACIDS

Indirect evidence for transport of γ-glutamyl amino acids (1, 2) has been supplemented by direct observation of γ-glutamyl amino acid uptake after their administration to mice. Uptake of L-γ-glutamyl-L-methionine sulfone, a poorly metabolized compound, by kidney was much greater than that of the corresponding free amino acid, and uptake was inhibited by γ-glutamyl amino acids but not by free amino acids (47). Appreciable amounts of L-γ-glutamyl-L-methionine sulfone were found in the kidney, and the data suggest that other tissues (liver, pancreas) may also transport γ-glutamyl amino acids. Under physiological conditions, γ-glutamyl amino acids might be hydrolyzed at the membrane and the amino acid products of such hydrolysis may be effectively transported.

However, in vivo studies show transport of intact γ-glutamyl amino acids (47) and there is increased formation of 5-oxoproline after suspension of rat kidney slices in media containing γ-glutamyl amino acids (48). This shows that γ-glutamyl amino acids are transported as such under these conditions, but does not exclude some membranous hydrolysis. Kidney and possibly other cells thus have a transport system for γ-glutamyl amino acids that is not shared by free amino acids. In the kidney, γ-glutamyl amino acid uptake takes place predominantly on the lumenal side rather than the basolateral side (28). As discussed later, there is good evidence that γ-glutamyl amino acids are formed by transpeptidation under physiological conditions.

Patients with transpeptidase deficiency exhibit glutathionuria and glutathionemia, as do animals given transpeptidase inhibitors (see above). When the urine of these patients and animals was treated with dithiothreitol and 2-vinylpyridine, chromatographic analysis showed, in addition to the vinylpyridine derivative of GSH, appreciable quantities of vinylpyridine derivatives of cysteine and γ-glutamylcysteine (16). The discovery of increased urinary excretion of GSH, γ-glutamylcysteine, and cysteine in both human and experimental animal γ-glutamyl transpeptidase deficiency suggests that the physiological function of transpeptidase is closely associated with the metabolism or transport (or both) of these compounds. Further studies showed that urinary γ-glutamylcyst(e)ine in animals treated with transpeptidase inhibitors is formed by the action of the residual γ-glutamyl transpeptidase (9). The patients are markedly deficient, but not altogether lacking in transpeptidase. Similarly, although the enzyme may be ~90% inhibited in the experimental animals, there is sufficient activity remaining to catalyze transpeptidation between GSH and cystine to form γ-glutamylcystine. It is relevant to note that cystine is an excellent acceptor substrate (K_m 30 μM) of transpeptidase (49–51).

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Thus, transpeptidation between GSH and cystine can occur in the presence of substantial transpeptidase deficiency or inhibition. An alternative explanation for the finding of urinary γ-glutamylcysteine, i.e. that cleavage of the cys-gly bond of GSH (or of GSSG) occurs, was rendered unlikely by studies with [^{35}S]GSH, and by failure to find such activity after an extensive search (9). When there is marked deficiency of transpeptidase, γ-glutamylcystine is apparently formed more rapidly than it is transported. The high extracellular levels of GSH that accompany inhibition of transpeptidase inhibit transport of γ-glutamyl amino acids (52); under these conditions, γ-glutamylcystine appears in the urine.

Additional evidence for transport of γ-glutamyl amino acids has come from studies in which administration of γ-glutamylcystine to mice increased renal GSH levels. The level of GSH in the kidney is regulated by feedback inhibition of γ-glutamylcysteine synthetase by GSH (53). When either γ-glutamylcystine or γ-glutamylcysteine disulfide was administered to mice, unusually high levels of renal GSH were found, evidently because the feedback-regulated step was bypassed (54). Thus, GSH levels of 6–7 mM were found in animals given γ-glutamylcystine or γ-glutamylcysteine disulfide as compared to values of 4.5–5.2 mM for untreated animals or controls given glutamate, cysteine and cystinyl-bis-glycine. These observations indicate transport of γ-glutamyl amino acids, and also suggest an alternative pathway of GSH biosynthesis.

Administration of γ-glutamylcystine or of γ-glutamylcysteine disulfide to animals previously treated with buthionine sulfoximine, to lower markedly the intracellular levels of GSH, had little effect on renal GSH levels, but when such animals were given γ-glutamylcysteine, a substantial increase in renal GSH occurred. When γ-glutamylcystine labeled selectively with ^{35}S in either the external or internal S atom were given to normal mice, the specific radioactivity of the renal GSH 15 min after giving the internally labeled compound was much greater than that found after giving the externally labeled compound (55). This result, obtained both with large doses and with tracer doses of the labeled γ-glutamylcystines, indicates that the administered γ-glutamylcystine is reduced to cysteine and γ-glutamylcysteine; the latter is converted directly to GSH. The experiments in which the animals were pretreated with buthionine sulfoximine indicate an apparent requirement for intracellular GSH for reduction of the administered disulfides. The findings support a metabolic-transport scheme (Figure 2) in which GSH is transported to a membrane site containing transpeptidase where it interacts with cystine to form γ-glutamylcystine. γ-Glutamylcystine is transported and reduced by transhydrogenation involving GSH. Extracellular GSH can inhibit γ-glutamylcystine transport, and intracellular GSH inhibits utilization of cysteine by γ-glutamylcysteine syn-

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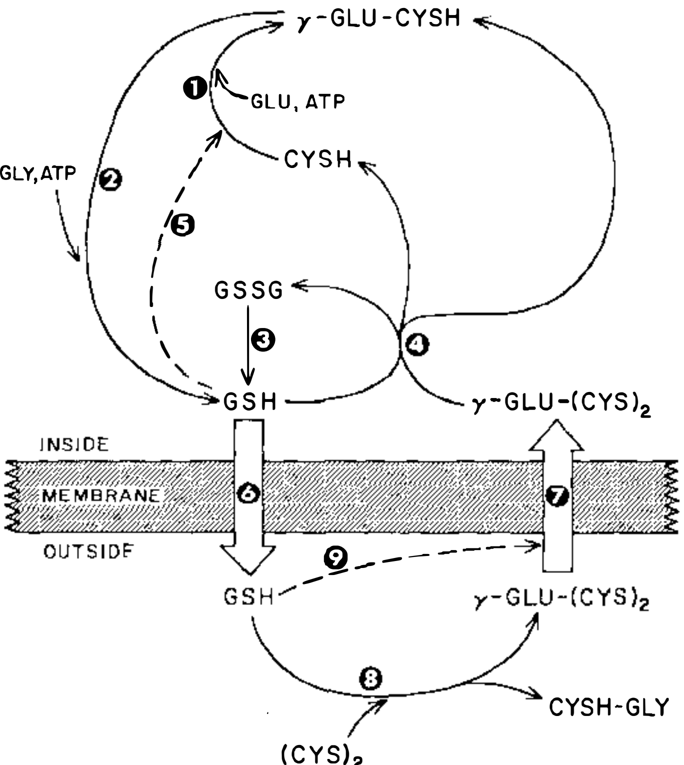

Figure 2 Metabolic-Transport Scheme (see text): 1. γ-glutamylcysteine synthetase; 2. GSH synthetase; 3. GSSG reductase; 4. transhydrogenation [GSH + γ-GLU-$(CYS)_2$]; 5. feedback inhibition by GSH of 1; 6. transport of GSH; 7. transport of γ-GLU-$(CYS)_2$; 8. γ-glutamyl transpeptidase; 9. inhibition by GSH of transport of γ-GLU-$(CYS)_2$.

thetase; thesc phenomena may function in regulation of GSH synthesis and transport.

FUNCTIONS OF THE γ-GLUTAMYL CYCLE

Since the cycle accounts for the biosynthesis of GSH, its functions are intimately associated with those of GSH, which include its roles in a variety of protective, metabolic, transport, and catalytic processes, in the regulation of GSH synthesis and utilization, and in the storage and transport of cysteine.

The hypothesis that the γ-glutamyl cycle functions in amino acid transport is based on the idea that γ-glutamyl amino acids are formed in or on the cell membrane through interaction of γ-glutamyl transpeptidase, intracellular GSH, and extracellular amino acids. That this mechanism

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constitutes one of the pathways that mediates transport of amino acids is strongly supported by the data reviewed above that demonstrate in vivo formation of γ-glutamyl amino acids and their transport. This pathway has not been proposed to function in all cells, or in a given cell for all amino acids (1, 2). Cystine and glutamine are the most likely candidates for this type of transport, but other amino acids may also participate. Amino acids are transported by "systems" (56). Although little is known about the mechanisms involved, there is evidence, based largely on competition studies, for several amino acid transport systems that overlap in specificity. Presently available data indicate that the γ-glutamyl amino acid pathway plays a role in amino acid transport; its relationship to other "systems" that have been postulated is not yet clear.

The arguments against the γ-glutamyl amino acid transport idea include: (*a*) equivalence between GSH turnover and amino acid transport has not been observed; (*b*) the transpeptidase functions in vivo solely as a hydrolase; (*c*) the distribution of transpeptidase activity in the renal tubule is not parallel to amino acid uptake; (*d*) blocks of the cycle are not always associated with defective amino acid transport; and (*e*) too much energy is needed.

The considerations reviewed above indicate that one would not expect equivalence between GSH turnover and amino acid transport, which some investigators have expected (57). With respect to the energy utilized, the export of GSH from cells, apparently an obligatory step in GSH turnover, followed by its extracellular or membranous breakdown, must require at least an amount of energy equivalent to the cleavage of two molecules of ATP to ADP. Cleavage of another molecule of ATP would be required if the process involves formation and utilization of 5-oxoproline. Possibly less energy than this would be needed if intracellular or membranous hydrolysis of γ-glutamyl amino acids occurs. The need for energy in this system is not unusually great when considered in relation to processes such as protein synthesis and urea formation (see 1).

Despite ample data indicating that transpeptidation is a physiologically significant function of γ-glutamyl transpeptidase, it has nevertheless been concluded (58, 59), that the sole function of this enzyme is to hydrolyze GSH and GSSG. This conclusion, which rests on invalid assumptions, has been dealt with in detail (60), but studies in two laboratories (58, 60) indicate the participation of about half of the GSH utilized by transpeptidase in transpeptidation. One study was based on an estimate (58). In another (60), the enzyme was incubated with GSH or GSSG and a mixture of amino acids approximating the amino acid composition of plasma, and the relative extents of transpeptidation and hydrolysis were determined by measuring the products formed. At pH 7 in the presence of 50 μM GSH and the amino acid mixture, about half of the GSH utilized participated in

transpeptidation; L-cystine and L-glutamine are the most active acceptors and other amino acids are also active. Thus, a major fraction of the GSH transported from cells participates in transpeptidation. Since membrane-bound transpeptidase must always be in close contact with amino acids and GSH, γ-glutamyl amino acids must be formed continuously; although they can also serve as substrates of transpeptidase, there is direct evidence for their transport into cells. There is a high concentration of transpeptidase in the renal brush border (about 0.1 mM), which is of about the same order as that of many amino acid acceptors. It is doubtful that parameters derived from Michaelis-Menten kinetics can be validly applied here. Nevertheless, it is of interest that the apparent K_m for L-cystine is lower than the plasma level of this amino acid.

Studies on the distribution of transpeptidase in segments of renal tubules showed the enzyme to be present in considerable concentration in both the proximal convoluted and straight segments, as well as in other regions of the nephron and elsewhere in the kidney (61, 62, 63). It is therefore unlikely that amino acids would escape the action of transpeptidase in the proximal convoluted tubule. Other considerations that support the conclusion that the transpeptidase functions in transpeptidation include the finding of γ-glutamyl amino acids in blood plasma (64; D. Wellner, unpublished), urine (65, 66), and various tissues (67–71), and the formation of γ-glutamyl amino acids by isolated renal tubules (72).

Dramatic defects in amino acid transport have not always been observed in humans or animals blocked at various steps of the cycle; this is not unexpected because the inhibitions thus far achieved are far from complete. Furthermore, there are multiple amino acid transport systems with overlapping specificity that can therefore supplement each other. Multiple overlapping mechanisms seem to have evolved for amino acid transport. There are other examples (e.g. deoxyribonucleotide synthesis) (see p. 738) in which multiple pathways have developed for important functions. Generalized aminoaciduria occurs in patients with severe γ-glutamylcysteine synthetase deficiency (73); cystinuria and possibly other defects in amino acid transport occur in γ-glutamyl transpeptidase deficiency (1, 16).

Renal GSH levels decrease when there is an appreciable increase in transpeptidation. Rats treated with glycylglycine, an excellent acceptor substrate, showed a marked decline in renal GSH levels (74). Administration of moderate amounts of amino acids to mice also led to a decline in the level of kidney GSH (11).[3] Such effects were not found when

[3] Administration of α-aminoisobutyrate, which is not a substrate of transpeptidase, also led to lower renal GSH levels and to increased plasma amino acid levels. The latter might contribute to a decrease in renal glutathione, but the effect of α-aminoisobutyrate was only partially blocked by serine plus borate suggesting that α-aminoisobutyrate may also act by another mechanism (11).

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mice were also treated with a mixture of serine and borate, a combination that effectively inhibits transpeptidase (75). Similar observations were made on isolated renal cells; suspension of the cells in media containing amino acids led to depletion of GSH (76). The GSH content of perfused kidneys decreases when the perfusate contains amino acids (77). Similar findings were made on the mammary gland (see below). The rate of turnover of liver GSH in intact animals increases after amino acid administration (78).

The rate at which the intracellular renal GSH level declines after giving an inhibitor of GSH synthesis is slowed by giving inhibitors of transpeptidase (11, 14). This finding, and those summarized above on the effects of amino acids on GSH levels and turnover indicate significant connections between amino acids, export of GSH, and transpeptidase. A full explanation of these phenomena will require, minimally, more detailed understanding of the membranous orientation of the transpeptidase, the mechanisms of GSH and γ-glutamyl amino acid transport, and associated cellular and membrane functions.

Several recent studies are relevant to the findings discussed above. An apparent association between amino acid transport and localization of transpeptidase in the intestine has been noted (79, 80); thus, the transpeptidase activity of villus tip cells is far greater than that of crypt cells, which are much less active in transport. The pattern of relative rates of uptake of various amino acids closely resembles the acceptor specificity pattern of the transpeptidase. Transpeptidase, 5-oxoprolinase, and γ-glutamylcysteine synthetase activities increase during lactogenesis in the rat mammary gland (81), and factors that affect transpeptidase activity in the gland induce parallel changes in amino acid transport (82, 83, 84). Plasma arterial-venous amino acid differences measured across the mammary gland were decreased by treatment with serine plus borate, AT-125, and L-γ-glutamyl-(*o*-carboxy)-phenylhydrazide; this effect was greatest on amino acids that are good acceptor substrates of transpeptidase. Incubation of isolated acini from lactating glands with high concentrations of amino acids led to decreased GSH levels, an effect prevented by serine plus borate. A correlation was found between length of lactation, increase of the amino acid arterial-venous difference, and transpeptidase activity. Similar correlations were noted in studies on the effects of milk accumulation and of hormones.

Sensitivity of certain cell lines to azaserine correlates with transpeptidase activity (85). Azaserine-resistant cells are less efficient in concentrating azaserine than the sensitive parental lines. Human Wilms' tumor cells are sensitive to azaserine, but azaserine-resistant strains derived from these cells have greatly reduced transpeptidase activity. The findings suggest that azaserine may be transported into certain cells as the γ-glutamyl derivative.

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Kalra and colleagues (86–88) incorporated detergent-isolated renal transpeptidase into phospholipid vesicles containing entrapped GSH; the vesicles took up glutamate, but not proline, and uptake, which was inhibited by inhibitors of transpeptidase, was associated with internal γ-glutamylglutamate formation. Transpeptidase was also incorporated into human erythrocyte membranes, and such modified cells took up alanine and glutamate but not methionine; uptake was inhibited by transpeptidase inhibitors. This system seems to be an interesting and potentially valuable model for further study of transpeptidase-mediated transport.

Several modifications of the γ-glutamyl cycle have been considered (2). For example, a model was suggested in which γ-glutamylcysteine (or another γ-glutamyl amino acid formed by transpeptidation) might function in an exchange diffusion system or in an active transport system (1).

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ENZYMES OF THE γ-GLUTAMYL CYCLE

γ-Glutamylcysteine Synthetase

This enzyme has been purified from several sources (89, 90), and many of its structural and catalytic properties have been examined, expecially in work on the rat kidney enzyme (91). The enzyme is inhibited nonallosterically by GSH under conditions similar to those that prevail in vivo; this indicates a physiologically significant feedback mechanism (53). GSH also inhibits the γ-glutamylcysteine synthetase activities of fetal liver and Novikoff hepatoma (92). Treatment of rats with sodium selenite increases the activity of liver γ-glutamylcysteine synthetase, an apparent effect of increased enzyme synthesis (93). Decreased γ-glutamylcysteine synthetase occurs in a human inborn error of metabolism (see p. 747) and also in the erythrocytes of certain GSH-deficient sheep (94, 95).

The acceptor amino acid specificity of the enzyme is rather broad, and it also interacts with several glutamate analogs including β-aminoglutarate (91), and α-aminomethylglutarate (96). The mechanism of the reaction involves formation of enzyme-bound γ-glutamyl phosphate and interaction of the latter with the amino group of cysteine (89, 97). Kinetic investigations are consistent with this possibility, but did not demonstrate γ-glutamyl phosphate as a discrete covalent complex (98). Methionine sulfoximine is an effective irreversible inhibitor of the enzyme and in the presence of MgATP is converted to methionine sulfoximine phosphate, which binds tightly to the enzyme (99). Of the four stereoisomers of methionine sulfoximine, only L-methionine-S-sulfoximine inhibits, the same stereoisomer that irreversibly inhibits glutamine synthetase and causes convulsions in animals (100). Chemically synthesized L-methionine sulfoximine phosphate inhibits γ-glutamylcysteine synthetase; the mechanism of inhibition is thus similar to

that previously shown for glutamine synthetase. However, methionine sulfoximine phosphate binds less tightly to γ-glutamylcysteine synthetase than it does to glutamine synthetase, and under certain conditions the inhibitor can be released from the inhibited enzyme with restoration of catalytic activity. The findings are consistent with intermediate formation of enzyme-bound γ-glutamyl phosphate, since phosphorylation of methionine sulfoximine seems to reflect phosphorylation of glutamate in the normal reaction. α-Ethylmethionine sulfoximine induces convulsions in mice and inhibits glutamine synthetase irreversibly, but it does not inhibit γ-glutamylcysteine synthetase (101). The reciprocal goal of inhibiting γ-glutamylcysteine synthesis without perturbing glutamine synthesis was achieved by preparing inhibitors in which the methyl moiety of methionine sulfoximine was replaced by propyl and butyl moieties, i.e. prothionine sulfoximine (102) and buthionine sulfoximine (103). A number of other sulfoximine analogs of methionine sulfoximine were also prepared and studied (103). Buthionine sulfoximine inhibits the enzyme more effectively than prothionine sulfoximine and at least 100 times more effectively than methionine sulfoximine. The data suggest that the S-alkyl moiety of the sulfoximine binds at the enzyme site that normally accepts L-cysteine. α-Methylbuthionine sulfoximine is almost as effective as buthionine sulfoximine (103). The higher homologs, penta-, hexa- and heptathionine sulfoximine, also inhibit the enzyme (104). The hexa- and heptathionine derivatives are unexpectedly toxic for reasons not yet understood. Buthionine sulfoximine, like methionine sulfoximine (99) is phosphorylated at the active site of γ-glutamylcysteine synthetase (104).

The enzyme is also inhibited by cystamine (105, 106), the optical isomers of 3-amino-1-chloro-2-pentanone (107) (the α-chloroketone analogs of L- and D-α-aminobutyrate), and L-2-amino-4-oxo-5-chloropentanoate (108). A sulfhydryl group at the active site interacts with the chloroketone inhibitors and also with cystamine; these compounds bind to the enzyme as glutamate analogs. The possibility that a γ-glutamyl-S-enzyme intermediate may be formed in the reaction needs to be considered. There is evidence that divalent metal ions play a role in the binding of amino acid substrates.

γ-Glutamylcysteine synthetase binds covalently to cystamine-Sepharose, an interaction facilitated by ATP and inhibited by Mg^{2+} plus glutamate (109). A large fraction of the enzyme applied to such columns binds apparently by forming a disulfide bond between cysteamine-Sepharose and a sulfhydryl group at or near the active site. The enzyme may be released by treatment with dithiothreitol. The enzyme does not bind to columns of S-(S-methyl)cysteamine-Sepharose, whereas free S-(S-methyl)cysteamine is a potent inhibitor. A cysteamine-S disulfide moiety derived from the external cysteamine residue of cystamine-Sepharose

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seems to be the critical group recognized by the enzyme. Studies with a number of cystamine analogs supported this conclusion and led to the further conclusion that a disulfide (or diselenide) moiety and a single free amino group are required for inhibition (G. F. Seelig, A. Meister, see 110).

γ-Glutamylcysteine synthetase from rat kidney has a single disulfide bond and two free sulfhydryl groups per M_r 100,000. Only one SH-group of the native enzyme is titrable with DTNB, and such reaction does not affect enzyme activity (G. F. Seelig, A. Meister, see Ref. 110). The enzyme (M_r 100,000) can be dissociated by treatment with SDS and thiol into heavy ($M_r \sim 74{,}000$) and light ($M_r \sim 24{,}000$) subunits; after the enzyme is cross-linked with dimethylsuberimidate, a species of $M_r \sim 100{,}000$ is obtained (108). Dissociation of the native enzyme by treatment with dithiothreitol yields a heavy subunit that exhibits full enzymatic activity and inhibition by GSH. Reassociation of the subunits has also been achieved (G. F. Seelig, A. Meister, see Ref. 111); the function of the light subunit needs additional study.

Glutathione Synthetase

This enzyme has been purified from several sources (89). There is evidence that enzyme-bound γ-glutamylcysteinyl phosphate is formed in the reaction, whose mechanism is thus similar to those catalyzed by γ-glutamylcysteine and glutamine synthetases. The most highly purified preparation was obtained from rat kidney by a method involving ATP-affinity chromatography (112). The enzyme (M_r 118,000) has two apparently identical subunits. The rat kidney enzyme is about 20 times more active than the apparently homogeneous preparation of this activity from erythrocytes. The regions of the active site that bind glycine and the cysteine moiety of L-γ-glutamyl-L-cysteine are highly specific. On the other hand, the enzyme acts on substrates in which the L-γ-glutamyl moiety of the dipeptide substrate is replaced by D-γ-glutamyl, β-aminoglutaryl, monomethyl (α-methyl, β-methyl, γ-methyl, *N*-methyl) glutamyl, glutaryl, and *N*-acetyl moieties.

γ-Glutamyl Transpeptidase

γ-Glutamyl transpeptidase (113, 114), an enzyme of major importance in GSH metabolism, initiates GSH degradation. It can catalyze three types of reactions: (*a*) transpeptidation, in which the γ-glutamyl moiety is transferred to an acceptor; (*b*) autotranspeptidation, in which the γ-glutamyl moiety is transferred to GSH to form γ-glutamyl-GSH; and (*c*) hydrolysis, in which the γ-glutamyl moiety is transferred to water. GSH, GSSG, S-substituted GSH, and other γ-glutamyl compounds are substrates. The L-isomers of

cystine, methionine, and other amino acids, as well as many dipeptides, especially aminoacylglycines, are good acceptors.

Histochemical studies indicate substantial transpeptidase activity in the membranes of cells that exhibit secretory or absorptive functions such as the epithelial cells of proximal renal tubules, jejunum, biliary tract, epididymis, seminal vesicles, choroid plexus, ciliary body, retinal pigment membrane, bronchioles, thyroid follicles, canalicular region of hepatocytes, pancreatic acinar and ductule cells (61); it is present in microvillus membranes. Such activity has been found on human lymphoid cell membranes (115), endoplasmic reticulum and Golgi, and there is evidence for its presence in renal basolateral membranes (24).

Highly purified enzyme preparations have been obtained from kidney of several species and from other tissues; kidney exhibits the highest activity, followed by pancreas, epididymis, seminal vesicle, jejunal epithelial cells, liver, and spleeen (114). This activity has also been found in soluble form in urine, seminal fluid, and pancreatic juice (29); there is little present in rat bile. The blood plasma of rats and mice is devoid of activity, but the enzyme is detectable in human plasma, where its elevation is of some clinical value in detection of liver disease (116, 117). Several γ-glutamyl substrates suitable for convenient determination of its activity have been developed. The most widely used is L-γ-glutamyl-*p*-nitroanilide (118). Useful spectrophotometric methods based on S-derivatives of GSH are also available (1, 119), and the fluorogenic substrate, L-γ-glutamyl-7-amino-4-methylcoumarin also provides a sensitive assay (120).

The enzyme has a donor site that interacts with both L- and D-γ-glutamyl compounds. The acceptor site consists of subsites for the cysteinyl and glycine moieties of cysteinylglycine; L-amino acids bind to the former, which prefers amino acids such as the L-isomers of cystine, glutamine, and methionine. Branched chain amino acids and aromatic amino acids are bound with less affinity. Kinetic studies are consistent with a ping-pong mechanism involving a γ-glutamyl-enzyme (1). The various complexities of the catalytic reaction have led to a number of interesting kinetic studies. Recently, it was found that compounds such as maleate (1), hippurate (121), various bile acids and their conjugates (S. J. Gardell, S. S. Tate, see Ref. 122; W. Abbott, A. Meister, see Ref. 123), as well as phospholipids (124), can modulate relative extents of hydrolysis and transfer.

The enzyme may be solubilized by treatment with a detergent or organic solvent or with proteinases (113, 114, 125, 126). The purified preparations are glycoproteins and exhibit heterogeneity associated with the presence of isozymes containing different amounts of sialic acid. The proteinase-solubilized rat kidney enzyme (M_r 68,000) consists of two subunits (M_r 46,000, 22,000). The M_r 22,000-subunit contains the active site residue

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(probably on OH group) involved in formation of the γ-glutamyl-enzyme intermediate. Although there is relatively little species variation in size of the light subunit, the transpeptidases fall into two groups with the respect to the heavy subunit: (M_r 46,000–50,000) rat and rabbit; $M_r > 60,000$ (bovine, sheep, hog, and human). Treatment of the rat kidney enzyme with urea at neutral pH leads to digestion of the heavy chain (owing to latent proteinase activity of the light subunit (127)); however, if treatment with urea is carried out in the presence of acetic acid, the subunits are not significantly degraded and their reconstitution may be achieved (128) with return of activity.

The heavy subunit of the detergent-solubilized enzyme differs from that of the protease-treated enzyme; the former contains a short, relatively hydrophobic, protease-sensitive amino terminal segment that probably anchors the enzyme to the cell membrane (125, 126). The molecular weights of the corresponding heavy subunits are estimated to be 51,000 and 46,000. The light subunit, which does not appear to interact directly with the lipid bilayer, is bound noncovalently to the heavy subunit. The biosynthesis of the enzyme has been studied in kidney slices suspended in media containing L-[^{35}S]methionine (129). In studies involving immunoprecipitation with antitranspeptidase antibodies, a protein (M_r 78,000) was identified that appears to be a precursor of the two enzyme subunits. The evidence suggests that this protein is initially synthesized in renal cells and that its cleavage occurs prior to transport of the enzyme to the brush border membrane (130).

The enzyme is inhibited by L-serine plus borate, owing to formation of a tetrahedral borate complex, a transition state analog (75). Other inhibitors include γ-glutamylhydrazones of α-keto acids (131) and various γ-glutamylphenylhydrazides (14). The L- and D-isomers of γ-glutamyl-(*o*-carboxy)phenylhydrazide (14, 132) are effective competitive inhibitors. Glutamine antagonists such as 6-diazo-5-oxo-L-norleucine, L-azaserine, and L-(αS, 5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid (AT-125) are effective irreversible inhibitors of the enzyme and serve as affinity labels of the γ-glutamyl site (16, 133, 133a, 134, 113, 114). Of these compounds, AT-125 is the most effective. As discussed above, when potent transpeptidase inhibitors are given to mice, glutathionuria occurs. Glutathionuria also results from administration of γ-glutamyl amino acids, presumably an effect of competitive inhibition (135).

γ-Glutamyl Cyclotransferase

This soluble enzyme, which catalyzes Reaction 4 (Figure 1), is widely distributed in mammalian tissues (1), and is highly active toward the L-γ-glutamyl derivatives of glutamine, alanine, cysteine, cystine, methionine, and several other amino acids. In general, its substrate specificity parallels

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the amino acid acceptor specificity of γ-glutamyl transpeptidase, supporting the idea that the cyclotransferase and transpeptidase function in sequence in metabolism. The enzyme also acts on di-γ-glutamyl amino acids; indeed, it is much more active toward certain di-γ-glutamyl amino acids than toward the corresponding γ-glutamyl amino acids. For example, the enzyme acts on di-L-γ-glutamyl-L-proline, but has no detectable activity toward L-γ-glutamyl-L-proline. Similar findings were made with the corresponding derivatives of valine, tyrosine, and leucine. On the other hand, the enzyme exhibits a high order of activity toward both the L-γ-glutamyl and di-L-γ-glutamyl derivatives of glutamine and alanine.

The enzyme has been purified from human and sheep brain (136), hog (137) and rat (138) liver, human erythrocytes (139) and rat kidney (140). Several forms of the enzyme, separable by ion exchange chromatography and electrophoresis, have been found. The enzyme is relatively unstable and undergoes substantial changes in physical and catalytic properties during preparation and storage. The most highly purified preparation, obtained from rat kidney (140), contains several readily accessible SH groups, whose modification is associated with appearance of multiple enzyme forms. The enzyme was obtained, after 1000-fold purification, in apparently homogeneous form by a procedure involving treatment with dithiothreitol followed by chromatography on thiol-Sepharose. It was also isolated in highly active stable form after reduction and treatment with iodoacetamide. It appears that most of the cyclotransferase activity of rat kidney is associated with a single protein. Apparent multiple forms may be produced by mechanisms involving intramolecular disulfide bond formation, perhaps associated with conformational changes in the protein, and also by mixed disulfide formation with low-molecular-weight thiols. Such changes might be involved in regulation of the enzyme.

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5-Oxoprolinase

This enzyme catalyzes the ATP-dependent cleavage of 5-oxoproline to glutamate:

$$\text{5-Oxo-L-proline} + \text{ATP} + 2\text{H}_2\text{O} \underset{\text{K}^+(\text{NH}_4^+)}{\overset{\text{Mg}^{2+}}{\rightleftharpoons}} \text{L-glutamate} + \text{ADP} + \text{P}_i$$

The ATP requirement is mandated by the unusual stability of the 5-oxoproline amide bond. 5-Oxoprolinase has been found in mammalian tissues (141), plants (142), and microorganisms (143). 5-Oxo-L-proline is formed by the action of γ-glutamyl cyclotransferase on γ-glutamyl amino acids, by enzymatic cleavage of amino terminal 5-oxo prolyl residues of

peptides and proteins, and by the action of γ-glutamylamine cyclotransferase (144) on ε-(L-γ-glutamyl)-L-lysine derived from the degradation of proteins containing transglutaminase-generated cross-links. 5-Oxoproline may also arise from dietary sources and be formed by nonenzymatic cyclization of γ-glutamyl compounds such as glutamine. 5-Oxoprolinase is found in virtually all mammalian tissues except the erythrocyte and the lens.[4] The enzyme, which was isolated in apparently homogenous form from rat kidney in 50% yield after 1700-fold purification (M_r 325,000), is composed of two apparently identical subunits (146). [An earlier report (147) of M_r 420,000 is probably incorrect.] The enzyme contains 27 SH groups per monomer, six of which can be titrated in the native enzyme, and two of which are required for catalysis (146). At least one SH is at or close to the nucleoside triphosphate (NTP) binding site. The enzyme can bind 5-oxo-L-proline in the absence of NTP, and it can bind NTP in the absence of 5-oxo-L-proline. When ATP is replaced by other NTP's such as ITP or UTP, there is rapid cleavage of the NTP in the absence of 5-oxoproline (148, 149). When 5-oxoproline is added to enzyme catalyzing hydrolysis of NTP, there is a slow time-dependent decrease in the V_{max} and an increase in the K_m for NTP. These findings suggest that 5-oxoprolinase is a hysteretic enzyme, and that in the normal reaction, binding of both ATP and 5-oxoproline induces a conformational change that brings the substrates into a juxtaposition that facilitates the reaction.

The 2 hydrolytic activities of 5-oxoprolinase may be uncoupled by: (*a*) substitution of ATP by certain other NTP's; (*b*) replacement of 5-oxoproline by certain analogs; or (*c*) substitution for Mg^{2+} by Mn^{2+} (in excess), Ca^{2+}, or Co^{2+} (150, 146). Modification of the enzyme by treatment with *N*-ethylmaleimide, 5,5′-dithiobis-(2-nitrobenzoic acid), 5′-*p*-fluorosulfonylbenzoyl adenosine, or 5′-*p*-fluorosulfonylbenzoyl inosine leads to loss of all catalytic activities; inactivation is reduced by either ATP or ITP (146). The enzyme does not catalyze ATP-ADP exchange reactions. Studies with ^{18}O-labeled substrates show that: (*a*) all three oxygens of 5-oxoproline are recovered in glutamate; and (*b*) the two water molecules used contribute one oxygen atom to P_i and one to the γ-carboxyl of glutamate (151). The enzyme also catalyzes the intrinsically exergonic hydrolysis of α-hydroxyglutarate lactone, a reaction that is ATP-dependent. Although conversion of 5-oxo-L-proline to L-glutamate by 5-oxoprolinase is essentially irreversible, very slow reversal was shown by

[4] Evidence for the occurrence of 5-oxoprolinase in rabbit lens has been reported (145); since this activity was reported not to require ATP, thermodynamic considerations suggest that the observed reaction may not be due to 5-oxoprolinase and that another explanation for the findings should be sought.

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measuring ATP formation in the presence of high glutamate concentrations. The most probable mechanism is one in which 5-oxoproline is phosphorylated by ATP on the amide carbonyl oxygen followed by hydrolysis of the resulting intermediate to yield γ-glutamyl phosphate, which is then hydrolyzed to glutamate and P_i.

The reaction has been studied with a variety of 5-oxoproline analogs, which fall into three categories (150, 152): (*a*) analogs that promote ATP cleavage but are not themselves hydrolyzed; (*b*) analogs whose hydrolysis is partially coupled to cleavage of ATP, i.e. molar formation of ADP exceeding that of imino acid; and (*c*) analogs that participate in fully coupled reactions. Mapping studies show that binding of imino acid substrate requires a 5-carbonyl (or =NH) moiety, an unsubstituted N_1 atom, and a C_2 atom of L-configuration; substantial modification of the 5-oxo-L-proline molecule in the region of C_3 and C_4 atoms is possible with retention of binding properties. Uncoupling phenomena seem to be associated with significant differences in orientation of analogs at the active site, which facilitate access of water to the nucleoside triphosphate.

The 5-oxo-L-proline analog in which the 4-methylene moiety is replaced by S (i.e. L-2-oxothiazolidine-4-carboxylate) is a good substrate (152, 153, 154). It has high affinity for the enzyme and is rapidly converted to L-cysteine, presumably through formation and nonenzymatic breakdown of S-carboxy-L-cystine. When L-2-oxothiazolidine-4-carboxylate is administered to mice, liver GSH increases suggesting that administration of this thiazolidine would be an effective cysteine delivery system. Recent studies show that L-2-oxothiazolidine-4-carboxylate is more effective than *N*-acetyl-L-cysteine in stimulating GSH synthesis. Protection against acetaminophen toxicity (153, 154) is mediated through increased synthesis of GSH. Thus, administration of buthionine sulfoximine inhibits the increase in hepatic GSH found after L-2-oxothiazolidine-4-carboxylate is given. This thiazolidine may be useful as a component of amino acid mixtures used in diets and in parenteral administration. Most such preparations do not now contain cysteine because of the toxicity of this amino acid (155–158) and because it is rapidly oxidized to the very insoluble cystine. The thiazolidine may also be useful for the growth of cells sensitive to the toxic effects of cysteine (158), and as a safener in agriculture to protect crop plants against herbicides.

Dipeptidase

Few studies have been carried out on the activities that cleave cysteinylglycine, the disulfide form of this dipeptide, and S-substituted cysteinylglycine derivatives. Dipeptides of this type are split by aminopeptidase M, an activity concentrated in the kidney and jejunal microvillus membranes

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(159, 160, 161). This enzyme splits dipeptides and a variety of larger peptides. The finding of relatively little aminopeptidase M in rat epididymis led to the search for another activity that might be responsible for the cleavage of dipeptides released in the course of GSH metabolism (162). A membrane-bound dipeptidase was isolated from rat kidney that exhibits a specific activity toward L-alanylglycine, which is about 70-fold greater than that shown by aminopeptidase M. This dipeptidase is much more active than aminopeptidase M in catalyzing hydrolysis of substrates such as S-methylcysteinylglycine, cystinyl-bis-glycine and leukotriene D4 (10, 162). The enzyme is present in the microvillus membranes of rat kidney, jejunum, and epididymis. The kidney enzyme (M_r 100,000) has two apparently identical subunits; it contains about two gram atoms of Zn per subunit. Neither this dipeptidase nor aminopeptidase M exhibits much activity toward L-cysteinylglycine. This dipeptide, formed in transpeptidation of GSH, may be transported and hydrolyzed intracellularly. Several studies show that there is substantial cytosolic dipeptidase activity (e.g. 163). Extracellular oxidation of L-cysteinylglycine may also occur.

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INTERCONVERSION OF GLUTATHIONE AND GLUTATHIONE DISULFIDE

Glutathione Oxidation

Elvehjem et al (164) found that mouse kidney homogenates catalyze O_2-dependent conversion of GSH to GSSG, that this reaction is inhibited by cyanide, and that kidney homogenates are much more active than those of liver. Orrenius et al (165, 166) confirmed these findings in isolated rat kidney and liver cells and obtained evidence for stepwise enzymatic breakdown of GSSG. Conversion of GSH to GSSG was ascribed to a "GSH oxidase". Tate et al (167) found such oxidase activity in kidney, epididymis, jejunum, choroid plexus and other tissues, and that this activity is membrane-bound and associated with renal and jejunal brush borders; its distribution follows a pattern similar to that of γ-glutamyl transpeptidase. Efforts to separate the two renal activities failed. Highly purified preparations of rat kidney transpeptidase were found to exhibit oxidase activity, as did each of the 12 isozymic forms of this enzyme (167, 168). Griffith & Tate (169) showed that the apparent GSH oxidase activity of transpeptidase is mediated by the cysteinylglycine formed in the transpeptidase-catalyzed degradation of GSH. This dipeptide is rapidly and nonenzymatically oxidized to form cystinyl-bis-glycine. The oxidation of GSH takes place by nonenzymatic transhydrogenation between GSH and cystinyl-bis-glycine (or between GSH and the mixed disulfide between cysteinylglycine and GSH). Such oxidation, which is inhibited by EDTA, is undoubtedly mediated by metal

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ions. In support of this mechanism, it was found that a covalent inhibitor of transpeptidase essentially abolishes both transpeptidase and oxidase activities of purified transpeptidase as well as of kidney homogenates. Notably, spontaneous oxidation of cysteinylglycine is more rapid than that of cysteine; in comparison, GSH reacts sluggishly with oxygen. This mechanism accounts for the oxidation of GSH observed with purified preparations of transpeptidase; thus, oxidation is *not* mediated by the transpeptidase, but rather by cysteinylglycine. Only a very low concentration of this dipeptide is needed to mediate GSH oxidation, and therefore there may or may not be a direct relationship between "oxidase" activity and the amount of transpeptidase. Indeed, a small amount of transpeptidase is sufficient to generate enough cysteinylglycine to catalyze rapid GSH oxidation; in the presence of large amounts of transpeptidase, oxidation of GSH may be greatly reduced because of substantial enzymatic degradation of GSH. These considerations are relevant to subsequent studies in which the finding of partial separations of transpeptidase and "GSH oxidase" activities were considered as evidence for existence of a separate renal GSH oxidase (170, 171, 172). The finding of GSH oxidation in kidney perfusion studies led to the conclusion that the oxidase is localized on the basolateral side of the renal cell (173), but this region apparently has transpeptidase (24). The reported resolution of a renal sulfhydryl oxidase from transpeptidase (170) is of doubtful validity because the fraction exhibiting "oxidase" activity was not shown to contain protein. Whether copper is involved in this oxidation, as suggested (171), could be determined if the enzyme had been isolated and analyzed.

Although GSH oxidation can be explained by nonenzymatic phenomena, kidney and other tissues may contain proteins capable of catalyzing this reaction; there is evidence consistent with this. Sulfhydryl oxidases have been isolated from milk (174, 175), kidney (176), and rat seminal vesicle secretions (177). Although even the most purified of these (176) exhibits some transpeptidase activity, these proteins may oxidize thiols directly. Trace metals and heme compounds can oxidize thiols rapidly, as might several metalloproteins as well. These considerations indicate that in all probability the cysteinylglycine-mediated mechanism (169) explains the oxidation of GSH catalyzed by purified transpeptidase as well as that observed in kidney homogenates (164) and with suspensions of kidney cells (165, 166). An aspect of this problem that needs more attention relates to the specificity for various thiols.

Since 10–15% of the total glutathione of rat and mouse blood plasma may be GSSG, some oxidation of GSH seems to occur in vivo, but the significance of this is not clear. The H_2O_2 formed could serve a protective function by destroying microorganisms. GSSG formation may promote

GSH translocation (14, 15) and provide a substrate for cystine formation (25).

Glutathione Peroxidase

This enzyme, present in a number of tissues, catalyzes GSH-dependent reduction of H_2O_2 (Figure 1, Reaction 9). The reduction of H_2O_2 in erythrocytes in the presence of GSH and GSH peroxidase is coupled with oxidation of glucose-6-phosphate and of 6-phosphogluconate, which provides NADPH for reduction of GSSG by GSSG reductase. This is a major pathway of H_2O_2 metabolism in many cells, and one which also catalyzes reduction of other peroxides (7, 178, 179, 180). It is thus important for the protection of membrane lipids against oxidation. Intermediates such as O_2^- and H_2O_2 are formed extensively in biological systems, and these produce reactive oxygen species that can lead to organic peroxide formation (181). Increased oxygen tension leads to increased formation of reactive oxygen species, and this may cause an increase in formation and release of GSSG. This subject has been extensively reviewed (179, 181, 182). Reactive oxygen species may produce other deleterious effects including those associated with mutagenesis. GSH peroxidase also plays a role in the biosynthesis of prostaglandins and in the regulation of prostacyclin formation (180).

GSH peroxidase of beef erythrocytes (M_r 85,000), consists of four apparently identical subunits, each of which contains one atom of selenium (180). It has long been known that Se is an essential nutrient. Se-deficient animals have markedly decreased GSH peroxidase activity. The reduced form of GSH peroxidase appears to contain selenocysteine selenol (–SeH) at the active site (180, 183–185a).

Another type of GSH peroxidase activity found in rat liver (186, 187) is unaffected by Se deficiency and exhibits much less activity toward H_2O_2 as compared to organic hydroperoxides. Studies with a hemoglobin-free liver perfusion system led to evidence that the Se-independent GSH peroxidase (a GSH S-transferase) reduces organic hydroperoxides, but does not function in reduction of H_2O_2. In this system, both Se-deficient and control liver preparations released GSSG after infusion of tert-butyl hydroperoxide.

Glutathione Transhydrogenases

Many metabolic and physiological functions involve thiol-disulfide exchange, e.g. protein synthesis, protein degradation, activation and inactivation of enzymes, synthesis of the deoxyribose intermediates required for DNA synthesis, reduction of cystine. It has long been thought that many such reactions involve participation of GSH, since it is the major

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intracellular thiol, and because GSSG reductase is widely distributed. Early reports described a beef liver activity that catalyzes GSH-dependent reduction of homocystine (188), and a similar system in yeast that catalyzes reduction of cystine and related compounds (189). Later, evidence was obtained for several apparently separate enzymes of low specificity that catalyze thiol-disulfide interchange between GSH and low-molecular-weight disulfides, as well as between GSH and protein disulfides (190). Attention has also been directed toward systems that catalyze reduction and rearrangement of protein disulfide bonds. GSSG reductase, contrary to early reports, does not directly catalyze such reductions, which seem to be mediated by separate transhydrogenases. Transhydrogenase activities are widely distributed, and the task of sorting out and identifying the individual catalysts involved, which are undoubtedly of major physiological importance, is being pursued in several laboratories. Because many varied substrates participate in thiol-disulfide interchange reactions, there may be many transhydrogenases. On the other hand, the very large number of reactions involved in the assembly of proteins, regulation of enzymes, and reductive degradation of proteins, makes it necessary to consider that the number of individual transhydrogenases may be much smaller than the number of reactions catalyzed. Freedman (191) has considered the idea (among others) that there may be separate enzymes for each of four reaction types.

Protein disulfide isomerase activity was first observed in studies on reactivation of reduced pancreatic ribonuclease (192–198). Several investigators (199–205) purified an activity from liver that catalyzes reductive cleavage of the disulfides of insulin in the presence of GSH. Both protein disulfide isomerase and GSH-insulin transhydrogenase activities were found to copurify (206–208), although some differences in the activity ratios were noted (209, 210). A recent characterization of thiol-protein disulfide oxidoreductase from rat liver (198) was based on an earlier procedure for purification of this activity from bovine liver (211). Comparison of the physicochemical properties of these enzymes with those previously isolated from the same and other sources showed substantial similarity, and suggests that thiol protein disulfide oxidoreductase, protein disulfide isomerase, and GSH-insulin transhydrogenase may be identical.

The physiological functions associated with this protein are not yet clear. Evidence that the widely distributed GSH-insulin transhydrogenase activity functions in insulin degradation has been published (212–216). Thiol protein oxidoreductase of liver and plasmacytoma catalyzes formation of interchain disulfides of monomeric immunoglobulin M (217), and the liver enzyme catalyzes activation of choleragen by reductive cleavage of the disulfide bond linking A_1 and A_2 peptides of the toxin (218). The enzyme

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has been localized in the endoplasmic reticulum of all sources examined. The findings are consistent with a role in the synthesis of protein disulfide bonds (197), but the enzyme may also be involved in enzyme regulatory mechanisms (219) and in protein degradation (212–216).

Although GSH transhydrogenases probably have important functions that relate to the synthesis, structure, degradation, and function of proteins, other systems that affect the thiol-disulfide status of cells are also of considerable importance. These include the widely distributed thioredoxin system, which exhibits high reducing potential (220, 221). Formation of cellular disulfides is also an important process in which GSSG might play a role. Ziegler and collaborators (222–224) proposed that oxidation of cysteamine to cystamine, catalyzed by a membrane-bound monoxygenase, might serve as a source of cellular disulfides.

Reichard and collaborators (220, 225), showed that deoxyribonucleotides are formed by direct reduction of ribonucleotides, and isolated the proteins involved, i.e. ribonucleotide reductase, thioredoxin, and thioredoxin reductase. A mutant of *Escherichia coli* was later found that lacked thioredoxin, but which is active in NADPH-dependent ribonucleotide reduction (226). In this mutant, GSH and glutaredoxin (which has been purified to homogeneity from cells of the wild type), function in the reduction (226–229). Glutaredoxin is an acidic protein (M_r 12,000) that exhibits high and specific transhydrogenase activity. *E. coli* ribonucleotide reductase is composed of two nonidentical subunits, protein B1 (M_r 160,000), and protein B2 (M_r 78,000), neither of which is active separately (220). The subunits combine in the presence of Mg^{2+} to yield active enzyme, which can reduce all four ribonucleoside diphosphates to the corresponding deoxyribose compounds. Protein B1 contains two ribonucleotide binding sites and binding sites for four allosteric effectors (nucleoside triphosphates) as well as thiol groups which, together with protein B2, mediate stoichiometric reduction of substrate in the absence of GSH. Protein B2 contains two nonheme iron atoms and an organic-free radical on a tyrosine residue. The catalytic site of the reductase is formed from both subunits and involves the free radical moiety and a dithiol moiety of protein B1 (228). Chemically reduced glutaredoxin is enzymically active in conversion of CDP to dCDP. Holmgren et al (229) showed that, in the presence of glutaredoxin, the substrates for ribonucleotide reductase are GSH and each of the four ribonucleoside 5′-diphosphates. Glutaredoxin contains about 89 amino acid residues including two half-cystine residues that form a single disulfide bridge that is readily reduced to a dithiol by GSH, NADPH and GSSG reductase. Such reduction does not occur with NADPH and thioredoxin reductase. Glutaredoxin and thioredoxin are structurally unrelated proteins, and apparently are separate gene products.

Highly purified glutaredoxin has GSH-disulfide dehydrogenase activity toward 2-hydroxyethyl disulfide, but this accounts for less than 1% of this type of transhydrogenase activity of crude extracts of *E. coli.*

Glutaredoxin has also been purified to homogeneity from calf thymus (230, 231). This protein ($M_r \sim 11{,}000$) is similar but not identical to the *E. coli* protein. It also exhibits GSH-disulfide transhydrogenase activity. However, thymus, in contrast to *E. coli*, appears to have only one cytoplasmic type of transhydrogenase, which is active with glutaredoxin, 2-hydroxyethyl disulfide, L-cystine, and several other substrates.

Interestingly, loss of the thioredoxin system in a mutant of *E. coli* does not lead to loss of ribonucleotide reduction (226), and GSH-deficient mutants grow at normal rates (see p. 745); thus, either pathway alone can mediate ribonucleotide reduction.

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Glutathione Reductase

This widely distributed flavoprotein, which catalyzes Reaction 11 (Figure 1) was first observed in the 1930s and later purified from several sources (7). Reaction 11 is essentially irreversible; this reaction accounts for the very high GSH:GSSG ratios found in cells. Although early studies suggested that the enzyme can catalyze reduction of several mixed disulfides between GSH and other compounds including proteins, it now appears that only GSSG, mixed disulfides between GSH and γ-glutamylcysteine (16), and between GSH and coenzyme A (232) are significant substrates. New methods have been described for the isolation of the enzyme from liver [calf (233), mouse (234), rabbit (235), rat (236)], and erythrocytes [pig (237), human (238–240)]. Extensive amino acid sequence homology was found between nine tryptic peptides of pig heart lipoamide dehydrogenase and that of human erythrocyte GSSG reductase. The homology apparently extends throughout the molecules, which have both mechanistic and structural properties in common. Both contain a cystine residue that undergoes reduction and oxidation during the catalytic cycle, and the sequences adjacent to the active cystine residue are highly homologous (241). The three-dimensional structure of human erythrocyte glutathione reductase at 2-Å resolution has appeared (242). The amino acid sequence was fitted unambigiously to this map. This enzyme (M_r 104,800) has two identical subunits, each of which can be divided into four domains and a flexible segment of 18 amino acid residues at the *N*-terminus. The X-ray analysis indicates that the binding positions for NADPH and GSSG are at the opposite sides of one subunit. Transfer of reduction equivalents is mediated by the ring of FAD, which is located at the center of each subunit, and by the adjacent active cystine residue. Each subunit contributes to both of the sites at which GSSG is bound and reduced. Continuation of these

studies should facilitate elucidation of the reaction mechanism and other aspects of this important intracellular catalyst.

CONJUGATION OF GLUTATHIONE

Exogenous Compounds; Glutathione S-Transferases

It was reported in 1879 that administration of bromobenzene or of chlorobenzene to dogs was followed by urinary excretion of compounds called mercapturic acids. Later work established that GSH reacts with a very large number and variety of foreign compounds to form GSH conjugates (3, 243–249). Compounds with an electrophilic center can readily conjugate with GSH; in some instances an electrophilic center is introduced by another reaction, for example, by action of microsomal oxygenase to form an epoxide that reacts with GSH. The interaction of foreign compounds with GSH may be spontaneous or catalyzed by GSH S-transferases. GSH conjugates are typically converted to mercapturic acids by a series of reactions initiated by γ-glutamyl transpeptidase in which the γ-glutamyl moiety of the conjugate is transferred to an acceptor; the resulting cysteinylglycine conjugate is converted by the action of dipeptidase to the corresponding cysteinyl conjugate which is *N*-acetylated to form an *N*-acetylcysteine conjugate (a mercapturic acid). The pathways of GSH conjugate metabolism and the mode of ultimate excretion vary with different compounds and the species; a voluminous literature is available (3, 247, 248).

GSH S-transferases occur in substantial quantities in liver and other mammalian tissues, e.g. erythrocytes (250), intestine (251–252); such enzymes also exist in plants and in insects. There are multiple forms of GSH S-transferase (253, 254), and there has been much interest in their purification and characterization, especially those of mammalian liver. The GSH S-transferases account for about 10% of the soluble protein of rat liver. They are identical with ligandins, first recognized as proteins that bind a variety of anionic compounds (255–258). It was suggested that ligandins function in the transport of these compounds from blood plasma into liver cells (255). Ligandins bind bilirubin, certain carcinogens, steroids, and azo dyes (259). The ligandin-GSH S-transferase family appears to have 3 detoxication functions: (*a*) catalysis, (*b*) binding of ligands which are not substrates; and (*c*) covalent bond formation with very reactive compounds leading to inactivation and destruction of the protein (259–264).

GSH-S-transferases, from liver, are dimeric enzymes that may contain four types of subunits, designated as Ya (M_r 22,000), Yb (M_r 23,500), Yb′ (M_r 23,500) and Yc (M_r 25,000) (253, 254, 265, 266). These may combine to form six isozymes that were originally named A, B, C, D, E, and AA on the basis

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of chromatographic properties. Two groups of GSH S-transferases may be distinguished by reaction with either antibody to GSH S-transferase B (subunits Ya and Yc) or to GSH S-transferase C (subunits Yb and Yb′). For each group there are three possible combinations of the two subunits: YaYa, YaYc, YcYc or YbYb, YbYb′, Yb′Yb′. Enzymes containing Ya or Yc exhibit high GSH peroxidase activity. A recent study of the subunit composition of rat liver GSH S-transferases, in which the poly (A) — RNA species for the S-transferase subunits were detected by a cDNA plasmid containing partial coding sequences for one of the rat liver subunits, has led to the conclusion that the minimum number of subunits for the liver isozymes is four (267).

The physiological functions of GSH S-transferases are undoubtedly diverse (see below); they may have a relationship to ethanol metabolism (268), heavy metals (269), reduction of peroxides (270, 187) and drug metabolism (249). These activities may be induced by drugs such as phenobarbital (271, 272).

Endogenous Compounds

Although most studies of GSH conjugation have dealt with detoxication of foreign compounds, there is increasing interest in conjugation reactions involving endogenous metabolites. For example, conversion of Δ^5-3-ketosteroids to the corresponding α,β-unsaturated Δ^4-3-ketosteroids is catalyzed by proteins of human and rat liver that appear to be identical with GSH S-transferases (273). Δ^5-3-Ketosteroid isomerase activity of human liver was found in several of the GSH S-transferases of this tissue. GSH S-transferases may have additional catalytic functions; there is evidence that form B of GSH S-transferase accounts for the nonselenium-containing GSH peroxidase activity. Estradiol-17-β also conjugates with GSH in rat liver preparations and in vivo (274–279). When the GSH conjugate of 2-hydroxy-estradiol-17-β was administered to rats, there was biliary excretion of the corresponding derivatives of cysteinylglycine, cysteine, and *N*-acetylcysteine. There are also reports on the formation of GSH conjugates of prostaglandins (280–282). GSH seems to function in several aspects of prostaglandin metabolism (283–285).

5-S-Glutathione-3,4-dihydroxyphenylalanine produced in melanocytes appears to be an intermediate in incorporation of cysteine sulfur into certain malanins (286). Interestingly, 5-S-cysteinyl-3,4-dihydroxyphenylalanine is excreted in large amounts in the urine of some patients with widespread malignant melanoma (287).

Conjugation with GSH also functions in the metabolism of the leukotrienes (288, 289). In this pathway, leukotriene A, an epoxide derived from arachidonic acid, reacts with GSH to form the conjugate leukotriene

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C. The γ-glutamyl moiety of this conjugate is removed by the action of γ-glutamyl transpeptidase, a reaction that proceeds more rapidly in the presence of amino acids (10), and leads to formation of leukotriene D, a slow reacting substance of anaphylaxis. Leukotriene D is further metabolized by the action of dipeptidase (10, 162) to the corresponding S-substituted cysteine, leukotriene E. A new leukotriene was recently discovered in studies in which γ-glutamyl transpeptidase was incubated with GSH and leukotriene E (10). The new compound, γ-glutamyl leukotriene E, was subsequently named leukotriene F (see review by S. Hammarström, this volume).

The apparent K_m of leukotriene C4 for the transpeptidase is about the same as that for GSH, which would probably compete in vivo with leukotriene C4 (10). The complexity of this system is further suggested by the possible conversion of leukotriene D4 by the transpeptidase, under physiological conditions, to the C4 derivative, to di- or poly-γ-glutamyl leukotriene derivatives, and to leukotriene F4. Notably, two enzymes of the γ-glutamyl cycle (transpeptidase and dipeptidase) are involved in the transformations of the leukotrienes. The enzyme that catalyzes formation of leukotriene C from the epoxide leukotriene A has not yet been identified.

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OTHER ASPECTS OF GLUTATHIONE FUNCTION

Coenzyme Functions

The activities of many enzymes are influenced by GSH and by other thiols, and such effects may reflect significant physiological regulatory phenomena. There are several enzymatic reactions in which GSH participates as a coenzyme. The best known is the glyoxylase reaction in which the hemimercaptal formed by nonenzymatic interaction of methylglyoxal and GSH is converted to S-lactyl GSH by glyoxylase I, which is hydrolyzed (by glyoxylase II) to D-lactate and GSH. Many studies (e.g. 7, 290–297a) have been reported on this enzyme, whose physiological function needs study. The *cis-trans* isomerization of maleylacetoacetate to fumarylacetoacetate (298, 299), and the analogous reaction of maleylpyruvate (300, 301) also require GSH. The dehydrochlorination of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) is catalyzed by a GSH-requiring enzyme found in houseflies; DDT apparently reacts with GSH to give an alkylated intermediate which breaks down to GSH, HCl, and the dehydrochlorinated product (302–305). The reactions catalyzed by prostaglandin endoperoxide D-isomerase and E-isomerase (284, 285, 306–309) also exhibit specific GSH involvement. Formaldehyde dehydrogenase catalyzes reversible conversion of formaldehyde, GSH, and NAD^+ to NADH, H^+, and S-formyl-GSH; the latter is cleaved by a hydrolase to formate and GSH.

Dehydrogenase and hydrolase activities have been found in many animal tissues and plants (310–320). The function of formaldehyde dehydrogenase apart from detoxication of formaldehyde is not yet clear.

Radiation and Oxyen Toxicity

Guzman-Barron (321) noted that thiols are easily oxidized, and postulated in 1944 that ionizing radiations would rapidly oxidize the thiol groups of cells. Later, it became apparent that radiation can produce a decrease in the cellular concentration of GSH and lead to formation of GSSG, and also that administration of various thiols can protect animals against the effects of radiation. Many observations show that the radiosensitivity of cells depends on the intracellular thiol level. The effects of radiation in air are generally greater than those found under hypoxic conditions, and the "oxygen effect", expressed as "O_2-enhancement ratio (OER)" is about three. The oxygen effect has been explained in terms of competition between thiol and O_2 for radicals produced by irradiation (322). Thus, ionizing radiation abstracts a hydrogen from a molecule to form a radical; thiols restore hydrogen to the radical, whereas reaction of radical with O_2 "fixes" damage by virtue of further chemical transformation. Although complex, the oxygen effect might be explained by the fact that in O_2, cells produce reactive oxygen compounds, which are normally destroyed by reaction with GSH. Under anoxic conditions, more GSH would be available to react with radiation-induced radicals. Thus, there would be less radiation damage in anoxia than in air. This predicts that the OER would decrease in the absence of GSH.

Recent relevant research includes studies on human lymphoid cells depleted of GSH by suspension in media containing buthionine sulfoximine (18), and experiments on fibroblasts from a patient (323, 324) with severe GSH synthetase deficiency (see p. 746). Lymphoid cell viability was markedly reduced by γ radiation of cells containing about 5% of the control level of GSH (18). The in vitro clonogenic survival of GSH-deficient fibroblasts and controls treated with X rays was studied under oxic and anoxic conditions (325); the OER was 1.5 (controls, 2.9). When irradiated in 95% air–5% CO_2 the GSH-deficient cells were slightly more radiosensitive than the controls. With 100% oxygen, there was a moderate effect on controls, but only a very low effect on the GSH-deficient cells. Misonidisole, a radiosensitizing agent, had a much greater effect on controls than on GSH-deficient cells; misonidisole seems to act, at least in part, by interacting with GSH (326; see also 327–331).

V79 fibroblasts depleted of GSH with buthionine sulfoximine gave OER values of 2.2; control 2.9 (332). These and other findings indicate the potential usefulness of buthionine sulfoximine as a drug. Since the rate of

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GSH depletion with this agent depends on the rate of GSH turnover, it is important to monitor the GSH levels during such experiments and therapy.

The role of GSH in the prevention of oxygen toxicity is closely related to phenomena associated with radiation (333, 334). Increased oxygen tension leads to increased formation of H_2O_2, other reactive oxygen species, free radicals and GSSG. Rats fed a low protein diet show increased susceptibility (decreased survival time) to 98% oxygen; this effect was associated with deficiency of dietary sulfur amino acids (335). Rats on such a diet (in contrast to controls) did not develop increased levels of lung GSH in response to hyperoxia. The significant relationship between dietary cysteine and GSH synthesis has been examined (335a). The implications of these studies with regard to oxygen therapy are clear, and the observations also reflect a significant function of GSH in protection against reactive oxygen compounds.

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Cancer

Potentially significant connections between GSH and carcinogenesis have attracted attention; see for example (336–343). Administration of certain carcinogens increases levels of GSH and of γ-glutamyl transpeptidase. Some tumors (e.g. skin, liver, colon) have unusually high levels of transpeptidase. It has been concluded that increased transpeptidase is a marker for very early, as well as late, putative liver preneoplastic and neoplastic cells (337). A recent study of transpeptidase in the developing mouse tooth, intervertebral disc, and hair follicle concluded that this enzyme is "a marker of cell differentiation, cell aging, and/or reduced cellular proliferation," and that it can be a marker of cell fetalization (338).

The reactive ultimate carcinogenic forms of chemical carcinogens are electrophiles, and thus good candidates for detoxication by reactions catalyzed by GSH S-transferases. A number of studies have been carried out on compounds that inhibit carcinogenesis by apparently increasing GSH S-transferases; elevations of other enzymes and of GSH levels have also been observed (344–351). For example, butylated hydroxyanisole, a food additive, has been found to protect against chemical carcinogenesis, and to increase the levels of liver microsomal epoxide hydratase, GSH S-transferase, and GSH.

A finding of great potential interest was reported by Novi (343), who treated rats bearing aflatoxin-induced liver tumors with large oral doses of GSH, and noted substantial regression of the tumors. Treatment was started three months after withdrawal of aflatoxin, at which time the tumors were presumably well established. Further investigations of this phenomenon will be of interest.

Calcium Metabolism

Orrenius and colleagues (352, 353) reported the formation of blebs in isolated hepatocyte membranes due to toxic compounds (e.g. bromobenzene) to be associated with GSH depletion and loss of extramitochondrial Ca^{2+}. Bleb formation is related to changes in Ca^{2+} level that affect the cytoskeleton and cell surface morphology. Evidence that the level of cellular thiols controls the Ca^{2+} pool suggests a significant link between GSH and Ca^{2+} homeostasis. This would be consistent with finding that Ca^{2+} efflux from perfused liver is stimulated by diamide (353a).

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GLUTATHIONE DEFICIENCY AND DEPLETION

Effects of Inhibition of Glutathione Synthesis

Studies on the effects of GSH depletion should yield significant information about the physiological functions of this compound. The use of oxidants including hydroperoxides and Kosower's reagent, diamide (354), led to interesting findings. However, such compounds have certain disadvantages; their effects are short lived, are invariably associated with large increases in GSSG, and are nonspecific since these reagents oxidize other cellular components. Depletion of GSH has also been attempted with compounds that conjugate with GSH such as 1-chloro-2,4-dinitrobenzene (355) and diethylmaleate, which is converted to maleate in tissues (for review see 355a). These compounds, however, are also not specific for GSH; their effects may also be of short duration, since GSH synthesis increases rapidly after acute depletion.

An excellent method for depletion of intracellular GSH is administration of sulfoximine inhibitors of γ-glutamylcysteine synthetase (102, 103); buthionine sulfoximine is a potent and selective inhibitor of GSH synthesis that is highly effective in vitro and in vivo. When present in high concentrations this compound may also have other effects; it may be considered a γ-glutamyl amino acid analog, and may compete with γ-glutamyl amino acids for transport (47). Injection of mice with buthionine sulfoximine produces a rapid decrease in the GSH levels of the kidney, liver, plasma, pancreas, and muscle; other tissues were also affected to a lesser extent (15). When the sulfoximine was given orally for 15 days, the GSH levels of virtually all tissues were greatly decreased. In these studies, the rate at which tissue GSH levels decline after administration of the inhibitor provides a measure of GSH utilization, which is about equivalent to the export of GSH. As discussed above, the marked decrease in the plasma level of GSH is a reflection of its inter-organ transport. When buthionine sulfoximine was given to mice and rats, the level of GSH in liver and kidney declined rapidly to about 20% of the control, and further decrease occurred

much more slowly; this may reflect existence of an intracellular pool of GSH, perhaps in the mitochondria, that turns over relatively slowly.

Studies of this type were also carried out on suspensions of human erythrocytes (356), macrophages grown in culture (20), and human lymphoid cells (17, 18, 45). Essentially exponential decline of the intracellular GSH levels was observed and substantial amounts of GSH were found extracellularly (18). Human lymphoid cells depleted of GSH exhibited increased sensitivity to irradiation. It was suggested earlier that GSH depletion by sulfoximine inhibitors renders tumor cells more susceptible to irradiation and to chemotherapeutic agents that are detoxified by reactions involving GSH (336). Tumor cells depleted of GSH by treatment with buthionine sulfoximine exhibit increased susceptibility to cytolysis by reactive oxygen intermediates (357). Treatment of mice infected with trypanosomes with buthionine sulfoximine led to prolonged survival of the animals (358); trypanosomes, which lack catalase and have high intracellular H_2O_2 levels, are evidently more susceptible to the effects of GSH depletion than are the host cells. Treatment of macrophages with buthionine sulfoximine led to decreased synthesis of leukotriene C and prostaglandin E_2 (359).

These studies have provided significant evidence for the proposed functions of GSH in destroying free radicals, and in the reduction of reactive oxygen intermediates. The findings are consistent with current thinking about the effects of radiation; they indicate that buthionine sulfoximine may be, as suggested (336), an important adjuvant in a wide range of chemotherapy and radiation therapy. Such an approach requires knowledge of the GSH status of the host cells and of the invading cell.

This approach may also be useful in elucidating additional functions of GSH, for example, the possibility that it is involved in thyroid hormone metabolism (360), melanin information (286, 287), the immune system (361), neurotransmission (362, 363), microfilament structure and function (364), and in other biological processes.

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Mutant Microorganisms Deficient in Glutathione Synthesis

Mutants of *E. coli* K 12 deficient in the activities of γ-glutamylcysteine synthetase or of GSH synthetase were isolated by Apontoweil & Berends (365, 366). No GSH could be detected in two mutants; two others had 1% and 12% of the control level of GSH. The mutants, which grew normally, did not show increased sensitivity to X rays, but were more susceptible than their parent strains to a number of sulfhydryl reagents and antibiotics.

Fuchs & Warner (367) isolated a mutant of *E. coli* deficient in GSH synthetase that accumulates γ-glutamylcysteine at a level about equal to

that of GSH in the parent line. The activity of the B1 subunit of ribonucleotide reductase in this mutant was also greatly reduced. This mutant grew at a normal rate, but was much more sensitive to diamide and methylglyoxal than the parent; it was more sensitive to X rays under anoxic conditions. Mutants deficient in GSSG reductase were also isolated (368, 369); they grew normally and contained normal amounts of GSH, indicating that this organism can reduce GSSG by another pathway, presumably involving thioredoxin. As noted above, GSH and the thioredoxin system can participate in alternate pathways of ribonucleotide reduction.

Saccharomyces cerevisiae, in contrast to *E. coli*, contains all of the γ-glutamyl cycle enzymes (370), and a mutant of this organism deficient in GSH synthetase exhibited decreased uptake of glycine (371). Further studies on this mutant, which might have some properties similar to those found in 5-oxoprolinuria (see below), would be of interest.

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Human Diseases Involving Defects of Glutathione Synthesis and Metabolism

Several human diseases are associated with deficiencies of specific enzymes of GSH metabolism. Since this subject has recently been reviewed (372), only the salient features are considered. Severe GSH synthetase deficiency (also known as 5-oxoprolinuria and pyroglutamic aciduria) is characterized by massive urinary excretion of 5-oxoproline, increased blood and cerebrospinal fluid levels of 5-oxoproline, severe metabolic acidosis, tendency to hemolysis, and defective central nervous system function. Severe generalized deficiency of GSH synthetase leads to GSH deficiency. Since GSH regulates its biosynthesis by inhibiting γ-glutamylcysteine synthetase, when there is a marked reduction in the intracellular level of GSH, there is increased formation of γ-glutamylcysteine. This compound is converted to 5-oxoproline and cysteine by γ-glutamyl cyclotransferase, and the overproduction of 5-oxoproline exceeds the capacity of 5-oxoprolinase, so that a substantial amount (about (30%) of the 5-oxoproline formed is excreted. The biochemical and clinical aspects of this condition have been considered in detail (372–375). There are now 12 reported cases, most of whom have developed central nervous system damage even when maintained on constant bicarbonate therapy for chronic acidosis.

Several patients have been found with decreased erythrocyte GSH levels and well-compensated hemolytic disease associated with a milder form of GSH synthetase deficiency without substantial 5-oxoprolinuria. The genetic lesion appears to lead to synthesis of an unstable GSH synthetase molecule; the rate of replacement of this defective but active enzyme is sufficiently rapid so that most tissues can compensate for the defect, but

such compensation is not possible in erythrocytes, which do not synthesize protein.

Patients with γ-glutamylcysteine synthetase deficiency experience hemolytic anemia, spinocerebellar degeneration, peripheral neuropathy, myopathy, and aminoaciduria. Patients with apparently generalized γ-glutamyl transpeptidase deficiency exhibit marked glutathionemia, urinary excretion of GSH, γ-glutamylcysteine and cysteine moieties, and may have defective renal amino acid transport.

5-Oxoprolinase deficiency has recently been reported in three individuals who present somewhat different clinical problems; they do not have obvious abnormalities of amino acid transport nor do they suffer acidosis probably because the accumulation of 5-oxoproline is not large. They excrete 29–71 mmoles of 5-oxoproline per day in their urine, substantially less than found in patients with severe GSH synthetase deficiency. Cultured skin fibroblasts of these patients contain about 2% of the control level of 5-oxoprolinase (375a).

5-Oxoprolinuria has also been observed in homocystinuria (375), in which 5-oxoproline formation appears to involve accumulation of homocysteine, a substrate of γ-glutamylcysteine synthetase (376). γ-Glutamylhomocysteine, a substrate of γ-glutamyl cyclotransferase, is converted to homocysteine and 5-oxoproline (375). Some patients with homocystinuria do not excrete 5-oxoproline; 5-oxoproline formation seems to be associated with a plasma level of homocysteine that is at least about 0.2 mM. Transient moderate 5-oxoprolinuria has been observed in a patient with an inherited defect of tyrosine metabolism in which a cysteine derivative [(2-L-cysteine-S-yl-1,4-dihydroxycyclohex-5-en-1-yl)-acetic acid] named hawkinsin appears in the urine; the mechanism by which 5-oxoproline is produced is not yet clear (377). 5-Oxoprolinuria may also arise from diets high in this compound (378), in end-stage renal disease (379), and in severe burns or allergic disease (379). The depletion of GSH that occurs in hereditary tyrosinemia (380, 381) may be related to accumulation of maleyl- or fumarylacetoacetate (whose cleavage is genetically blocked); these compounds may be produced in sufficient quantity to interact with and deplete GSH. Such phenomena may be associated with increased incidence of cancer in this condition. Therapy with thiol compounds, including GSH, has been tried (381).

Some patients with moderate degrees of erythrocyte GSH peroxidase deficiency have increased hemolysis, but the mechanisms involved require further study (382). Moderate erythrocyte GSSG reductase deficiency, correctable by administration of riboflavin, is not generally associated with symptoms (383). Severe GSSG reductase activity, unaffected by administration of riboflavin or by addition of FAD to the enzyme assays, has been

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observed in three siblings whose symptoms include hemolysis and cataracts; their parents exhibit intermediate levels of reductase (384).

ANALYTICAL PROCEDURES

Progress in almost any area of biochemistry depends importantly on reliable analytical methods. That there are many methods for the determination of glutathione suggests that such analyses are indeed not always satisfactory. In early work, total nonprotein thiols were determined, e.g. by iodometric titration or by reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). *o*-Phthaldehyde (OPA) has been used (385, 386), but it reacts with many primary amines (387). It was proposed that the fluorophor formed on reaction of GSSG with OPA in alkali could serve as the basis of a method in which *N*-ethylmaleimide (NEM) was used to alkylate the GSH present. However, the procedure gives erroneously high values of GSSG (388).

Enzymatic determination of GSH has been accomplished using glyoxylase (389), and by a method using both glyoxylase and GSSG reductase (390) in which GSH is determined from A_{240nm} (lactoyl-GSH), and a second assay with the reductase (based on NADPH oxidation) measures GSSG. The requirement for GSH by maleylpyruvate isomerase (391) and by formaldehyde dehydrogenase (392) has been used in spectrophotometric assays. Methods using GSH S-transferase are also available (393, 394). In one, the sample is incubated with GSH S-transferase and *o*-dinitrobenzene and the nitrite released on conjugation is determined with *N*-(1-naphthyl)ethylene diamine (394).

In addition to methods involving stoichiometric reactions, there are recycling assays which offer higher sensitivity. Thus, the rate of formation of 2-nitro-5-thiobenzoic acid is measured in a system containing DTNB, GSSG reductase, phosphate buffer, EDTA, NADPH, and sample (35, 37, 395, 397). The GSH + GSSG present is determined by comparison of the result with an appropriate standard curve. It is of great importance to determine the standard curve under the same conditions, for example, in the presence of the protein precipitant used in preparation of the sample; other important details have been described (35, 36). The procedure can be made specific for GSSG by masking GSH with NEM (37, 398–400) or 2-vinylpyridine (35); NEM inhibits GSSG reductase, so it must be removed before assay.

Many column chromatographic procedures have been described (16, 396, 401–405); most are lengthy. HPLC is more rapid. Electrochemical detection has been used (406, 407); the electrodes thus far used are sensitive to other reducing substances (406). Precolumn derivatization followed by HPLC has been used with fluorescent reagents such as *N*-(9-

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acridinyl)maleimide, but difficulties arise, owing to fluorescence of these reagents and their products (408, 409). Precolumn derivatization with DTNB has been used with HPLC (410), however, detection at 280 nm is troublesome because other compounds absorb. An HPLC method in which thiols are converted to S-carboxymethyl derivatives followed by reaction with 1-fluoro-2,4-dinitrobenzene to yield 2,4-dinitrophenyl derivatives has been described (411). Several postcolumn HPLC methods are available (412--414); one is based on reaction of GSH with *N*-chloro-5-dimethylaminonaphthalene-1-sulfonamide (412). In others, OPA is used (413, 414). A sensitive and selective precolumn derivatization procedure has been developed by Fahey et al (415) using Kosower's monobromobimane reagent (416). Experience with this method in the authors' laboratory has been satisfactory.

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Several investigators have reported data suggesting occurrence in tissues of mixed disulfides between proteins and GSH or other thiols (e.g. 417–424). In evaluating these findings, it is important to consider such factors as freshness of the sample (e.g. 418), and the procedures used for homogenization and deproteinization. Samples must be worked up promptly. Rat blood plasma was found to contain 19–23 μM GSH and 1.5–2 μM GSSG in samples worked up within 3 min. Samples examined 7 min. later contained about 11 μM GSH and 3 μM GSSG; under these conditions, some of the GSH that disappeared was found in the form of mixed difulfides (36). Homogenization at pH values higher than about 2 (e.g. 420, 421) is undesirable because of the likelihood of thiol-disulfide interchange and thiol oxidation. The nature of the protein precipitant is of great importance. Perchloric acid, trichloroacetic acid, and metaphosphoric acid are generally unsuitable because GSH:GSSG ratios are not maintained. In the authors' experience, sulfosalicylic acid and picric acid are more satisfactory. Although acid homogenization decreases its rate, disulfide interchange may still occur. The possible effects of anesthesia also need to be considered (423, 424). Treatment of tissue extracts with metal hydrides and other reducing agents may liberate thiols from thiol ester linkage. The various manipulations used may also liberate noncovalently bound GSH and GSSG from proteins, e.g. GSH S-transferases. That the extent of artifactitious GSH binding can be substantial is indicated by rapid binding of added [^{35}S]GSH to plasma proteins (36). When kidney homogenates were treated with the internal standard norleucine, about 20% of the norleucine (and therefore a similar fraction of the free GSH and GSSG present in the homogenate) remained in the pellet. No evidence for significant amounts of protein-GSH mixed disulfides was found (O. W. Griffith, unpublished). Several enzymes do form mixed disulfides with GSH and other thiols, but the significance of such findings is not yet clear (e.g. 425); they may represent

artifacts. We believe that GSH probably does form mixed disulfides with certain proteins under physiological conditions. Tissues may also contain thiol esters of GSH and may bind GSH and GSSG noncovalently. However, the published data on forms designated as mixed disulfides between proteins and low-molecular-weight thiols including GSH are difficult to interpret and should be given serious critical attention. Studies suggesting that mixed disulfides between proteins and low-molecular-weight thiols are increased after treatment with certain drugs, or after oxidative stress, while potentially interesting, require refinement.

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CONCLUDING REMARKS

The development of selective enzyme inhibitors (e.g. the sulfoximine inhibitors of γ-glutamylcysteine synthetase), and their effective use in vivo has facilitated biochemical dissection of this complex system. Apart from their usefulness in research, these inhibitors and compounds that increase GSH synthesis have promise in therapy (425a). As one example, approaches involving use of toxic agents together with L-2-oxothiazolidine-4-carboxylate might be considered in situations in which the cell to be killed and the cell to be spared exhibit appropriate quantitative differences in GSH S-transferase and 5-oxoprolinase activities (154).

Despite much progress in defining the biochemistry that underlies the functions of GSH, many important questions remain. We need to learn more about the mechanism of GSH transport, the factors that regulate it and its significance in cell membrane function. Further work is required on the metabolic interactions between amino acids and GSH, and on the formation, transport, and utilization of γ-glutamyl amino acids. The voluminous literature on γ-glutamyl transpeptidase does not contain answers to many questions about its structure, membranous orientation, and induction in neoplastic cells.

There is good evidence that GSH has the important function of destroying reactive oxygen intermediates and free radicals that are constantly formed in metabolism, and in larger amounts after administration of certain drugs, oxygen, and X rays. However, many aspects of the interconversion of GSH and GSSG require study, e.g. the significance of extracellular GSH oxidation by O_2, and the nature and number of GSH transhydrogenases. Although GSH is involved in many intracellular reductive processes, there is good evidence that the thioredoxin system is also important in such reactions. Both GSH and the thioredoxin system may perform some of the same functions. Similarly, the existence of an oxidizing system that promotes disulfide formation requires further

attention. The occurrence of mixed disulfides between GSH (and other thiols) and proteins needs careful investigation.

The several GSH S-transferases require further sorting out; more data are needed about the functions of these widely distributed enzymes in normal metabolism. Explorations of the biochemical functions of GSH in protein synthesis and degradation are needed, as are explorations of its probable functions in other processes such as the immune system and neurotransmission. Further biochemical study of the GSH-induced feeding response of hydra (426–428) might be productive. The metabolic functions of several of the enzymes that use GSH as a coenzyme, including glyoxylase and formaldehyde dehydrogenase need more study. The decrease in tissue GSH levels with age (429–430) is another area of potentially fruitful study.

Much less is known about GSH metabolism in bacteria and plants than in mammalian systems; certain phases of GSH metabolism in microorganisms have been reviewed (431, 431a). Observations on human diseases associated with GSH deficiency indicate that GSH is essential for normal health. However, a number of bacteria, including certain anaerobes, do not contain GSH (432), and at least one organism (*E. coli*) that normally contains large amounts of GSH does not seem to require it for growth. Interestingly, both microorganisms and plants (433, 434) seem to export GSH, at least in the few instances thus far studied. *E. coli* accumulates the novel compound glutathionyl-spermidine (435), and homoglutathione (γ-glutamyl-cysteinyl-β-alanine) has been found in mung beans (436). Thioredoxin, and possibly also GSH, function in photosynthesis (437). Plants contain GSH S-transferases (438). Clearly much potentially important research on GSH remains to be done.

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Annual Review of Biochemistry
Volume 52, 1983

Annu. Rev. Biochem. 1983.52:711-760. Downloaded from www.annualreviews.org
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Biochemical Pharmacology 64 (2002) 1037–1048

Biochemical Pharmacology

Glutathione, iron and Parkinson's disease

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Received 11 February 2002; accepted 22 April 2002

Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disease involving neurodegeneration of dopaminergic neurons of the substantia nigra (SN), a part of the midbrain. Oxidative stress has been implicated to play a major role in the neuronal cell death associated with PD. Importantly, there is a drastic depletion in cytoplasmic levels of the thiol tripeptide glutathione within the SN of PD patients. Glutathione (GSH) exhibits several functions in the brain chiefly acting as an antioxidant and a redox regulator. GSH depletion has been shown to affect mitochondrial function probably *via* selective inhibition of mitochondrial complex I activity. An important biochemical feature of neurodegeneration during PD is the presence of abnormal protein aggregates present as intracytoplasmic inclusions called Lewy bodies. Oxidative damage *via* GSH depletion might also accelerate the build-up of defective proteins leading to cell death of SN dopaminergic neurons by impairing the ubiquitin–proteasome pathway of protein degradation. Replenishment of normal glutathione levels within the brain may hold an important key to therapeutics for PD. Several reports have suggested that iron accumulation in the SN patients might also contribute to oxidative stress during PD.
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Keywords: Parkinson's disease; Oxidative stress; Mitochondrial dysfunction; Lewy bodies; Glutathione; Iron

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease affecting approximately 1% of the human population aged 65 and above [1,2]. PD is a slowly progressive neurodegenerative disorder and is clinically manifested by defective motor function, a decline in cognitive function and depression [3]. Physiologically, PD is characterized by dopamine deficiency owing to the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) region of the midbrain [4]. Its pathological features also include the presence of intracytoplasmic inclusions known as Lewy bodies [5]. Research in the recent years has accumulated substantial evidence supporting the hypothesis that oxidative stress triggers a cascade of events leading to the death of neuronal cells during PD [6].

During different processes of cellular aerobic metabolism such as mitochondrial oxidative phosphorylation, reactive oxygen species (ROS) like superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and peroxynitrite (ONOO–) are generated. Excess production of these molecules can potentially damage different macromolecules such as proteins, nucleic acids and lipids thereby leading to cellular degeneration [7]. To counter this, the cells maintain a battery of detoxifying enzymes and small molecule antioxidants a list of which is provided in Table 1. When there is either an increased production of ROS or a decrease in the levels of antioxidant defenses or both, the toxic effects of such a scenario can be summed as oxidative stress [4,8].

Although the body in general has evolved several defense mechanisms to counteract oxidative stress, the brain appears to be more susceptible to this damage than other organs. Dopaminergic neuronal cells of the SNpc are particularly vulnerable to such insult due to the ROS generated during dopamine metabolism. Levels of both

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Abbreviations: PD, Parkinson's disease; SNpc, substantia nigra pars compacta; ROS, reactive oxygen species; MAO, monoamine oxidase; GSH, glutathione; GSSG, oxidized form of glutathione; γ-GCS, gamma-glutamyl cysteine synthetase; GPx, glutathione peroxidase; GR, glutathione reductase; γ-GT, gamma-glutamyl transpeptidase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine; 4HNE, 4-hydroxynonenal; MPP+, 1-methyl-4-phenyl pyridium; Ub, ubiquitin; LIP, labile iron pool.

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PII: S0006-2952(02)01174-7

Table 1
A brief list of the various antioxidant molecules present in the cell [8]

Antioxidant molecules
Glutathione
Ascorbic acid
Lipoic acid
α-Tocopherol
Ubiquinol
Carotenoids
Uric acid
Flavanoids
Antioxidant enzymes
Catalases
Superoxide dismutase
Glutathione peroxidase
Glutathione-*S*-transferase
GSSG reductase
Repair enzymes like DNAses, RNAses, lipases and proteases
Thioredoxin reductase

lipid peroxidation and the DNA oxidation by-product 8-hydroxy-deoxyguanosine (8-OHDG) have, for example, been demonstrated to be elevated in the SN of PD patients compared with age-matched controls [9–12]. The nonenzymatic oxidation of dopamine leads not only to the generation of quinone and semiquinone molecules but also ROS such as H_2O_2. Enzymatically, both the synthesis of dopamine by tyrosine hydroxylase and catabolism by monoamine oxidase (MAO) can also lead to H_2O_2 production [13,14]. Nigral neurons contain neuromelanin that can bind iron and initiate its reaction with H_2O_2 *via* the "Fenton reaction" to form the highly reactive $^{\bullet}OH$. Hydroxyl radicals can in turn extract methylene hydrogens from polyunsaturated fats in neural membrane phospholipids, initiating lipid peroxidation and cell death. Increases in iron levels have been implicated in inducing cytotoxicity during PD by promoting excessive accumulation of $^{\bullet}OH$ [15]. The sequence of oxidative events within the nigrostriatal system becomes interlinked because neurodegeneration due to oxidative stress in turn increases dopamine turnover leading to increased H_2O_2 production. An important player in protecting dopaminergic SN cells against oxidative stress is the antioxidant molecule glutathione (GSH).

2. GSH, the universal antioxidant

The tripeptide GSH (γ-L-Glu-L-Cys-Gly) is the most abundant intracellular nonprotein thiol compound in mammalian cells [16]. GSH is also present as glutathione disulphide (GSSG), the oxidized form of GSH and as GSSR representing GSH-cysteine disulphides on proteins. Most importantly, GSH functions as an antioxidant with a crucial role as a scavenger of toxic free radicals and detoxification of xenobiotics. Other functions include maintenance of thiol redox potential in cells by reducing the thiol groups of proteins, transport and storage of cysteine and as a cofactor in certain isomerization reactions [17,18]. Recent research data suggest GSH may also have a role in signal transduction, cell proliferation, regulation of gene expression and apoptosis [19–21]. The nitroso form of GSH (GSNO) is considered to be a storage and transfer form of nitric oxide [16,22,23]. Furthermore, GSH also appears to play a role in various cellular processes such as DNA metabolism, protein synthesis, activation of certain enzymes and enhancement of immune function [24,25].

3. Regulation of GSH levels

De novo GSH synthesis in all cell types *in vivo* occurs from the constituent amino acids in two consecutive steps catalyzed by γ-glutamyl cysteine synthase (GCS) and GSH synthase. GCS catalyzes the formation of γ-glutamyl cysteine, the rate-limiting step in GSH synthesis, and the synthase completes the tripeptide by adding glycine. Both the reactions occur in the cytosol and require ATP (see Fig. 1 for the scheme of GSH metabolism). Mammalian γ-GCS is a heterodimer consisting of a catalytic heavy subunit (γ-GCS$_H$; 73 kD) and a regulatory light subunit (γ-GCS$_L$; 31 kD). Mammalian GSH synthetase is a homodimer (monomer MW 52 kD) [26]. The synthesis of GSH also includes GSSG reductase-mediated regeneration. GSSG reductase is a flavoenzyme, which catalyzes the transfer of the reduction equivalent from NADPH onto GSSG [27]. On the other hand, cellular levels of GSH are diminished when it is either consumed during the formation of GSH-*S*-conjugates by GSH-*S*-transferases (GST) [28], by conversion to GSSG by GSH peroxidase (GPx) [27] or by the release of GSH from cells [29,30]. Approximately 10% of total cellular GSH is transported to the mitochondria through an energy dependent-mechanism. Recently, Shi *et al.* [31] have shown that knockout mice harboring a null mutation in the heavy chain of γ-GCS were embryonic lethal and that the animals failed to gastrulate demonstrating that GSH synthesis is indispensable during the early stages of mammalian development. In cell culture, permanent cell lines derived from the mutant blastocysts could be rescued by the addition of GSH to the medium. These cells also remained healthy and proliferated indefinitely if grown in GSH-free medium supplemented with *N*-acetyl cysteine (NAC). These results show that GSH or an antioxidant equivalent, NAC, can rescue cells suggesting that GSH-like molecules are essential for cell growth. This is likely *via* GSHs crucial role as part of the primary cellular defense against oxidative stress. GSH reacts with toxic free radicals both nonenzymatically and also acts as an electron donor in the reduction of peroxides catalyzed by GPx. The resultant GSSG is acted upon by GSH reductase thus recycling the GSH. GPx plays a major role in the recycling of GSH as suggested by the data that GPx knockout mice challenged with toxins like

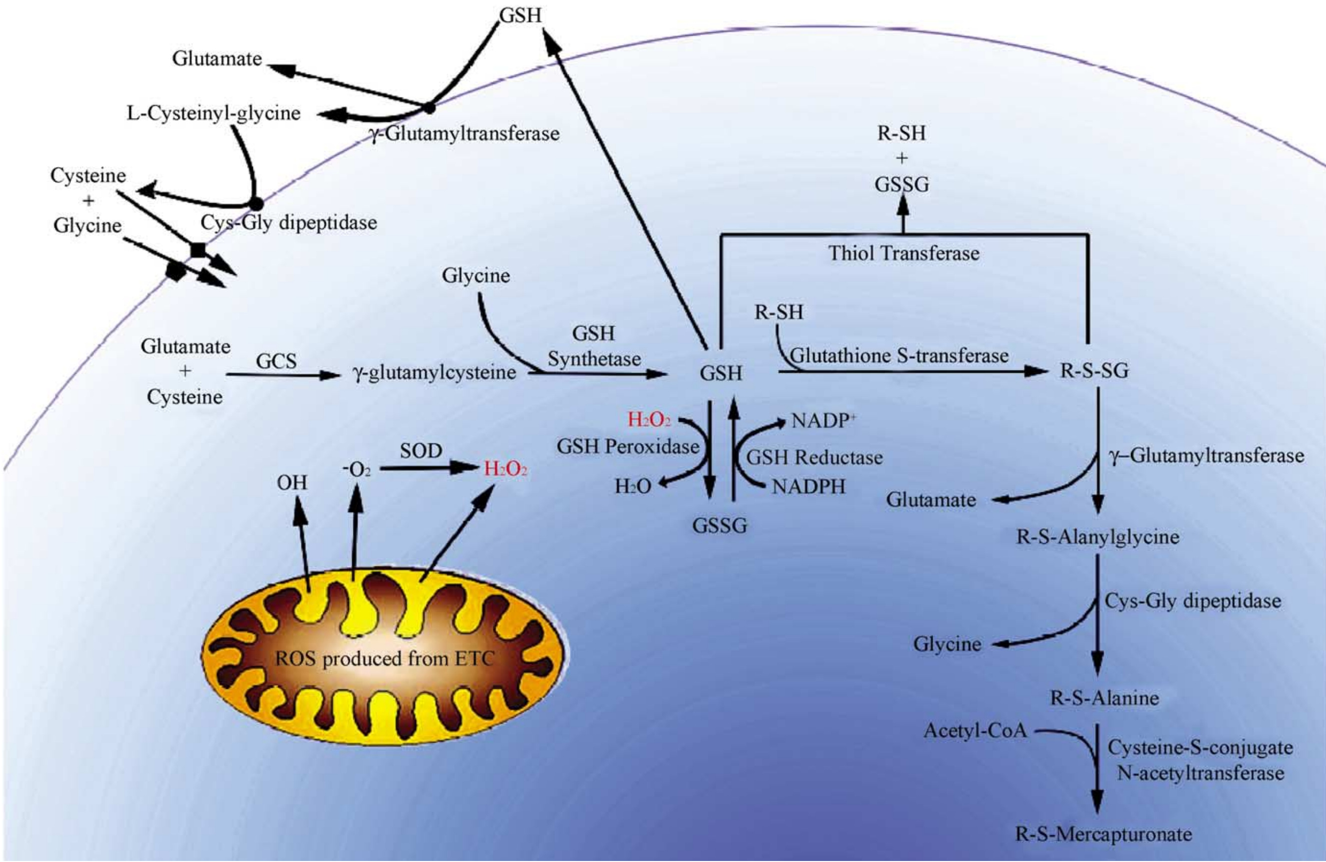

Fig. 1. A schematic representation of the different pathways involved in GSH metabolism. GSH is synthesized *de novo* in the cytoplasm from its constituent amino acids in a two-step reaction. The cellular GSH pool is also contributed to by conversion from GSSG, the oxidized form of GSH. H_2O_2 produced in the mitochondria is detoxified by the conversion of GSSG to GSH. GSH also participates in the formation of mixed disulfides with thiol-proteins (RSH), which can further become metabolized. GSH that is secreted from the cell is broken down into its constituent amino acids which can be taken up by the cells to be used for *de novo* GSH synthesis. For more details, see references [16,17,27].

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exhibited greater depletion of dopamine compared to age matched control mice [32].

A steady-state balance between the synthesis and depletion of GSH maintains its cellular levels. The synthesis of GSH depends, among other things, on the expression levels and posttranslational modification status of γ-GCS, availability of the substrates and feedback inhibition by GSH on the enzyme [26]. GSH is a nonallosteric feedback inhibitor of the γ-GCS enzyme. In the presence of thiols like GSH, the disulfide bridge connecting the heavy and light subunits of GCS heterodimer is reduced inducing a conformational change within the substrate binding site of GCS_H. The relaxed substrate-binding site can accommodate tripeptides like GSH with greater affinity thereby inhibiting substrate binding leading to decreased synthesis of GSH. Studies suggest that phosphorylation of serine/threonine residues on the γ-GCS_H molecule can lead to decreased activity of the enzyme thus lowering the synthesis of GSH [26].

The pathways resulting in loss of GSH mainly involve the conversion to GSSG by GPx, conjugation reaction with proteins [33] and 4-hydroxynonenal (4HNE) [34,35] and transport of GSH/GSSG across the plasma membrane to the outside of the cell. Once outside the cell, γ-glutamyl transpeptidase (γ-GT), a membrane bound enzyme initiates the degradation of extracellular GSH by catalyzing the transfer of the γ-glutamyl moiety from GSH/GSSG or a GSH conjugate onto an acceptor molecule. The product of this reversible reaction is further hydrolyzed into the constituent amino acids cysteine and glycine, which are taken up by cells *via* a peptide transporter and utilized for synthesis of GSH [36]. Hence an increase in γ-GT activity should theoretically lead to a decrease in the cellular pool of GSH. In fact Sian *et al*. [37] have proposed that the decrease in GSH observed in the PD SN is due to an increase in γ-GT activity.

4. GSH in the brain

Compared to other organs in the body, the brain is more susceptible to oxidative damage due to several factors which include high oxygen utilization [38], high iron content [39], presence of excess unsaturated fatty acids which are targets for lipid peroxidation [40], and decreased

activities of detoxifying enzymes like superoxide dismutase (SOD), catalase and GR [18]. Mitochondria, nitric oxide synthase, arachidonic acid metabolism, xanthine oxidase, MAO and P450 enzymes are all sources of ROS in the brain. GPx is the major enzyme for the detoxification of H_2O_2 in the brain since the brain has reduced catalase activity.

GSH is present in the brain in millimolar concentrations [18]. Although there are some reports favoring the transport of GSH across the blood brain barrier and their uptake into brain cells, it still needs to be established whether these play a role in GSH homeostasis in the brain [41,42]. Nevertheless, the constituent amino acids of GSH may cross the blood brain barrier and be utilized for GSH synthesis in the brain following the same pathway as described earlier [27]. Several reports employing different techniques have shown the presence of GSH both in neurons and glial cells [27,43,44]. The concentration of GSH appears to be higher in brain astrocytes compared to neurons [45]. Although there are reports which suggest that GSH is released from brain cells, very limited information is available about the exact mechanism [46–48]. Apart from the antioxidant functions of GSH in the brain, extracellular GSH has been hypothesized to have additional functions as a neurotransmitter [49], neurohormone, in the detoxification of glutamate and in leukotriene metabolism [27]. According to recent evidence accumulated based on coculture experiments, brain astrocytes and neurons appear to interact metabolically with one another in terms of GSH metabolism [18].

It has been observed that there is an age-dependent depletion in intracellular GSH of many organisms including humans [50]. In humans, there appears to be a decline in the GSH levels in the cerebrospinal fluid during aging [51]. Studies have shown that aged mice have a 30% decrease in levels of GSH compared with younger animals [52,53]. Since the brain requires extensive ROS detoxification, it is evident that a decrease in GSH content could increase oxidative damage making the brain more susceptible to neurological disorders.

5. GSH and PD

GSH plays an important role in the adult brain by removing H_2O_2 formed during normal cellular metabolism. In general, SN has lower levels of GSH compared to other regions in the brain. Previous experiments from our laboratory [54] have demonstrated that the relative variations in levels of GSH in different brain regions are cortex > cerebellum > hippocampus > striatum > SN which is consistent with previous reports [55,56]. However, the GSH profiles of all regions are the same through the lifespan, namely, high values during growth dropping to a maturation plateau and then decreasing 30% during aging [56]. It has been observed that during PD, there is a further reduction in GSH levels within the SNpc [57,58]. In fact, GSH depletion is the first indicator of oxidative stress during PD progression suggesting a concomitant increase in ROS. Although GSH is not the only antioxidant depleted during PD, the magnitude of GSH depletion appears to parallel the severity of the disease and occurs prior to other hallmarks of the disease including decreased activity of mitochondrial complex I (described later) [59,60]. GSH levels are not altered in areas of the brain other than SN or in other diseases affecting dopaminergic neurons. In a related work from our laboratory [61], systemic administration of buthionine sulfoximine (BSO), a GCS inhibitor resulted in neurodegenerative effects on the dopaminergic SN neurons that were not generalized to other neuronal cell populations in the brain. BSO treatment has been shown to potentiate the effects of both MPTP and 6-hydroxydopamine (6-OHDA), toxins used to model PD in the SN and striatum [62,63]. These data suggest that these neurons may be especially susceptible to the effects of disturbance in cellular redox *via* GSH depletion. Early depletion in nigral GSH levels observed in Parkinsonian brain is not explainable by increased oxidation of GSH to GSSG as levels of both are found to be decreased. Furthermore, there is no failure of GSH synthesis because the activity of γ-GCS in the SN is normal in PD [37]. GSH losses have rather been suggested to be due to increased activity of the enzyme γ-GT resulting in increased removal of both GSH and GSSG from cells [37]. The increased activity of γ-GT may be the initial step in the pathogenesis of PD.

Mitochondria are responsible for generating 90% of the ATP required for all cellular functions, for detoxifying ROS produced *via* mitochondrial respiration, for controlling the cellular redox state, and for regulating cytoplasmic calcium levels by acting as the major intracellular sink for this ion. Oxidative damage to the mitochondria might interfere with all of these functions. Mitochondrial dysfunction appears to play a major role in the neurodegeneration associated with the pathology of PD [64]. Decreased GSH availability in the brain is believed to promote mitochondrial damage most likely *via* increases in levels of oxidative stress to this organelle. Depletion of brain GSH has been shown to result in decreases in mitochondrial enzyme activities in preweaning rats as well as losses in ATP production in the aging murine brain [65,66]. Previous studies have shown that complex I in bovine heart submitochondrial particles is particularly affected by oxidative stress [67,68]. Reduction in the activity of mitochondrial complex I in the SN have been reported as a major biochemical feature in the pathogenesis of PD [60,69,70]. Interestingly, complex I has been found to be one of the most severely affected mitochondrial enzymes during oxidative stress [71]. In synaptic mitochondria, complex I exerts a major control over oxidative phosphorylation such that a decrease in its activity by 25% drastically affected ATP synthesis and the overall energy metabolism within the cell. On the other hand, inhibition of

complex III and IV up to 80% was necessary to show similar effect [72]. To understand the consequences of GSH depletion on mitochondria in dopaminergic neurons of the SN during PD, we constructed a dopaminergic PC12 cell line model system wherein the levels of the γ-GCS enzyme were inducibly decreased resulting in a reduction in GSH synthesis [73]. A decrease in the mitochondrial GSH in these genetically engineered cells resulted in increased oxidative stress and impaired mitochondrial function as reflected by decreased pyruvate-mediated mitochondrial respiration and ATP synthesis. Although there is no reported decrease in the activity of the γ-GCS enzyme in PD brains, the ultimate effect of the genetic manipulation mimics that which is seen in the Parkinsonian brain, that is a decrease in the GSH levels with no corresponding increase in GSSG levels. An interesting feature in our experiments was that GSH depletion in these cells led to selective inhibition of mitochondrial complex I activity. These results suggest that the early observed GSH losses in the SN may be directly responsible for selective inhibition of mitochondrial complex I activity and the subsequent mitochondrial dysfunction which ultimately leads to dopaminergic cell death associated with PD. One of the possible reasons for the susceptibility of complex I to oxidative damage is thiol oxidation and the presence of accessible oxidation sensitive iron-sulfur centers within this enzyme complex [74]. GSH is the chief molecular player in maintaining the SH groups of protein in reduced state thus controlling the activity of thiol dependent proteins [75]. GSH is known to protect proteins from oxidation by conjugating with oxidized thiol groups to form protein-SS-G mixed disulfides which can then be re-reduced to protein and GSH by GR, thioredoxin or protein disulfide isomerase [76]. In dopaminergic cells *in vivo*, GSH can also bind to quinones formed during oxidation of dopamine and prevent these compounds from reacting with protein sulfhydryl groups [77,78]. Sriram *et al*. [79] have demonstrated that thiol oxidation and loss of mitochondrial complex I activity precede excitatory amino acid mediated neurodegeneration. Both could be prevented by treatment with antioxidant thiol agents. GSH has been suggested to be involved in the repair of oxidized iron-sulfur centers of mitochondrial complex I [80]. In a recent study, when GSH was administered to PD patients by i.v. injections daily for up to a month, a significant improvement in disease related disability was observed [81]. Whether such treatment is effective in actually altering GSH levels in the brain and can have a prolonged effect in retarding the progression of the disease is unclear, however it can be implied that maintenance of thiol homeostasis is critical for the protection of dopaminergic SN neurons against neurodegeneration.

Selective inhibition of complex I *via* systemic administration of either MPTP or rotenone give similar patterns of morphological damage as that observed in the Parkinsonian brain. MPTP is a protoxin which causes selective destruction of dopaminergic neurons in SNpc [82,83]. The active form of MPTP is 1-methyl-4-phenyl pyridium (MPP+) which is formed within glia catalyzed by MAO B (MAOB). MPP+ diffuses out of glia and is actively taken up by SN dopaminergic neurons through a receptor-mediated mechanism where it exerts its toxic effects at least in part by accumulation and direct inhibition of mitochondrial complex I activity resulting in reduction in ATP synthesis thus contributing to mitochondrial dysfunction. Experiments involving SOD transgenic and GPx knockout mice indicate that this agent mediates its deleterious effects at least in part through induction of oxidative damage [32,84]. MPP+ has been shown to produce superoxide, H_2O_2 and $^{\bullet}OH$ [85–87]. Apart from oxidative damage *via* ROS production, MPTP also contributes to oxidative stress by depleting the levels of GSH [88,89]. This amplifies the damage caused because depletion of GSH causes further increase in ROS levels thereby contributing synergistically to mitochondrial dysfunction perhaps *via* direct inhibition of complex I activity.

PD is also characterized by the presence of proteinaceous neuronal inclusions in the midbrain known as Lewy bodies, which may also contribute to subsequent neurodegeneration [5,90]. Rare familial forms of PD exist which involve genetic mutations in various components of the ubiquitin (Ub)-proteasome degradation pathway which could contribute to the accumulation of proteins leading to the formation of Lewy bodies [91–94]. Ub belongs to a family of heat shock proteins (HSPs) involved in various stress response pathways. The protein Ub is covalently attached through thiol groups to misfolded or damaged proteins and aids in their degradation by transporting them to the 26S proteasome complex. The first step in this process is the activation of Ub by an activating enzyme called E1 through formation of a thiol ester bond. Ub is then transferred onto a thiol group of one of several specific Ub carrying enzymes (E2s). From the E2 enzyme, the Ub molecule is next transferred *via* one of several specific Ub ligases (E3) onto a lysine residue on the protein substrate to be degraded [95]. In familial forms of PD, the protein α-synuclein accumulates in the SN forming aggregates and is found to be a major component of Lewy body deposits [96,97]. The gene *Parkin*, which is mutated in some rare early onset PD cases, was found to contain sequences with striking homology to Ub. Recent evidence suggests that *Parkin* is an E3 ligase which acts to ubiquitinate α-synuclein. Furthermore, *Parkin* is also one of the components of the proteinaceous deposits which make up the Lewy bodies [98]. In a work recently published from our laboratory, we have demonstrated that GSH depletion in dopaminergic cells results in decreased E1 activity and subsequent disruption of the Ub pathway [99]. These data suggest that GSH is essential for preventing cysteine residues at the active sites of these enzymes from being oxidized leading to decreased proteolysis *via* the Ub-proteasome pathway. Taking all these data into consideration, it can be implied

that the early loss of GSH in sporadic PD might play an important role in the subsequent accumulation of protein aggregates in the SN in sporadic PD. During different kinds of stresses including oxidative stress, Ub and other related HSPs are expressed to help in the removal of defective proteins thus preventing formation of aggregates [95]. Results suggest that administration of MPTP might induce several HSPs *in vivo* [100]. It might be possible that in the early stages of PD, due to depletion of GSH in the SN, there is an elevated level of oxidative stress leading to the activation of expression of HSP and HSP-related chaperone molecules to protect the neuronal cells against the accumulation of oxidized proteins and aggregation. But during progression of the disease, there is a further increase in oxidative stress with a concomitant decrease in the levels of GSH thus leading to a scenario where the existing defenses against accumulation of defective proteins cannot control the build-up of protein aggregates. The depletion in GSH may also interfere with the ability of the Ub–proteasome system to degrade proteins leading to proteinaceous build-up and deposits [99].

During oxidative damage of lipids, aldehydes are formed as by-products, the most prevalent of these being 4HNE. 4HNE has been proposed to integrate into membranes affecting *in vivo* membrane fluidity. It has been found that GSH forms conjugate with 4HNE in a reaction catalyzed by GST preventing it from incorporating into the membranes [34,35]. Additionally, 4HNE may also form adducts with important proteins like Na^{+}/K^{+} ATPase rendering them inactive. GSH has also been observed to prevent 4HNE from conjugating with proteins [33]. In the PD brains, the levels of 4HNE adducts have been reported to be elevated which might be reflective of the loss in GSH in the SN [11].

Lastly, by serving as the major storage form for cysteine in the cell, GSH may help reduce the levels of free cysteine which can bind to dopamine quinones to form adducts which have been proposed to inhibit complex I activity [101]. Therefore, changes in GSH levels may affect the ability of the cell to protect against toxic levels of dopamine quinones and cysteine which can form various deleterious adducts by conjugating to one other or to thiol groups on proteins. Fig. 2 highlights the functions of GSH in counteracting against oxidative stress during PD.

6. Role of iron in oxidative stress and PD

Besides decreased levels of GSH and impaired mitochondrial complex I, a third component supporting the role of oxidative stress in PD is iron. Sofic *et al*. [102] have demonstrated that total iron levels in the substantia nigra

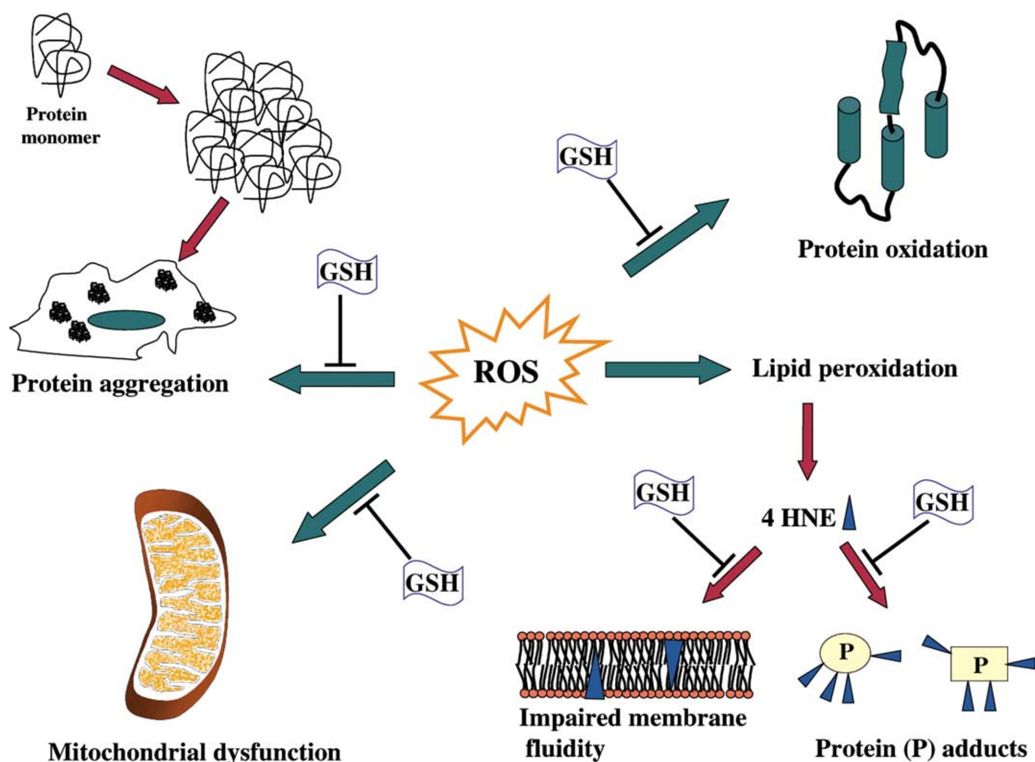

Fig. 2. The different roles of GSH: a schematic representation of the antioxidant properties of GSH as relevant to SN dopaminergic neuronal cells in PD. Apart from the detoxification of ROS themselves [16,17], GSH may protect neurons against the build-up of protein aggregates which form Lewy bodies within the cell [99]; mitochoindrial dysfunction due to inhibition of complex I activity [73]; the deleterious effects of the lipid peroxidation by-product 4HNE [33–35]; and protein oxidation [75,76].

(SN) of PD patients are higher than age matched controls [103]. Similar findings utilizing various methods have been reported in literature [104–111]. Additionally, what gives credence to the role of iron in pathogenesis of the disease is its ability to generate $^{\bullet}$OH *via* the Fenton reaction. Intracellular iron levels are stringently regulated as a labile iron pool (LIP), which provides optimum iron levels for vital biochemical reactions and limits the availability of free iron for generation of ROS. Ferritin is the major iron storage protein in the body which maintains iron in a nonreactive form in the cell. It is uncertain whether the excess iron observed in the PD brains is in a free form or bound to ferritin [106,112–115]. Griffiths *et al.* [105] demonstrated that in PD patients, ferritin is heavily loaded with iron implying that even if there is an increase in ferritin levels to counter excess iron levels, the ferritin molecules are saturated with iron. In the event of a superoxide or catechol-mediated release of iron from loaded ferritin pool, there could be an increase in LIP causing an increase in reactive iron available for generation of ROS [116]. Furthermore, iron promotes autooxidation of dopamine in SN neurons, releasing additional H_2O_2 [117]. Iron also catalyses the conversion of excess dopamine to neuromelanin, an insoluble black-brown pigment that accumulates in all dopaminergic neurons with age in humans [118]. Neuromelanin in general is neuroprotective and sequesters redox active ions in the cell with a high affinity for Fe^{3+} ions. However, when bound to excess Fe^{3+}, neuromelanin tends to become a prooxidant and reduces Fe^{3+} to Fe^{2+}, which then gets released from neuromelanin owing to weak affinity [119]. This increases the neuronal LIP and also the fraction of iron capable of reacting with H_2O_2. Under normal conditions, GSH constantly clears H_2O_2, thus preventing the production of $^{\bullet}$OH radicals. GSH also conjugates with quinones formed during dopamine oxidation and prevent them from facilitating release of iron from ferritin [120]. However under conditions of GSH depletion as in PD, this protection is attenuated leading to oxidative stress. It has been suggested that liberation of iron from ferritin or neuromelanin alters the homeostasis of mitochondrial Ca^{2+} with subsequent depletion of tissue GSH, resulting in oxidative stress [121]. A typical neuron of the SN in a PD patient containing dopamine, neuromelanin, high levels of iron and depleted GSH levels thus has a very conducive environment for generation of ROS. Hence oxidative stress produced during PD is likely the consequence of H_2O_2 production due to a combination of dopamine oxidation, GSH depletion and Fe^{3+} generated by neuromelanin or released from ferritin, thus allowing the Fenton reaction to proceed at a considerable rate resulting in neuronal death. This hypothesis gains support from the observation that pigmented neurons are preferentially lost during the course of PD [122–126]. It remains to be elucidated whether iron accumulation precedes injury of pigmented neurons or occurs as a consequence of neuronal degeneration. Intranigral iron injection in rats produces a selective lesioning of dopaminergic neurons, resulting in behavioral and biochemical Parkinsonism [127]. The biochemical changes due to oxidative stress resulting from tissue overload of iron (referred to as siderosis) are similar to those identified in the SN of PD brains [128].

It has been proposed that the catecholaminergic neurotoxin 6-OHDA can cause Parkinsonian symptoms in animal models and that oxidative stress, enhanced by iron, may play a key role in its toxicity. Neurotoxicity *via* 6-OHDA has also been linked to the release of iron from its binding sites in ferritin [130]. During dopamine metabolism, many toxic products such as hydrogen peroxide, oxygen-derived radicals, semiquinones, and quinones are generated which can exert neurotoxic effects. Pretreatment of rats with desferrioxamine, an iron chelator, attenuates 6-OHDA lesioning of nigrostriatal dopamine neurons [131]. Ben-Shachar *et al.* [132] have demonstrated that dopamine administration in rats pretreated with the MAO inhibitor pargyline caused mortality in a dose-dependent manner. Desferrioxamine proved to also be neuroprotective against this dopamine-induced neurodegeneration. It has been proposed that catecholamines can exert neurotoxic effects not only by inducing oxidative stress but also by affecting the mitochondrial electron transport chain. Glinka *et al.* [129] have demonstrated that 6-OHDA but not its oxidative products could reversibly inhibit complex I in isolated brain mitochondria.

Although these observations support the role of iron as a neurotoxin, it remains to be established whether accumulation of iron in PD is primary or secondary to other known events such as GSH depletion and complex I inhibition. It has been found that in patients with incidental Lewy body disease (ILBD) exhibiting preclinical PD symptoms, GSH depletion in SNpc are similar to that seen in PD patients. Since these patients exhibit physiological iron levels and normal mitochondrial complex I activity, it has been suggested that GSH depletion might be an upstream biochemical event in nigral neurodegeneration [133].

It is also important to take into account the lack of specificity of iron accumulation and its association with neuronal degeneration in various pathological conditions. Alterations in iron levels have been described in many neurodegenerative diseases such as multiple system atrophy, progressive supranuclear palsy, Huntington's disease, Alzheimer's disease, multiple sclerosis and spastic paraplegia [134]. Furthermore, iron levels are increased in the SN of 6-OHDA lesioned rats [135] and MPTP treated monkeys [136,137] suggesting that iron accumulation can occur as a secondary event during neuronal degeneration, irrespective of the causative agent. These nevertheless do not undermine the role of iron in disease progression. Recent findings that iron-related oxidative stress might promote α-synuclein aggregation strengthens the putative role of iron as an important link between the biochemical lesions and Lewy bodies during PD progression [138–141].

7. GSH as a therapy for PD

Based on all of the information described above, we can conclude that one of the most important events during pathogenesis of PD is depletion of GSH within the SN dopaminergic neurons. Hence it is plausible that one of the ways to counteract this problem is to replenish the GSH pool either by increasing the synthesis of the tripeptide or by slowing its degradation. There are several studies where administration of precursors of GSH metabolism such as γ-glutamyl cysteine have been used to increase the levels of GSH in rat brains [142]. Similarly, precursors of cysteine synthesis have been administered in various animal models with the view to accelerate cysteine production in the brain thus increasing GSH levels [142]. These efforts have been hampered by the problem that elevating levels of cysteine in the brain itself may be toxic to cells. GSH replacement can also be achieved by administering thiol reagents such as GSH itself or GSH analogs. GSH cannot easily penetrate the blood brain barrier due to the presence of the cysteine SH group and is not efficiently absorbed into neuronal cells in the brain [27,41,42]. Hence rather than GSH, modified forms of GSH such as GSH analogs (e.g. GSH esters) have generally been used *in vivo*. Yamamoto *et al*. [143] have shown that YM 737, a GSH analog, has protective properties against cerebral ischemia in rats. It would be interesting to test such compounds in animal models of PD to see if they are effective.

Gene expression analyses in animal models of PD using cDNA microarray approaches have suggested that neurodegeneration in PD is a complex process [144,145]. Although the exact molecular events leading to neurodegeneration have not been elucidated yet, the results of microarray analyses indicate the role of genes related to oxidative stress, glutaminergic excitatory, neurotrophic factors, nitric oxide-mediated and inflammatory processes. Since each of these events is complex and involves several biochemical mechanisms, it could be surmised that a single drug may not be completely effective against PD. Hence, drugs to counteract oxidative stress such as thiol reagents (GSH esters/GSH analogs) might be more effective if administered as a part of multi-drug therapy involving a cocktail of drugs [146].

8. Conclusions

In the last decade, there has been significant progress towards establishing that PD neuropathology may be at least in part attributed to depletion of the cellular GSH pool within SN dopaminergic neurons. This has been shown to affect various cellular processes including mitochondrial function which has been suggested to be the main source of neurodegeneration in the disease. GSH depletion might also affect protein degradation thus contributing to the build-up of defective proteins such as proteinaceous deposits which form Lewy bodies. Accumulation of iron in the SN has also been demonstrated to contribute to oxidative stress during PD. There have been several efforts in the recent years involving the use of GSH or related molecules in GSH metabolism as therapeutics in the treatment of PD to elicit an increase in brain GSH levels.

Acknowledgments

This work was supported by National Institute of Health (NIH grant no. AG12141).

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Negative Correlation between Brain Glutathione Level and Negative Symptoms in Schizophrenia: A 3T ^{1}H-MRS Study

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Abstract

Background: Glutathione (GSH), a major intracellular antioxidant, plays a role in NMDA receptor-mediated neurotransmission, which is involved in the pathophysiology of schizophrenia. In the present study, we aimed to investigate whether GSH levels are altered in the posterior medial frontal cortex of schizophrenic patients. Furthermore, we examined correlations between GSH levels and clinical variables in patients.

Methods and Findings: Twenty schizophrenia patients and 16 age- and gender-matched normal controls were enrolled to examine the levels of GSH in the posterior medial frontal cortex by using 3T SIGNA EXCITE ^{1}H-MRS with the spectral editing technique, MEGA-PRESS. Clinical variables of patients were assessed by the Global Assessment of Functioning (GAF), Scale for the Assessment of Negative Symptoms (SANS), Brief Psychiatric Rating Scale (BPRS), Drug-Induced Extra-Pyramidal Symptoms Scale (DIEPSS), and five cognitive performance tests (Word Fluency Test, Stroop Test, Trail Making Test, Wisconsin Card Sorting Test and Digit Span Distractibility Test). Levels of GSH in the posterior medial frontal cortex of schizophrenic patients were not different from those of normal controls. However, we found a significant negative correlation between GSH levels and the severity of negative symptoms (SANS total score and negative symptom subscore on BPRS) in patients. There were no correlations between brain GSH levels and scores on any cognitive performance test except Trail Making Test part A.

Conclusion: These results suggest that GSH levels in the posterior medial frontal cortex may be related to negative symptoms in schizophrenic patients. Therefore, agents that increase GSH levels in the brain could be potential therapeutic drugs for negative symptoms in schizophrenia.

Citation: Matsuzawa D, Obata T, Shirayama Y, Nonaka H, Kanazawa Y, et al. (2008) Negative Correlation between Brain Glutathione Level and Negative Symptoms in Schizophrenia: A 3T ^{1}H-MRS Study. PLoS ONE 3(4): e1944. doi:10.1371/journal.pone.0001944

Editor: Bernhard Baune, James Cook University, Australia

Received February 19, 2008; **Accepted** February 27, 2008; **Published** April 9, 2008

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Funding: This study was supported in part by grants from the Minister of Education, Culture, Sports, Science, and Technology of Japan (to K.H.).

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Accumulating evidence suggests that oxidative stress associated with impaired metabolism of the antioxidant glutathione (GSH) plays a key role in the pathogenesis of schizophrenia [1,2]. First, activity of glutathione peroxidase (GSH-Px), a key antioxidant enzyme, was found to be decreased in red blood cells [3,4] and plasma [5] of some, but not all schizophrenic patients [6,7]. Furthermore, plasma GSH-Px levels were significantly and positively correlated with psychosis rating scores in schizophrenic patients [8]. Second, it has been reported that the activity of glutamate cysteine ligase (GCL), the rate-limiting enzyme for GSH synthesis, as well as expression of the catalytic GCL subunit (GCLL) protein in cultured skin fibroblasts from schizophrenic patients were significantly decreased compared to those in comparison subjects, and that decreased GCL activity was correlated with decreased GCLL protein expression [9]. Third, Do et al. [10] reported that levels of GSH in the cerebrospinal fluid of drug-free patients of schizophrenia were significantly decreased compared to those in normal comparisons. Furthermore, a study using postmortem brain samples demonstrated decreased levels of GSH, oxidized GSH (GSSG), GSH-Px, and GSH reductase in the caudate region of brains from schizophrenic patients, suggesting impaired GSH metabolism in schizophrenic brains [11]. Moreover, a 1.5T ^{1}H-magnetic resonance spectroscopy (MRS) study with double quantum coherence technique demonstrated significant reduction (52 %) in GSH levels in the medial frontal cortex of schizophrenic patients compared to comparisons [10]. However, Terpstra et al. [12] reported that levels of GSH in the anterior cingulated cortex, measured by 4T

^{1}H-MRS with MEGA-PRESS (MEscher-GArwood-Point RE-Solved Spectroscopy) sequence, did not differ in schizophrenic patients and comparisons. Fourth, several genes involved in GSH metabolism have been shown as potential candidate genes for schizophrenia. Association of the glutathione-S-transferase (GST) M1 gene was shown in schizophrenic subgroups in Japanese [13] and Korean populations [14]. Recently, Tosic et al. [15] reported that the levels of mRNA for GCLM and glutathione synthetase, which are responsible for GSH synthesis, were significantly decreased in the fibroblasts of schizophrenic patients in a Swiss population. Subsequently, they reported the GCLM gene as a susceptibility gene for schizophrenia in Swiss and Danish populations [9,15]. Taken together, these findings provide genetic and functional evidence that an impaired capacity to synthesize GSH under conditions of oxidative stress is a vulnerability factor for schizophrenia.

GSH plays a major role in the modulation of redox-sensitive sites on the N-methyl-D-aspartate (NMDA) receptors [16–18], which are implicated in the pathophysiology of schizophrenia [19–23]. Considering the NMDA receptor hypofunction hypothesis for schizophrenia [19–23], it is of great interest to study whether levels of GSH are altered in the brains of schizophrenic patients. In the present study, we aimed to investigate whether GSH levels are altered in the posterior medial frontal cortex of schizophrenic patients. Furthermore, we examined the correlations between GSH levels and clinical features including the severity of clinical symptoms (positive symptoms, negative symptoms and cognitive deficits). In addition, we performed genetic analysis for the genes involved in GSH metabolism: namely, GCLM, glutathione peroxidase 1 (GPX1), and several classes (GSTM1, GSTO1, GSTP1, GSTT1 and GSTT2) of glutathione-S-transferase (GST).

Table 1. Characteristics and clinical variables of subjects enrolled in this study

Variable	Controls (n = 16)	Schizophrenia (n = 20)	P values
Sex, Male/Female	12/4	12/8	0.481 a)
Age (year)	30.0±7.2 (21–41)	30.7±5.8 (20–39)	0.581 b)
Education (year)	15.2±2.9 (12–21)	13.5±1.7 (12–16)	0.04 b)
Estimated IQ*1	107.4±17.3 (90–128)	98.6±10.9 (80–114)	0.03 b)
age at onset of illness (year)		23.6±5.5 (11–31)	
Duration of illness (year)		7.30±5.2 (1–21)	
GAF scale		51.5±11.5 (29–71)	
Amount of medication*2		283.1±216(80–667)	
BPRS score		26.2±8.6 (13–43)	
BPRS positive score		12.2±5.7 (4–24)	
BPRS negative score		6.1±2.9 (2–12)	
SANS score		76.9±12.9 (60–103)	
DIEPSS score		0.41±0.15 (0.11–0.78)	
GSH (mM)	0.928±0.24 (0.608–1.465)	0.808±0.26 (0.432–1.250)	0.166 b)

All values are shown as mean±SD (range).
a)Chi-squire test,
b)Student t-test.
*1: Short form version of Wechsler Adult Intelligence Scale, Revised (WAIS-R)
*2: Chlorpromazine equivalent (mg)
GAF: Global Assessment of Functioning, BPRS: Brief Psychiatric Rating Scale, SANS: Scale for the Assessment of Negative Symptoms, DIEPSS: Drug Induced Extra-Pyramidal Symptoms Scale
doi:10.1371/journal.pone.0001944.t001

Materials and Methods

Subjects

This research was performed under approval of the ethics committee of Chiba University Graduate School of Medicine and National Institute of Radiological Science. The experiments were thoroughly explained to the subjects, and written informed consent was obtained from all. Twenty schizophrenic patients and 16 age- and gender- matched normal controls with no past history of psychotic disorders or drug dependence were enrolled in the study. Characteristics of subjects are shown in **Table 1**. Due to a few highly educated comparisons, the extent of education and estimated IQ were significantly different between the two groups, but the estimated IQ of all patients was within the normal range. All patients were outpatients meeting the DSM-IV criteria for schizophrenia [24] and having no other psychiatric disorders. All patients were taking second-generation neuroleptics: i.e., risperidone (2–12 mg/day, n = 9), olanzapine (5–20 mg/day, n = 5), aripirprazole (6–12 mg/day, n = 4), quetiapine (500 mg/day, n = 1) or perospirone (48 mg/day, n = 1), with no change in their medication for the past month. Of the patients, twelve were diagnosed as residual type and eight were as paranoid type.

^{1}H-MRS measurement and data analysis

All data were acquired using the 3T SIGNA EXCITE (GE) with a standard quadrature coil. GSH spectra were acquired by the MEGA-PRESS sequence [25]. A GSH peak at chemical shift 2.95 ppm originating from cysteinyl β-CH_2 was observed by editing pulse at 4.95 ppm α-CH resonance line J-coupled to the observed spins. Acquisition parameters for the measurement were as follows: echo time (TE) = 94 ms, repetition time (TR) = 1500 ms, number of excitations (NEX) = 512, band width 2.5 kHz, data point 4096. TE and TR were set experimentally as optimum for our system after confirming the GSH signal changes to be within a certain range (TE: 62–101 ms, TR: 1077 ms–12000 ms) with both phantom solutions and human subjects. The short TR enabled us to increase NEX and obtain a satisfying signal/noise (S/N) ratio in the human brains. For the quantification of GSH, we prepared eight phantom solutions containing different concentration of GSH (0.3–30.0 mM) with N-acetyl aspartate (NAA, 10 mM) and creatine (8 mM) to get the reference spectra. During the phantom data acquisition, the solutions were kept at 37±0.6°C.

For the acquisition of human spectra, an 18.6-ml (28×30×22 mm) volume of interest (VOI) was placed on the posterior medial frontal cortex under the guidance of T_2-weighed images (**Figure 1A**). The posterior medial frontal cortex was selected since reduction in the GSH levels in this region of schizophrenic patients has been reported previously [10]. To minimize variation in the positioning of the head, subjects were positioned by the same investigator. The overall examination time was 1 hour or less.

For all data acquisition, high-order shim followed by automatic local shim adjustment was used and repeated until the half linewidth was accomplished under 3 Hz (phantom) or 8 Hz (human). The raw data of both phantom solutions and human subjects were processed on GE analysis software (GE Medical Systems, Milwaukee, WI). Fourier transform was done with an exponential weighting function of 2 Hz. The area of the GSH signal was measured on Image J (http://rsb.info.nih.gov/ij/) software.

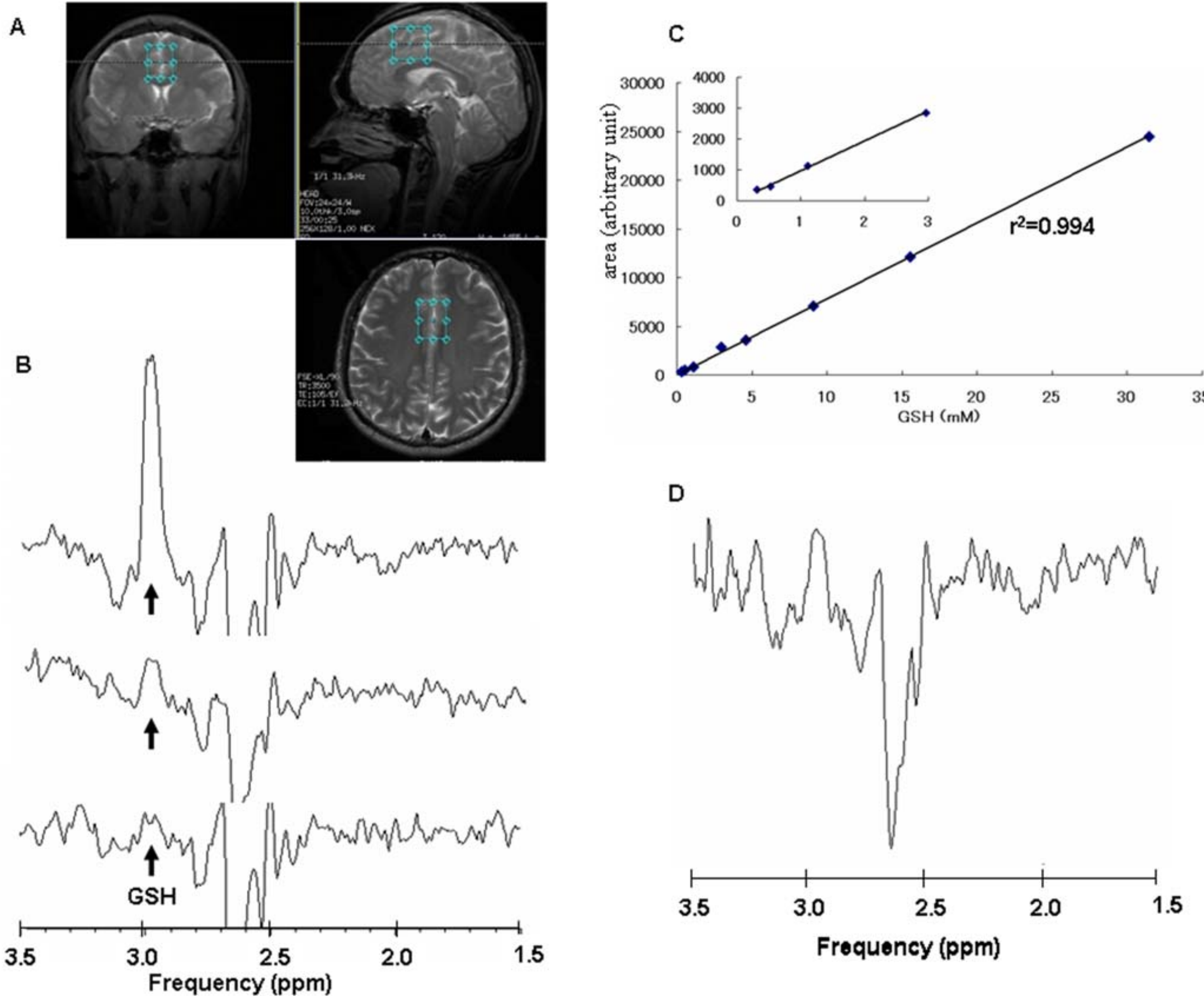

Figure 1. Proton MRS of GSH. (A): T2-weighed magnetic resonance imaging of the targeted region. The blue boxes show the voxel size (28 x 22 x 30 mm) in the posterior medial frontal cortex of a human brain. (B): representative data of reference phantom spectra of GSH (0.5, 1.0, 3.0 mM). Note that the GSH signal increases according to the phantom concentration. (C): Quantification of GSH. Plots showing a linear correlation (r2 = 0.994) between the GSH signal area at 2.95 ppm and the concentration of GSH. (D): Representative data of GSH signals of the posterior medial frontal cortex of a human subject. The GSH level was calculated as 0.735 mM by applying the linear concentration curve on (C).
doi:10.1371/journal.pone.0001944.g001

Evaluation of clinical variables

The Scale for the Assessment of Negative Symptoms (SANS) and Brief Psychiatry Rating Scale (BPRS) were used to evaluate the severity of negative symptoms and psychotic symptoms (positive and negative symptoms), respectively. The Drug-Induced Extrapyramidal Symptoms Scale (DIEPSS) was used to evaluate and exclude the effects of drug-induced extrapyramidal symptoms which could affect the severity of symptoms in schizophrenic patients. Functional disability was assessed using the Global Assessment of Functioning (GAF) scale.

Cognitive function tests

Several cognitive function tests were used. In the Word Fluency Task (letter, category), subjects were given an initial letter (letter fluency task) or a certain category (category fluency task) as a cue [26]. Both tasks consisted of three trials, and the number of words produced in one minute for each trial was recorded for evaluation. In the Stroop Test, a list of twenty four colored dots (D), a baseline test, and 24 colored words incongruent with the color (C) were used. The difference between the reaction time (C-D) was assessed [27]. In the Wisconsin Card Sorting Test (WCST), subjects were instructed to sort cards according to a rule (color, shape, or number). The numbers of achieved categories and perseverative errors were assessed [28]. In the Trail-Making Test part (TMT) A, subjects drew lines as quickly as possible to connect 25 consecutively numbered circles. In the TMT part B, subjects connected 25 consecutively numbered and lettered circles by alternating between the two sequences. The time taken to complete each part of the test was recorded in seconds [29]. In the Digit Span Distractibility Test (DSDT), subjects were asked to remember a tape-recorded string of digits read by a female voice while ignoring the digits read by a male voice (distracter) [30].The percentages of digits correctly recalled in conditions with and without distracters were assessed separately.

Genotyping

Genetic analysis for the genes involved in GSH metabolism-GCLM, glutathione peroxidase 1 (GPX1), and several classes of glutathione-S-transferase (GSTM1, GSTO1, GSTP1, GSTT1 and GSTT2)-as performed by the methods described previously [15,31–33].

Statistical analysis

All calculations were performed with SPSS software (SPSS version 12.0J, Tokyo, Japan). Student's t-test (unpaired) was employed for the comparison of GSH levels between schizophrenic patients and normal control subjects and of the scores of the cognitive function tests between the two groups. For the genotyping results, the differences between patients and controls were evaluated by Fisher's exact test. Pearson's correlation coefficients were examined to identify any correlations of GSH levels with the clinical severity (BPRS, SANS, and DIEPSS) of schizophrenic patients and with the scores of cognitive function tests of all subjects. A value of $p<0.05$ was used as the standard for statistical significance in all analyses.

Results

GSH concentration between schizophrenic patients and healthy comparisons

We used eight phantom solutions of different GSH concentrations (0.3–30 mM) to acquire reference spectra for quantification. As shown in **Figure 1B**, acquired GSH phantom spectra clearly increased their areas at chemical shift 2.95 ppm in a concentration-dependent manner. In **Figure 1C**, plots show a linear correlation ($r^2 = 0.994$) between the GSH signal area and the GSH concentration. The areas of GSH spectra acquired from human subjects *in vivo* were applied to the linear concentration curve for quantification (**Figure 1D**). As shown in **Table 1**, GSH concentration (0.808±0.26 mM (mean±SD)) in the posterior medial frontal cortex of schizophrenic patients (n = 20) did not differ (t = 1.416, df = 34, p = 0.166) from that (0.928±0.24 mM (mean±SD)) of age- and gender-matched normal healthy controls (n = 16)(**Table 1**). Furthermore, there were no correlations between GSH levels and clinical variables (age, education, estimated IQ, age at onset of illness, duration of illness, GAF, and amount of medication) in the subjects.

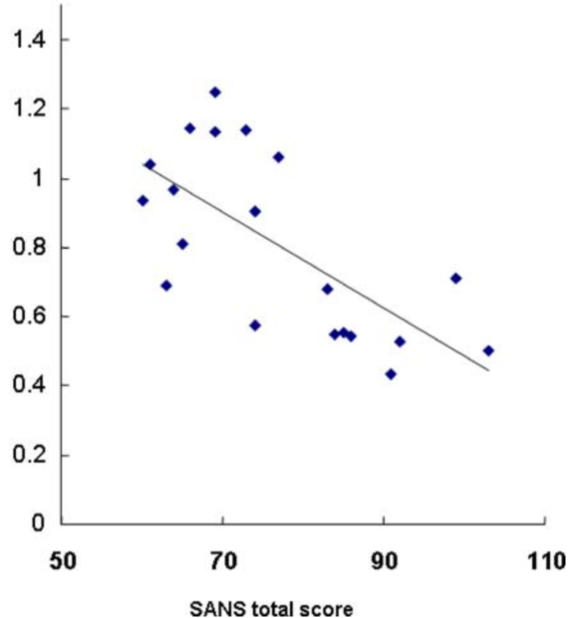

Figure 2. Correlation between GSH levels and the severity of negative symptoms in schizophrenia. There was a significant negative correlation (r = −068, p<0.001) between GSH levels and SANS total scores of schizophrenic patients (n = 20).
doi:10.1371/journal.pone.0001944.g002

Correlation between GSH concentration and clinical variables

We examined the correlation between GSH level and the severity of clinical symptoms (scores of SANS, BPRS and DIEPSS) in the schizophrenic patients (n = 20). Interestingly, there was a significant negative correlation (r = −0.68, p<0.001) between GSH level and SANS total score in schizophrenic patients (**Figure 2**). Of five subscale-symptom groups in SANS, significant negative correlations with GSH level were detected in four subscales (S1: affective flattering-blunting (r = −0.57, p = 0.009), S2: alogia (r = −0.67, p = 0.001), S3: avolition-apathy (r = −0.52, p = 0.02), S4: anhedonia-asociality (r = −0.62, p = 0.004)), but not in attention impairment (r = −0.27, p = 0.252). Furthermore, we also found a significant correlation (r = −0.60, p = 0.005) between GSH levels and the negative symptom subscore on BPRS. However, there were no significant correlations between GSH level and BPRS total score (r = −0.41, p = 0.076), BPRS positive symptom score (r = −0.43, p = 0.059) and DIEPSS score (r = −0.32, p = 0.167). Because these correlations might have been affected by medication, we controlled for the doses of antipsychotics using partial correlation coefficients. Even when the administered antipsychotics (chlorpromazine equivalents) were adjusted for using partial correlation coefficients, the relationships between GSH level and SANS score (partial correlation coefficient = −0.60, p = 0.007) or BPRS negative score (partial correlation coefficient = −0.52, p = 0.02) remained significant.

Correlation between GSH concentration and cognitive functions

As shown in **Table 2**, significant differences were observed between schizophrenia patients and normal controls in all cognitive function tests: Word Fluency (letters: t = 4.67, df = 34, p<0.001; category: t = 3.57, df = 34, p<0.01), Stroop Task (t = −3.47, df = 34, p<0.01), WCST (category: t = 3.95, df = 34, p<0.001; perseverative error : t = −4.61, df = 34, p<0.001), Trail Making Test (TMT-A: t = −3.21, df = 34, p<0.001; TMT-B: t = −3.43, df = 34, p<0.01; TMT-B-A; t = −2.17, df = 34, p = 0.03), and DSDT (without distracter: t = 1.35, df = 34, p = 0.18; with distracter; t = 3.23, df = 34, p<0.01).

Then, we examined the correlations between GSH levels and the scores of cognitive function tests. We found a significant negative correlation (r = −0.36, p = 0.03) between GSH level and TMT-A scores in all subjects (n = 36)(**Table 2**). There were no correlations between GSH levels and the scores of other cognitive function tests (**Table 2**).

Correlations between GSH concentration and the genotypes of enzymes related with GSH metabolism

There was a significantly (p = 0.017) different genotype distribution for the GSTT2 gene between schizophrenic patients and healthy controls. No different distribution was observed in other genes (**Table S1**). Then, we investigated whether or not these genotypes affected GSH levels in the posterior medial frontal

Table 2. Performance on cognitive function tests and their correlations with GSH level

Cognitive function test	mean scores±SD		Coefficients with GSH level (r)[a)]
	Control subjects (n = 16)	Schizophrenia (n = 20)	
Word Fluency (letter)	41.3±8.8	28.4±7.8***	0.15
Word Fluency (category)	48.9±8.4	39.8±6.9**	0.21
Stroop test (C-D, sec)	5.8±3.9	12.1±6.4**	−0.05
WCST (category)	5.1±1.9	2.7±1.8**	0.01
WCST (perserverative error)	2.1±2.5	11.5±8.4***	−0.23
TMT-A (sec)	21.8±6.7	32.0±8.4**	−0.36*
TMT-B (sec)	48.4±18.2	80.5±32.1**	−0.14
TMT B-A (sec)	26.6±13.5	53.6±51.3*	0.06
DSDT (without distractor)	87.9±12.8	80.3±20.8	0.18
DSDT (with distractor)	93.0±7.2	74.4±22.1**	0.31

*P <0.05, **P <0.01, ***P <0.0001 (vs.Control)
[a)]Pearson's coefficients between GSH level in all subjects (n = 36). *P <0.05
WCST : Wisconsin Card Sorting Test, TMT : Trail Making Test, DSDT : Digit Span Distractibility Test
doi:10.1371/journal.pone.0001944.t002

cortex. There were no significant differences in GSH levels relevant to those genotypes. However, we found a difference in GSH levels between patients (n = 15) and normal controls (n = 5) in subjects with the G/G genotype of the GSTT2 (Met139Ile) gene, although the difference only showed a trend toward statistical significance (p = 0.058) (**Figure S1**). We also found a difference in GSH levels between patients (n = 13) and normal controls (n = 11) in subjects with the C/C genotype of the GCLM (ss60297536) gene; again, the differences only showed a trend toward statistical significance (p = 0.099) (**Figure S1**).

Discussion

The major finding of this study was that GSH levels in the posterior medial frontal cortex of schizophrenic patients were significantly correlated with the severity of their negative symptoms. To the best of our knowledge, this is the first report demonstrating the negative correlation between brain levels of GSH and the severity of negative symptoms in schizophrenia.

The measurement of brain GSH levels by ^{1}H-MRS has been elusive up until now because GSH exists at a relatively low concentration and the cysteinyl β-CH_2 signal of GSH at 2.95ppm overlaps with other resonances such as those of aspartate, γ-aminobutylic acid (GABA), and especially creatine, with its high concentration in human brain. The MEGA-PRESS sequence is able to highlight the GSH signal by adding two editing pulses with a normal PRESS sequence. Sufficient GSH signal was obtained by setting an optimum condition with repeated preliminary measurements using both phantom solutions and human subjects, and the shorter TR than in previous studies [12,25,34] enabled us to increase the number of scans within the short examination time.

In this study, we found no alteration in GSH concentrations in the brains of schizophrenic patients, which was consistent with a previous report using the MEGA-PRESS sequence [12], but not a previous report using a double quantum coherence filter technique [10]. The reasons underlying this discrepancy are currently unclear. One possibility may be due to the difference of technique (MEGA-PRESS sequence vs. a double quantum coherence filter) for GSH measurement. Another possibility may be due to medication. The patients enrolled in the study of Do et al. [10] were first-episode patients whereas those in the present study and that of Terpstra et al. [12] were medicated. However, in this study, we found no effect of medication on GSH levels in schizophrenic patients. Therefore, it is unlikely that medication contributes to this discrepancy, although further study is necessary.

The present finding suggests that increasing the brain levels of GSH should be considered a potential therapeutic approach for negative symptoms in schizophrenia. It is well known that oral administration of GSH does not result in its effective increase in the brain because of its poor penetration through the blood-brain barrier, indicating that GSH is not a suitable agent for treating neuropsychiatric diseases such as schizophrenia. The antioxidant N-acetyl-L-cysteine (NAC) has been widely used as a donor of cysteine, the limiting precursor in the synthesis of GSH, and NAC has a good penetration through the blood-brain barrier. Recently, Lavoie et al. [35] reported that treatment of schizophrenic patients with NAC significantly improved impaired mismatch negativity, which is an auditory evoked potential component related to NMDA receptor function [36]. Furthermore, a multi-center double-blinded trial of NAC showed improvement of negative symptoms on the Positive and Negative Symptoms Scale after 6 months of treatment with NAC ([36], Berk et al, unpublished work). Interestingly, it has been reported that GSH-deficient mice showed enhanced dopamine neurotransmission, altered serotonin function, and augmented locomotor responses to low doses of the NMDA receptor antagonist phencyclidine, suggesting that the GSH deficiency produced alterations in monoaminergic function and behavior in mice relevant to schizophrenia [37]. Moreover, we reported that NAC could attenuate behavioral changes and neurotoxicity in rodents and non-human primates after repeated administration of the psychostimulant methamphetamine [38,39]. Taken together, the findings suggest that NAC has potential as a therapeutic drug for negative symptoms in schizophrenia.

In this study, we found a weak negative correlation between GSH levels in the posterior medial frontal cortex and TMT-A scores. There was also a positive correlation (r = 0.47, p = 0.024) between TMT-A scores and SANS total scores in schizophrenic patients. The posterior medial frontal cortex can be divided functionally into two parts: an upper half including Brodman areas 8 and 9 and a lower half including part of the anterior cingulate cortex, Brodman areas 24 and 32 [40]. Both parts are shown to play a role in self monitoring and control of action demanded in the context of social cognitive processes [40]. The relation between GSH level and cognitive symptoms might be assessed in more detail by setting smaller and more specific VOI in the brain, although it is currently difficult to get sufficient GSH signal with smaller VOI. Nonetheless, it seems that GSH levels in the posterior medial frontal cortex may be associated with cognitive impairment as well as negative symptoms in schizophrenia. Therefore, GSH levels in the posterior medial frontal cortex may be a predictive biological factor for the severity of cognitive impairment and negative symptoms in schizophrenia.

In this study, GSH levels were not affected by the genotypes of several genes related to GSH metabolism. The genotype distribution of GSTT2 was significantly (p = 0.017) different between patients (n = 20) and normal controls (n = 16), but this was considered to be a type I error due to the small sample size, as our study using a larger sample size (over 200 of both groups) revealed no significant difference (Matsuzawa et al, submitted). Interestingly, we found that brain GSH levels in patients with the C/C genotype of the GCLM

(ss60297536) gene were lower than those of controls with the C/C genotype of the GCLM (ss60297536) gene although the differences only showed a trend toward statistical significance ($p = 0.099$). Further study using a large sample will be necessary to study the relationship between GCLM gene polymorphism and GSH levels in schizophrenic patients.

In conclusion, the present study suggests a negative correlation between GSH levels in the posterior medial frontal cortex and the severity of negative symptoms in schizophrenia. Therefore, agents (e.g., NAC) that can increase brain GSH levels should be considered potential therapeutic drugs for negative symptoms in schizophrenia.

Supporting Information

Figure S1 GSH levels and the relevance with polymorphisms of GCLM and GSTT2 gene. The plots show GSH levels of controls and patients with each genotype of GCLM-588 (left) and GSTT2 (right). The bars represent mean GSH level ± standard deviation (mM).

Found at: doi:10.1371/journal.pone.0001944.s001 (0.20 MB TIF)

Table S1

Found at: doi:10.1371/journal.pone.0001944.s002 (0.05 MB DOC)

Author Contributions

Conceived and designed the experiments: KH DM. Performed the experiments: ES DM TO YS HN EY HI MI YK. Analyzed the data: KH DM YS. Contributed reagents/materials/analysis tools: TO YS HN EY JT TM HI MI YK. Wrote the paper: KH DM.

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Free Radic Biol Med. Author manuscript; available in PMC 2014 September 01.

Published in final edited form as:
Free Radic Biol Med. 2013 September ; 62: 13–25. doi:10.1016/j.freeradbiomed.2013.05.001.

Glutathione Metabolism and Parkinson's Disease

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Abstract

It has been established that oxidative stress, defined as the condition when the sum of free radicals in a cell exceeds the antioxidant capacity of the cell, contributes to the pathogenesis of Parkinson's disease. Glutathione is a ubiquitous thiol tripeptide that acts alone, or in concert with enzymes within cells to reduce superoxide radicals, hydroxyl radicals and peroxynitrites. In this review, we examine the synthesis, metabolism and functional interactions of glutathione, and discuss how this relates to protection of dopaminergic neurons from oxidative damage and its therapeutic potential in Parkinson's disease.

Keywords

Glutathione; Glutathione S-transferase; Parkinson's disease; Oxidative Stress; Substantia nigra

Introduction

Neurons are among the most metabolically active cells in the body, requiring the correct balance of oxygen and glucose to maintain healthy function. However, when the metabolic balance is overwhelmed and the sum of free radicals in a cell is greater than the capacity of the cell to detoxify these substances, oxidative stress is generated. Increased oxidative stress has been shown to contribute to the etiology or progression of a number of neurodegenerative diseases since the brain uses a disproportionate amount of oxygen per volume of tissue compared to other organs [1]. When free radicals of oxygen are present within the environment of the cell, they may damage lipid membranes, interfere with DNA integrity, and interrupt cellular respiration through alterations in mitochondrial complex I [2–4]. The reduction or detoxification of free radicals is handled by a number of homeostatic mechanisms, in normal physiological conditions.

Parkinson's Disease (PD) is one of the neurological disorders affected by changes in oxidative balance. PD is a progressive neurodegenerative disease with noticeable outward symptoms generally appearing in the sixth decade of life. The most common phenotypes of

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this disorder include progressive deterioration of autonomic and motor functions and in some cases, cognitive decline. Although the underlying etiology of Parkinson's disease is not completely understood [5, 6], the most common neuroanatomical pathology is the accumulation of misfolded alpha-synuclein into intracellular aggregates called Lewy Bodies, present throughout the enteric [7, 8], peripheral [9] and central nervous systems [10, 11]. Progression of the disease results in the significant loss of the dopaminergic neurons situated in the midbrain substantia nigra pars compacta.

Sources of Reactive Oxygen Species in the Substantia Nigra

The loss of dopaminergic neurons located in the substantia nigra pars compacta (A9) is the lesion most characteristic of Parkinson's disease, although other regions of the central, peripheral and enteric nervous systems also show considerable cell loss [12–15]. Within the CNS, it is not entirely clear why the substantia nigra is so significantly affected, although this region does have a number of characteristics that make it particularly vulnerable to oxidative stress. These factors include (but are not limited to) the presence of endogenous dopamine, iron, and neuromelanin [16–18]. Additionally, the intrinsic antioxidant defenses in this structure are more vulnerable than in other brain regions due to lower levels of glutathione (GSH) [19, 20] and glutamylcysteine ligase activity [21], and higher microglial:astrocyte ratios [22, 23].

Dopamine (DA), which is the most abundant neurotransmitter in the basal ganglia [24], is synthesized in the large diameter neurons of the substantia nigra and is released from the terminals that reside within the caudate and putamen nuclei (in rodents this is called the striatum) [25]. Functionally, dopamine modulates excitatory and inhibitory synaptic transmission ensuring smooth directed movement [26]. When released from presynaptic terminals, DA is actively taken up from the synaptic cleft through a number of monoamine transporters (i.e dopamine active transporter (DAT)), where it is packaged into intracellular vesicles by vesicular monoamine transporters (VMAT) [27]. In the SNpc dopaminergic neurons, the predominant VMAT is VMAT2 [28, 29]. When DA is produced in excess of capacity and cannot be transported into the cell through the DAT or packaged internally by VMAT, it remains in free form where it can be readily oxidized to DA quinone, or form superoxides and hydrogen peroxide [30–32]. These superoxides may damage cell and organelle membranes, leading to cellular dysfunction.

Inside the cell, DA quinones react with the sulfhydryl groups of the free amino acid cysteine, cysteine found in glutathione, and other cysteine residues to covalently modify proteins [31, 32] that cause cellular toxicity and in some cases, cell death [30, 31, 33, 34]. DA quinones have also been shown to react with neuromelanin to form eumelanin [35], which is present in DA neurons of the SN. DA may also autooxidize to form hydroxyl radicals ($OH^{\cdot}$) [30, 32, 36] or after oxidation to hydrogen peroxide, may react with iron, copper, or oxygen (O_2) to form hydroxyl radicals [37].

Iron metabolism is necessary for the function of some enzymes, including tyrosine hydroxylase (the rate limiting enzyme in DA biosysthesis) and for overall neuronal health [38–41]. Iron is transported into cells from the bloodstream while bound to transferrin, and stored intracellularly by binding to the protein ferritin [37]. Ferritin in the cytosol is comprised of heavy (H) and light (L) chain subunits. The H-subunit has ferroxidase activity, converting Fe^{2+} to Fe^{3+}, while the L-subunit stabilizes the complex of subunits to remain in iron storage form. The ratios of H- versus L-type subunits of ferritin vary among tissues and in different cell types within the brain. These differences can affect the interactions of iron with other cellular components and make some cell types more vulnerable to oxidative stress [37, 42].

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Within the CNS, the SN is the structure containing the highest level of iron [43, 44]. In a reduced state, iron (Fe^{2+}) readily reacts with hydrogen peroxide to form hydroxyl radicals via the Fenton reaction [37, 45]. The ratio of reduced iron (Fe^{2+}) to oxidized iron (Fe^{3+}) is approximately 1:1 in the normal SN [46, 47]. However, in PD patients the ratio of reduced to oxidized iron in the SN has been reported to increase [48], in one report to 1:3 [49]; a dysregulation not found in other tissues or regions of the brain [49, 50]. Since numerous studies have shown that the elevated levels of reduced iron in the SN can lead to cellular toxicity [51–54], it has been suggested that iron chelation may provide some level of neuroprotection in Parkinson's disease [55–58].

The SN contains another protein that may also contribute to oxidative stress. Neuromelanin, a brown, black insoluble substance that is formed from oxidative metabolites of dopamine and norepinephrine [59, 60], has been shown to interact with lipids, pesticides, other toxic compounds including paraquat [61, 62], and many heavy metal ions including iron [63–65]. Of the transition metals, neuromelanin binds most tightly with iron [62, 65]. Although these interactions may initially be protective [66], when this system is overwhelmed (i.e. iron is present in excess), neuromelanin may begin to catalyze the production of free radicals [67].

Glutathione: An Important Antioxidant in the Brain

Glutathione (GSH), a ubiquitous thiol tripeptide, provides protection from oxidative stress-induced damage through the reduction of reactive oxygen species (ROS). GSH acts alone or in concert with other enzymes to reduce superoxide radicals, hydroxyl radicals and peroxynitrites [3]. Additionally, GSH detoxifies xenobiotics, is a storage and transfer form for cysteine, and maintains cellular redox potential by keeping sulfhydryl proteins in a reduced state [68]. The antioxidant characteristics of GSH have been demonstrated in a number of models of oxidative stress including depletion of GSH with L-buthionine-(S,R)-sulphoximine (BSO) [69–73], ethacrynic acid [74], or reduction of GSH synthesis using antisense directed against gamma-glutamylcysteine synthetase (γGCS), hereafter referred to as glutamylcysteine ligase (GCL) (see section on GSH synthesis below) [75–78] or glutaredoxin 2 [79]. In these studies, diminished levels of GSH increase oxidative stress in whole cells as well as in mitochondrial fractions, and increase lipid peroxidation, intracellular calcium, and gamma-glutamyl transpeptidase (γGT) activity.

Several studies discussed below illustrate these points by utilizing dopaminergic systems. Depletion of GSH by BSO, an irreversible inhibitor of ***GCL***, that does not by itself induce nigrostriatal damage in vivo [80], potentiates the amount of MPTP–induced tyrosine hydroxylase-positive (TH^+) neuron death in the SNpc (48.6% cell death compared to 30.1% cell death) [69, 80]. Additionally, in conditions of increased oxidative stress such as when mesencephalic cells are placed in culture or during normal ageing in vivo, decreasing GSH levels causes neuron loss [76].

The reduction of GSH activity by ethacrynic acid (EA), an effective loop diuretic used in clinical practice [81], has also been shown to increase cell sensitivity to free radicals. Astrocytes exposed to EA and 3-morpholinosydnonimine (SIN1, a compound that generates peroxynitrates) show significant increases in lactate dehydrogenase (an indirect marker of cell death), decreased ATP levels and decreased mitochondrial membrane potential. The critical role of GSH in this system is shown by experiments where excess GSH monoester is included with EA and SIN1, and LDH activity is inhibited [74]. Treatment with a thiol ester compound (gamma-glutamylcysteinyl ethyl esther) or exogenous GSH while examining cellular models of diminished GSH levels in concert with Complex I inhibitors MPP^+, MPTP, or DHBT-1, restores mitochondrial Complex I activity, inhibits cell loss and protects against cell and striatal DA loss [73, 75, 82].

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Further support for the critical role of GSH as a free radical reducing agent is demonstrated in several studies that utilize siRNA knockdown of GSH modulating enzymes. Lee et al [79] used siRNA directed against glutaredoxin 2 (Grx2), a protein that is critical for controlling redox signaling in the mitochondria by modulating the interactions between the glutathione pools and protein thiols [83] to reduce Complex I activity [84]. The reduction in Grx2 results in a loss of Complex I (30%) and m-aconitase (60%) activity, decreased ferritin levels and a subsequent increase in mitochondrial iron [79]; each leading to increased oxidative stress. Similarly, a 50% reduction of GSH by antisense knockdown of GCL [77] increases cellular oxidative stress in PC12 cells. Using this paradigm, Jha et al [78] noted a reduction in mitochondrial performance using three different measures; a 70% reduction of ATP levels, a 65% reduction in mitochondrial pyruvate-dependent 5,5′-dithiobis (2-nitrobenzoic acid) (MTT), and a 60% reduction in oxygen consumption. It was also demonstrated that this effect was specific to complex I, and not complex II, III, or IV activity. Addition of dithiothreitol (a thiol reducing agent) restored complex I activity to control levels in GCL-diminished cells, suggesting that it is the oxidation of sulfhydryl groups that results in the inhibition of complex I. In PC12 cells, GSH depletion decreases TH activity and DA uptake [77]. Additionally, mitochondrial complex I activity, which is reduced in Parkinson's disease and is critical to DA neuron survival [85, 86] is compromised when GSH levels are reduced in combination with exposure to NO and generation of peroxynitrite radicals [74, 75].

In the SN, the reduced form of GSH is an important mediator of oxidative stress. Studies of postmortem CNS of PD patients reveal lower levels of GSH in the SN (40%), but not in other regions of the brain, compared to age-matched controls. In contrast, GSH levels in the SN from patients with other neurodegenerative diseases that involve the basal ganglia, such as Multiple System Atrophy and Supranuclear Palsy, are unchanged [87].

Glutathione Synthesis in the Brain

In non-nervous system mammalian cells, GSH is abundant and can be found at concentrations of 0.5–10 mM [68]. In the brain, however, GSH levels are often found at lower (1–3 mM) concentrations [88]; a protective mechanism itself, since the GSH precursor molecules may be toxic at high concentrations [89–92]. In general, the GSH tripeptide is synthesized as the product of two successive reactions (Figure 1). This synthesis occurs in neurons and glial cells, although astrocytes synthesize GSH more effectively than neurons based on their ability to utilize a wider variety of precursor substrates [93]. Neurons synthesize GSH using glutamine, glutamate, cysteine and glycine or conjugates of these amino acids provided by the breakdown of GSH by γ-glutamyl transpeptidase (γGT). Astrocytes, however, have the ability to utilize a far greater number of substrates in the synthesis of GSH including the amino acids: glutamate, cysteine, glycine, glutamine, aspartate, asparagine, ornithine, proline and cysteine, conjugates of these amino acids as well as glutathione disulfide (GSSG), 2-oxothiadolazine-4-carboxylate (OTC), N-acetylcysteine (NAC) and GSH itself [3, 94–96], although each of these components are ultimately converted to glutamate, cysteine or glycine prior to GSH synthesis [97].

The first step in GSH synthesis is the formation of γ-glutamylcysteine (γGC) from the substrates L-glutamate and L-cysteine by glutamylcysteine ligase (GCL) (also referred to as γ-GlutamylCysteine synthetase (γGCS)) [98]. GCL is a heterodimer containing both disulfide and noncovalent bonds between its heavy and light chain subunits [99] is the enzyme in the rate-limiting step of GSH synthesis. Intracellular GSH levels are regulated by negative feedback of G by GSH [100] as well as the availability of cysteine [68]. An intermediate product of this synthesis, γ-glutamylphosphate, is produced by the reaction of L-glutamate and ATP, which reacts with L-cysteine to form L-γ-glutamylcysteine. In the

second step of GSH synthesis, L-γ-glutamylcysteine and ATP combine to form L-γ–glutamyl-L-cysteinylphosphate that is combined with glycine in a reaction catalyzed by GSH synthase to form GSH [101, 102].

GSH is also metabolized to its component peptides that are recycled to regenerate GSH. In this reaction, the ectoenzyme γ-glutamyl transpeptidase (γGT) catalyzes the transfer of the γ-glutamyl moiety from GSH or a GSH conjugate to an acceptor molecule (CysGly or CysGly conjugate) [95]. As mentioned above, glutathione disulfide (GSSG), 2-oxothiadolazine-4-carboxylate (OTC), and N-acetylcysteine (NAC) can also serve as precursors for the synthesis of GSH [95]. Alternatively, glutathione reductase (GR) uses NADPH as an electron donor to reduce GSSG and regenerate GSH [99].

Reduction of Free Radicals by Glutathione Conjugating Enzymes

Glutathione Peroxidase

Glutathione peroxidases (GPXs) are a group of 8 (GPX1-8) enzymes that are important for reducing hydrogen peroxide to water. GPX's are selenocysteine enzymes that use GSH as a reducing agent and require selenium [103, 104] for their antioxidant function. In the brain, the selenoproteins GPX-1-3 exist as tetrameric proteins, composed of four identical subunits with each monomer having a molecular weight of 22–23 kDa, [105], while GPX4 has activity as a monomer [106]. GPX1 and 4 are found in the mitochondria, nucleus and the cytosol [107], and GPX1, also known as GSHPx, is present in both neurons and glial cells [108, 109]. Overexpression of GPx decreases the amount of neuron loss, reduces hydrogen peroxide accumulation and lipid peroxidation in neurotoxic conditions [110]. Glutathione peroxidase and catalase reduce hydrogen peroxide when acting alone, but more effectively decrease toxicity of exogenous hydrogen peroxide when acting together [111].

GPX proteins and Parkinson's disease

An immunocytochemical study of GPX1 expression shows that dopaminergic neurons in the SNpc express low levels of this protein, while other regions not affected in PD, such the ventral tegmental area, express higher levels [108]. In addition, GPX1 immunoreactive microglia were reported in samples of SN taken from patients with PD as well as those with Dementia with Lewy Bodies Disease (DLB) [109].

In an experimental model of PD, mice that lack GPX1 under normal oxidative conditions show no apparent neuropathological lesions compared to wild-type mice. However, when challenged with MPTP, a toxin that induces oxidative stress, DA, DOPAC, and HVA levels in these mice are decreased. [112]. Additionally, microarray analysis of mRNA expression in the lateral versus medial tiers of the SN reveals downregulation of *gpx1* and *gpx3* in the lateral tier compared to the medial tier of the SN [113]; this is complimentary to the observation of greater cell loss in the lateral SN of PD patients [114]. Savaskan et al. have shown that GPX4 protein level is increased following a neurodegenerative lesion of the entorhinal cortex. Subsequent immunocytochemical analysis shows that the location of this increase occurs in astrocytes [115]. In cortical samples taken from PD patients, GPX3 and GPX4 protein are also elevated compared to control subjects [116].

In DA neurons of the SN, GPX4 is colocalized with neuromelanin [113]. Examination of brains in SN from PD patients shows that volume-density immunolabeling of GPX4 is reduced; however, relative to the remaining cell density, GPX4 levels are increased compared to control subjects [113]. Further study of Selenoprotein 1 (Sepp1, a transport protein and source of selenium for selenoproteins) and GPX4 in the SN and putamen shows an association between Sepp1 and GPX4 localization in the putamen of control subjects

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while the correlation is lost in PD patients. No correlation of Sepp1 and GPX4 immunoreactivity is seen in the SN of PD or control samples [117].

Glutathione S-Transferases

Glutathione S-Transferases (GSTs) are a class of abundant proteins [118] that function as xenobiotic metabolizing enzymes [119–121] in eukaryotes. This class of enzymes may be viewed as a cellular defense against numerous artificial and naturally occurring environmental agents. GSTs function by catalyzing the conjugation of glutathione to various electrophiles and xenobiotics. Additionally, certain GST's have been shown to have other functions, including modulation of cell survival pathways [122, 123].

In humans, there are three distinct classes of GST's: cytosolic, mitochondrial and microsomal. Within the brain, the cytosolic forms of GST are predominant, and will be the focus here. Each of the cytosolic GSTs is configured with different combinations of monomers that are 199–244 amino acids in length [124, 125]. The ultimate composition of the holoenzyme is critical to the function, since each of the monomers contributes half of the overall GSH:electrophile active site [126, 127]; this dimerization occurs in the presence of increased levels of oxidative stress [128].

At this time, seven classes of cytosolic GST have been identified, and are named alpha, mu, pi, sigma, theta, omega, and zeta [129]. Within each GST molecule, there are two characteristic domains: I and II. Domain I comprises the N-terminal residues (1–80) of the protein whose structure consists of a series of beta pleated sheets and alpha helices. Domain II comprises the remaining residues (81–209±11) and is also referred to as the hydrophobic site (H site) [126]. The GSH binding domain is found in Domain I and is structurally conserved in each of the isoforms [130]. Structural differences among the isoforms are found within domain II. The variable residues in this domain contribute to the array of substrate specificity found among the GSTs [130, 131]. For instance, mu and pi classes contain more polar domain II regions that enable them to react with charged substrates [132] whereas the alpha isoform contains a more hydrophobic domain II [130].

In the brain, the active GSTs are composed of dimers containing alpha, mu, or pi class GST monomers [133–137]. GSTmu is the most highly expressed isoform in the brain in terms of abundance, followed by GSTpi, then GST alpha [135]. Cellular localization studies show that GSTpi and GSTmu are expressed in both neurons and astrocytes [135, 138, 139]. Interestingly, in the SN, the structure most affected in the CNS of PD patients, only GSTpi, but not GSTmu, is found in the A9 DA neurons [135]; a finding that may provide a clue why these neurons are particularly sensitive to oxidative stress. The distribution of GSTs in the brain appears to also be age dependent [136]. GSTpi is the only GST isoform that appears to be expressed in human fetal brain [136, 140], while the alpha, mu and pi classes are found in adult [136]. Unlike other components of the glutathione system, GSTs have also been implicated in signal transduction regulation, specifically in pathways involved in mediation of cell death. Mechanistically, GSTmu has been shown to inhibit apoptosis signal-regulating kinase 1 (ASK1) [141] while GSTpi has been shown to inhibit JNK signaling [128, 142–144].

ASK1, which is activated upon oxidative challenge [145–147], facilitates the downstream activation of JNK and the stress activated protein kinase p38 [148, 149]. GSTmu inhibits ASK1 signaling through a protein-protein interaction [141]. Furthermore, following stress, downstream activation of p38 requires the dissociation of GSTmu from ASK1, suggesting that GSTmu regulates the signal cascade mediated by ASK1 [141, 148].

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GSTpi has two distinct functions related to oxidative stress [123]. Under non-stressed conditions, GSTpi exists predominantly in a monomeric form [128]. In this conformation, sequences at the C-terminus of GSTpi have been shown to bind to JNK; which inhibits the interaction of JNK with cJUN and reduces downstream apoptotic signaling [142, 144, 150]. As oxidative stress increases within the cell, GSTpi subunits form dimers. This interferes with the C-terminus interaction with JNK, allowing progression of cell death signaling [128]. The GSH and GST systems collaborate to maintain oxidative homeostasis. When the cellular environment is stress free (i.e. there are low levels of free radicals), GSH levels alone are sufficient to maintain redox balance [151] and monomeric GSTpi bind JNK [128]. As the number of free radicals within the cell increases, GSTpi dimerizes and in this conformation interacts with GSH to more efficiently reduce these free radicals [152], bringing the cell back to homeostasis.

As stated above, GSTpi is the only GST isoform found in the A9 DA neurons [135]; a finding that may provide a clue why these neurons are particularly sensitive to oxidative stress. Suggested by evidence of a decrease in total GSH [20, 153] and increase in oxidative stress in postmortem analysis of PD brains [154–157], alterations in structure or sequence (polymorphisms) of GSTs (particularly GSTmu and GSTpi) may contribute to the disease susceptibility and progression of PD. A number of studies have examined the correlation of GST sequence polymorphisms with PD (Table 1) and with one exception, no associations were found (although see DePalma et al, 1998 [158].

Although polymorphisms in GSTs alone do not appear to correlate with PD etiology, they do appear to have some effect on other aspects of PD, including age of onset and interactions with environmental agents. For example, Golbe et al reported that age of onset in individuals carrying the A53T alpha-synuclein (PARK1) mutation is positively associated with a GSTpi G-for-A nucleotide substitution at position 313 [159], while McCormick et al identified polymorphisms in GSTpi that correlate with increased risk of PD after exposure to pesticides [160]. Additionally, DePalma et al reported a marked increase in association of PD with a GSTT1*0 polymorphism when correlated with other PD risk factors, such as rural living and well water consumption [158]. The lack of a consistent correlation between GST polymorphisms and PD does not rule out a significant role for this enzyme in the etiology of PD, as there could be other regulatory influences on these proteins, including those controlling synthesis and breakdown.

GSTpi and animal models of Parkinson's Disease

The role of GSTs in the protection of DA neurons has been examined using several models of Parkinson's disease. Several groups have mutated the Parkin gene in Drosophila, and find subtle but significant loss of neurons in the protocerebral posterior lateral (PPL) DA neuron cluster [161] as well as an increase in oxidative stress [162] and a downregulation of GSTO1, which has high thiol transferase activity [163]. Examination of these mutants reveals that when GSTS1 is deleted the subtle loss of DA neurons in the PPL is enhanced. Conversely, overexpression of GSTS1 ameliorates this neurodegeneration [161]. When GSTO1, which has two distinct alleles, GSTO1A and GSTO1B, is deleted, there is an increased sensitivity to the xenobiotic paraquat [162, 164]. The re-expression of the GSTO1A in the null mutant eliminates the sensitivity of the PPL DA neurons to paraquat and suppresses phospho-JNK activity, which is implicated in apoptosis [163].

A number of polymorphisms in the leucine rich repeat kinase 2 (LRRK2) gene have been shown to confer PD in humans [165–167]. The most common polymorphisms in the LRRK2 gene are the G2019S and R1441C alelles, which affect kinase signaling [168] and GTPase activity [169], respectively. Recently, Chen et al have demonstrated that expression of the G2019S polymorphic LRRK2 gene in SH-SY5Y cells induces apoptotic cell death and that

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overexpression of GSTP1 reduces this apoptosis. The mechanism for this induction is a G2019S-induced hypermethylation of the GSTP1 promoter that can be reduced by addition of NAC or catalase [170].

Smeyne et al [135] examined the effects of loss of GSTpi on MPP^+ or MPTP-induced dopaminergic neuron death. Using primary cultured dopaminergic cells harvested from the SN of MPTP resistant Swiss-Webster mice [171], inhibition of GST by ethacrynic acid or siRNA directed against GSTpi increases the amount of MPP^+-induced DA neuron death to levels that mimic neurons from an MPTP-sensitive C57BL/6 strain. Similarly, when MPTP is administered to Swiss-Webster mice carrying a null mutation of GSTpi [172] SNpc DA neuron loss increases to levels seen in the MPTP-sensitive C57Bl/6 strain [135]. Administration of MPTP to mice lacking GSTpi also shows cellular ubiquitination and increased susceptibility to ubiquitin proteosome system (UPS) damage and inactivation [173]. Shi et al [174] demonstrates that overexpression of GSTP1 in Neuro2A cells as well as in primary cortical neurons confers protection from oxidative stress induced by addition of rotenone.

GSH Transport in the Brain: Multidrug Resistance Proteins (MDRP) and the Blood Brain Barrier (BBB)

Free radicals that have been reduced by thiol conjugation are transported out of the brain through multidrug resistance proteins (MDRP) at the gliovascular interface [175–178]. MDRPs are a family of ATP-binding cassette (ABC) transporters that actively transport electrophilic substances across cell membranes [179]. This large family of proteins (MDR1 (also known as P-glycoprotein), MRP1-MRP6, MRP8, BSEP, and BCRP), is encoded by 10 genes (ABCB1, ABCC1-ABCC6, ABCC11, ABCB11, and ABCG2) [180, 181]. Each member of the ABC transporter family has a unique pattern of expression in tissue, which may vary between and within species [182–184]. In particular, the localization of transporters is specific to the luminal or basolateral membrane of endothelial cells and at astrocytic endfeet of the BBB [181, 183, 185]. In addition to the BBB, these ABC transporters are also found at the blood-cerebrospinal fluid interface, which forms both a physical and enzymatic barrier between the tight ependymal cell junctions of the choroid plexus and cerebrospinal fluid [186, 187], and also expresses proteins critical to modulating ROS detoxification. The transport of free radical conjugates through MDRPs across the BBB is both ATP and sodium dependent [175, 176].

P-glycoprotein (Mdr1), Mrp1, Mrp2 and Mrp5 are the most widely studied of the transporters in the brain. These transmembrane proteins alter their conformation by utilizing ATP to translocate substances, including xenobiotics conjugated with GSH, across membranes [188–193]. Traditionally, it was believed that the expression of these transporters was found solely on capillary endothelial cells [194–196]; however, confocal studies suggest that some of these transporters may be localized to astrocytes [197].

A number of experiments demonstrate that the expression of these efflux transport proteins are correlated to intracellular oxidative stress levels [176, 198–202]. For example, Hong et al [176] demonstrated that BSO depletion of GSH increases P-gp levels, and conversely, treatment with the antioxidant NAC decreases its expression. In hepatocytes, P-gp transports MPP^+ into the cell [203], suggesting that this pore contributes to xenobiotic sensitivity. P-gps can also transport the anti-Parkinsonian drug budipine [204], suggesting that it may also play a protective role in PD. Ziemann et al [198] demonstrated that addition of H_2O_2 to hepatocytes increases expression of *mdr1*, while Deng et al [205] showed a similar effect on the P-gp protein following genotoxic stress-inducing DNA damage. In models of the BBB, oxidative stress generated by hydrogen peroxide [202] and GSH depletion [176, 201] result

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in increased expression of P-gp. Each of these studies demonstrates the cellular coordination of ROS detoxification between GSH or GST regulation and cellular efflux. In conditions of high ROS, where free GSH levels are lower due to binding of electrophiles, MDRP expression is increased. Conversely, when cells are at homeostasis, free GSH levels are elevated and MDRP levels decrease.

MDRPs and GSTs often act in concert to remove free radicals as well as xenobiotic compounds from cells. Nitrogen monoxide (nitric oxide, NO) has been shown to induce iron release from activated macrophages during the cytotoxic defense against tumor cells [206]. Although necessary for cell signaling and enzyme activity, NO and iron are toxic when free and in excess concentrations within the cell [207–209]. Within the cell, free iron can complex with intracellular NO and GSH to form dinitrosyl-dithiol-iron complexes (DNICs) [206, 210]. A recent study has demonstrated the selective relationship between GSTP1, DNIC's and MRP1 [211]. In the presence of NO or iNOS and hyperexpressed MRP1, cells transfected with GSTP1, but not GSTA1 or GSTM1, have reduced iron efflux compared to cells transfected with the control vector. Cells with MRP1 hyperexpression and GSTP1 expression are also resistant to the cytotoxic effects of S-nitroso-glutathione (GSNO), which is a NO generator, demonstrating the critical role for GSTP1 and MRP1 as a mechanism for free radical detoxification. Investigation of DNICs, GSTp, and MRP1 as an effective storage and release mechanism for NO and iron in the SN may be useful to discover the elements at work in the balance of homeostasis versus oxidative stress leading to neurodegeneration.

Numerous studies demonstrate alterations in MDRPs in Parkinson's disease. Expression of MDR1 in blood vessels is reduced in the striatum of PD patients compared to control subjects [212]. Additionally, studies that examined the uptake of [$^{(11)}$C]-verapamil, a compound that is usually extruded from the brain by P-gp, demonstrate a significant elevation of [$^{(11)}$C]-verapamil in PD patients compared to controls [213, 214] suggesting that P-gp dysfunction contributes to PD pathogenesis [213, 214]. Polymorphisms in MDRPs have also been examined as they relate to PD risk. Westerlund [215] showed that a 1236C/T, but not 2677G/T/A or 3435C/T increases the risk of PD in a Swedish population. The lack of a direct effect of the 3435T is supported by Funke et al [216] and Furano et al [217]. The 3435T polymorphism, however, is implicated as a contributing risk factor in the development of Parkinson's disease when combined with exposure to pesticides [218, 219].

GST Expression as a Biomarker for Parkinson's Disease

One of the major obstacles in interrupting the process of pathogenesis in Parkinson's disease is the inability to detect pathology (i.e. SNpc dopaminergic neuron loss, striatal dopamine loss) prior to onset of observable symptoms (tremor). This has lead to a search for a presymptomatic Parkinson's disease biomarker. According to the NCI Dictionary of Cancer terms, a biomarker is "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease". Numerous functional tests (olfaction, gut motility) as well as imaging modalities (PET, fMRI) have been examined to identify persons with Parkinson's disease prior to symptom onset [220]. However, these are often time reliant on personal narrative or are quite expensive and/or invasive.

Since GSTs are modulated by a number of actions that induce oxidative stress, it is thought that measurement of this class of proteins may allow identification of individuals where this process is aberrant. The observation that certain GSTs are expressed in brain regions affected in Parkinson's disease and change expression in models of PD [135, 139, 174] make these particularly interesting enzymes for study. Werner et al used a proteomic method to examine protein expression in the substantia nigra of PD and age-matched controls and

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find increased expression of both GSTmu and GSTpi isoforms in PD patients [221]. Shi et al [174, 222] examined synaptosomal fractions isolated from the frontal lobes of PD patients and find significantly increased levels of GSTpi protein. Maarouf et al [223], also using a proteomic analysis, examined postmortem ventricular cerebrospinal fluid from PD patients and find a significant increase in GSTpi. Recently, Korff et al [224] measured changes in GSTpi protein in blood, comparing GSTpi levels at baseline and at various times after addition of MPP^+, as an inducer of oxidative stress. They demonstrated that 4 hours after MPP^+, GSTpi is significantly increased in the white blood cells of PD patients compared to control subjects, while no changes are seen when examining whole blood, plasma or the red cell fraction. These studies suggest that GSTpi and potentially other members of this and other anti-oxidant families may be viable biomarkers for PD.

GSH and Precursor Delivery as a Therapy for Parkinson's Disease

As discussed earlier, increased levels of oxidative stress and decreased levels of GSH have been described in a number of PD models [69, 73, 74, 76, 77, 80], as well as in the SN of PD patients [6, 19, 20, 85]. Based on these findings, it has been suggested that restoring the level of GSH in brains of Parkinson's disease patients may be a promising therapy to protect the affected DA neurons from further injury [225]. A number of therapeutic compounds have been examined, including GSH alone (via delivery in liposomes and nanoparticles), co-drugs, such as GSH:L-Dopa or GSH:DA conjugates, as well as GSH analogues, and other hybrid compounds. Optimally, successful candidates should be stable during gastrointestinal digestion, undergo bioconversion to constituent compounds that are transported into the brain, navigate to the desired site of action, and protect against the oxidative damage. Additionally, for effective treatment with these GSH analogues, they should be characterized by limited γ-GT metabolism, while also maintaining their reducing ability [226].

Several examples of these types of compounds have been generated and tested. Minimal improvement was reported in studies examining effect of GSH infusion on the amelioration of PD symptoms. Sechi et al [227] used iv infusion of GSH into nine patients with early untreated PD and found that during infusion patients showed a 42% decline in disability compared to vehicle treated controls. This effect lasted 2–4 months after discontinuation of the infusions. However, in a study that examined 21 patients, intravenous administration of GSH produced no significant improvement [228]. Another chemical method has also been used to increase GSH levels in the brain. Lee et al [229] coupled a hydrogen sulfide (H_2S)-releasing molecule to L-dopa. When injected intravenously, this compound reached the brain and increased dopamine levels by 2.2-fold and GSH by 1.4-fold.

The lack of significant improvement seen following administration of GSH leads to questions regarding the delivery and transport of GSH into the brain. A number of molecules have been used to improve transport through the BBB. Liposomes are artificially prepared vesicles with outer lipid bilayers that encapsulate aqueous materials, including GSH. These liposomes have low toxicity, do not induce an immune reaction and are protected from enzymatic digestion and metabolism [230]. Based on their solubility, liposomes can easily pass through cell membranes including the BBB [231–233]. Although Liposomal-GSH has not been used in human trials, Zeevalk et al show that these conjugates are 100-fold more effective at replenishing GSH levels and preventing degeneration in paraquat + maneb treated mesencephalic cultures than GSH alone. Additionally, the liposomal-GSH is not a substrate for GST's or γ-GT [234].

Nanoparticles provide another method for improving GSH transport into the brain. These are nanometer-scale polymeric substances with a structural and functional organization that

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delivers compounds that are encased by, or linked to the surface of the nanoparticle. Nanoparticles provide high capacity drug loading while maintaining resistance to enzymatic degradation, allowing prolonged drug delivery in the plasma with low toxicity side effects [226, 235]. One such nanoparticle, linking GSH and chitosan, maintains improved stability of GSH in oxidative conditions more effectively than free GSH [236]. Another type of nanoparticle used for drug-delivery across the BBB is dendrimers, a tree-like polymer, approximately 5nm in size, to which a number of functional groups can be attached [237]. These dendrimers are used to deliver the anti-inflammatory and anti-oxidant agent N-acetyl-l-cysteine (NAC, a reducing agent like GSH) to LPS stimulated BV-2 microglial cell cultures. The presence of these NAC-containing dendrimers is significantly more effective than NAC alone in reducing H_2O_2 (68% vs 41%) and TNF-α (77% vs 44%) [238].

Co-drug delivery of compounds, including GSH, links two different compounds that have similar or different modes of action in order to synergize their actions in the brain [239]. A number of these co-drugs have been developed as a potential treatment for Parkinson’s disease. The most frequent cofactor for these co-drugs is l-dopa. L-dopa has been conjugated to a number of other agents including entacapone (a COMT inhibitor marketed under the trade name Stalevo®) [240], cysteine [241], N-acetyl cysteine [242], l-Methionine [241], lipoic acid [243], caffeic acid and carnosine [244]. Co-drugs have been made that directly link GSH and L-dopa [245, 246]. Functionally, it is hypothesized that by joining L-dopa to GSH the exogenous GSH can be directed to the specific neurons within the SNpc that are affected in PD. A second application for co-drug development is to better target the protein of interest (in this case GSH) through the BBB, while protecting the protein from enzymatic degradation so that it can be released in a functional state once in the brain. To achieve this goal, More et al developed a series of metabolically stable urea analogues of glutathione [247], that are protected from 3GT cleavage [248].

Other recently generated codrugs include flavanoid compounds that enhance the uptake of cystine/cysteine by uncoupling their uptake from the cystine/glutamate antiporter, X(c)(–). Flavanoids are plant polyphenols with free radical scavenging capacity. Amino acid moities were added to flavanol compounds to test their effectiveness as neuroprotectants in conditions of glutamate toxicity [249]. Conjugation of the flavanoid epicatechin (EC) with cysteine, cysteamine-EC, increases cell survival and GSH level in a dose-dependent manner [250]. Support for the hypothesis that neuroprotection occurs through increasing cystine/cysteine availability is provided by experiments demonstrating the loss of this effect following BSO treatment or cystine depletion [250].

In addition to co-drugs, glutathione analogues called UPF peptides, each with anti-oxidative capacities, have been synthesized [251]. These compounds link GSH molecules to tyrosine derivatives by an amide bond and have better hydroxyl radical scavenging properties than glutathione alone. Two of these UPF proteins, UPF1 (4-methoxy-L-tyrosinyl-γ-L-glutamyl-L-cysteinyl-glycine) and UPF17 (4-methoxy-L-tyrosinyl-α-L-glutamyl-L-cysteinyl-glycine) are shown to increase free radical scavenging by 500-fold [252]. UPF1 is resistant to γGT activity and increased CuZnSOD activity, suggesting that UPF1 has antioxidant activity independent from GSH [253]. Although not yet tested in animals, or PD patients, these conjugated proteins may provide novel peptide based therapies to slow or ameliorate disease progression.

Conclusions

The cause of Parkinson’s disease is multifactorial, thought to have genetic and environmental etiologies. In each case, the mechanism underlying the disease includes alterations in pathways that increase oxidative stress. In this review, we have discussed the

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role of glutathione as well as facets of glutathione metabolism and function, which can be summarized in Figure 2. Further understanding of the mechanisms by which glutathione interacts with free radicals should lead to new approaches for slowing or ameliorating progression of this and other neurodegenerative diseases.

Abbreviations

ABC	ATP-binding cassette transporter
ASK1	apoptosis signal-regulating kinase 1
BBB	Blood Brain Barrier
BSO	L-buthionine-(S,R)-sulphoximine
COMT	Catechol-O-methyltransferase
DA	Dopamine
DAT	Dopamine transporter
DHBT-1	7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2H-1,4-benzothiazine-3-carboxylic acid
DOPAC	3,4-Dihydroxyphenylacetic acid
γGCS	γ-glutamylcysteine synthetase
γGC	γ-glutamylcysteine
γGT	γ-glutamyl-n-transpeptidase
GCL	glutamylcysteine ligase
GPX	Glutathione Peroxidase
GSH	Glutathione
GSSG	glutathione disulfide
GST	Glutathione S-Transferase
HVA	Homovanillac Acid
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
LPS	Lippopolysaccharide
MDRP	Multidrug Resistance Protein
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	mitochondrial pyruvate-dependent 59,5-Dithiobis (2-nitrobenzoic acid)
NAC	N-acetylcysteine
OTC	2-oxothiadolazine-4-carboxylate
PD	Parkinson’s disease
Pgp	P-glycoprotein
ROS	Reactive oxygen species
SIN1	3-morpholinosydnonimine

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SNP	Single nucleotide polymorphism
SNpc	Substantia nigra pars compacta
TH	Tyrosine hydroxylase
VMAT2	Vesicular monoamine transporter 2

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Highlighs

- Glutathione, a thiol tripeptide, is an important antioxidant in the brain.
- GSH is critical for protecting DA neurons in the SNpc from free radicals damage.
- GPx and GSTs catalyze the reduction of electrophiles using GSH
- GST mutations combined with environmental insults correlate with increased PD risk
- Maintaining GSH levels may provide a therapeutic treatment for PD

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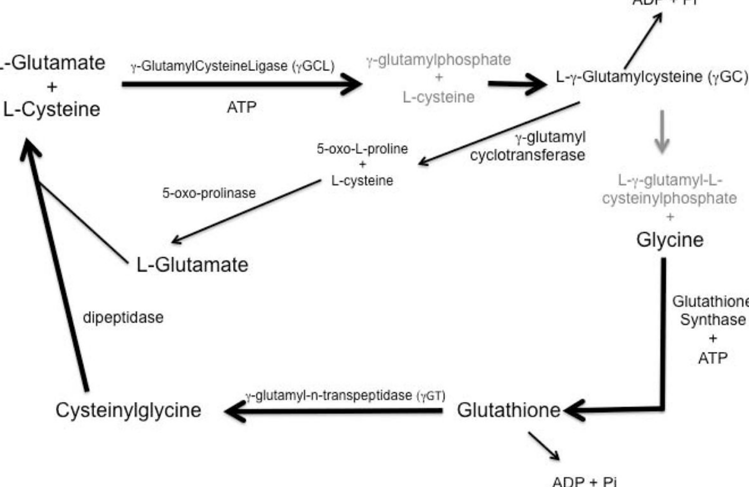

Figure 1.

Glutathione synthesis pathway. Glutathione is synthesized from L-glutamate and L-cysteine in a 2-step reaction catalyzed in an ATP dependent manner by γ-glutamylcysteine ligase (GCL) (also referred to as γ-glutamylcysteine synthetase) and the addition of glycine by glutathione synthase. Glutathione can be recycled to its constitutive amino acids by γ-glutamyl-n-transferase and dipeptidase.

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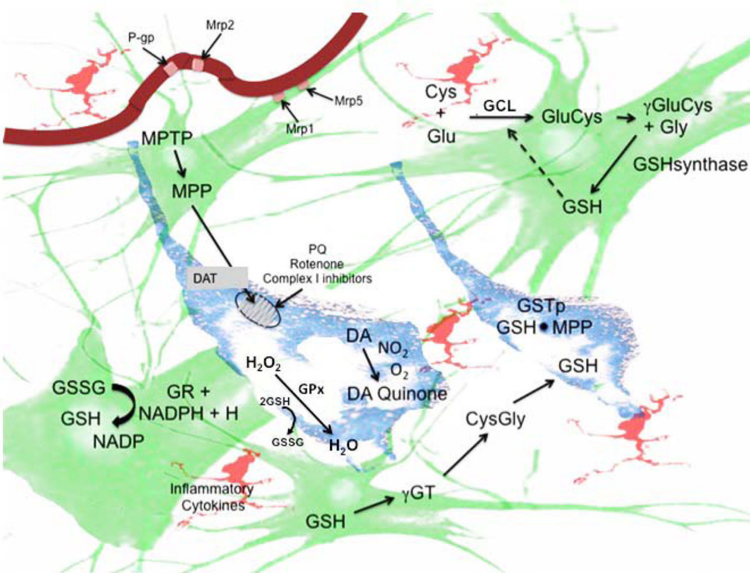

Figure 2.

Schematic representation of glutathione synthesis and catabolism in the substantia nigra. Glutathione (GSH) synthesis occurs in astrocytes (green) and dopaminergic (DA) neurons (blue). GSH is synthesized from L-glutamate (Glu) and L-cysteine (Cys) by γ-glutamylcysteine ligase (GCL) and the addition of glycine by glutathione synthase. Once generated, the oxidized form of GSH (GSSG) can be recycled to reduced GSH by glutathione reductase (GR) and NADPH. Additionally, GSH and/or its conjugates can be recycled by γ-glutamyl transpeptidase (γGT). GSH reduces ROS generated by a number of agents that are transported through the dopamine transporter (DAT), including MPTP, MPP^+, and rotenone, that block mitochondrial Complex I. GSH can also reduce direct redox agents such as paraquat (PQ) or DA adducts (DA quinone) and inflammatory cytokines released from microglia (pink). GSH maintenance and clearance of conjugated electrophiles requires energy in the form of ATP and NADPH. Hydrogen peroxide (H_2O_2) is reduced by glutathione peroxidase (GPx) to water using GSH. In DA neurons, the reduction of free radicals is catalyzed by conjugation of GSH to an electrophile by glutathione S-transferase pi (GSTp). Conjugated adducts are transported from the brain parenchyma through MDRPs, including Mrp1 and Mrp5 through the basolateral membrane into capillary endothelial cells. Once in these cells, other MDRPs, including P-gp and Mrp2 transport these to the bloodstream for excretion.

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Table 1

GST polymorphisms and Parkinson's Disease

GST isoform	association with PD	population	locus	Amino acid change	effect	Reference
GST O1	PD Risk	Caucasian	rs4925	Ala140Asp	$p = 0.034$	[254]
	PD Risk	Japanese	rs4925	Ala140Asp	none	[255]
	PD Risk	Japanese	rs11191972	C to T	none	[255]
	PD Risk with smoking	California			none	[256]
	PD Risk with smoking	California	rs4925	Ala140Asp	none	[256]
	PD Risk with smoking	California	rs2297235	Chr10: 10861 A to G	none	[256]
	Age of Onset	USA	rs4925	Ala140Asp	none	[257]
	PD Risk	Japanese	rs4925	Ala140Asp	none	[258]
	PD Risk,					
	Age of Onset	Australian	rs4925	Ala140Asp	none	[259]
	PD Risk, Age of Onset	Australian	rs4925	Ala140Asp	none	[259]
GST O2	PD Risk	Caucasian	rs156697	Asn142Asp	none	[254]
	PD Risk	Japanese	rs156697	Asn142Asp	none	[255]
	PD Risk	Japanese	rs2297235	-183A to G	none	[255]
	none	California			none	[256]
	PD Risk, Age of Onset	Australian	rs156697	Asn142Asp	none	[259]
	Age of Onset	USA	rs2297235	Chr10: 10861 A to G	none	[257]
GST M1	PD Risk	East Indian	null	null	none	[260]
	PD Risk	Japanese	null	null	none	[255]
	PD Risk	Chilean	null	null	$p = 0.0092$	[261]
	none	Portuguese	null	null	none	[262]
	age of onset	Greek A53T	null	null	none	[159]
	PD Risk, synergy with CYP2D6	French	null	null	none	[263]
	PD Risk	USA	null		none	[264]
	PD Risk	Japanese	null	null	none	[265]
	PD Risk, Age of Onset	Swedish	null		p=.03 for earlier age of onset	[266]
	PD Risk	UK	null		none	[267]

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GST isoform	association with PD	population	locus	Amino acid change	effect	Reference
	PD Risk with exposure to pesticides	Australian	null		none	[268]
GST M3	age of onset	Greek A53T	deletion	intron 6 3-bp deletion		[159]
GST A4	PD Risk	Italian	mutations	sequence 7 exons	none	[269]
GST T1	PD Risk	East Indian	null	null	none	[260]
	PD Risk	Japanese	null	null	none	[255]
	none	Japanese	null	null	none	[270]
	age of onset	Greek A53T	null	null		[159]
GST P1	PD Risk	East Indian	rs1695	Ile105Val	none	[260]
	PD Risk	Japanese	rs1695	Ile105Val	none	[255]
	none	Japanese	rs1695	Ile104Val	none	18327668
	PD Risk	Portuguese	rs1695	Ile105Val	odds ratio = 2.0	[262]
	PD Risk with smoking	California	rs947894	Ile105Val	none	[256]
	PD Risk with smoking	California	rs1799811	Ala114VAl	none	[256]
	PD Risk with smoking	California	rs1799811	Ala114VAl	none	[256]
	none	UK	rs947894	Ile105Val	none	[271]
	age of onset and exposure to pesticides	UK	rs1799811	Ala114VAl	p=.04	[271]
	age of onset	Greek A53T	rs1695	Ile105Val	p=.02	[159]
	age of onset	Greek A53T	rs1799811	Ala114Val	p=.05	[159]
	PD Risk	USA		Ile104Val	none	[264]
	PD Risk	USA		Ala113Val	none	[264]
	PD Risk and exposure to pesticides	Australian	rs1799811	Ala114Val	p=0.009	[268]
GST Z1	age of onset	Greek A53T	Lys32Glu	Lys32Glu		[159]
	age of onset	Greek A53T	Arg42Gly	Arg42Gly		[159]
	PD Risk	Australian		Glu32Lys	none	[272]
	PD Risk	Australian		Gly42Arg	none	[272]
	PD Risk and exposure to pesticides	Australian		Arg42Gly	none	[268]

Glutathione metabolism in brain

Metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species

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Glutathione metabolism in brain

Metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species

Abstract

The cells of the adult human brain consume ≈20% of the oxygen utilized by the body although the brain comprises only 2% of the body weight. Reactive oxygen species, which are produced continuously during oxidative metabolism, are generated at high rates within the brain. Therefore, the defense against the toxic effects of reactive oxygen species is an essential task within the brain. An important component of the cellular detoxification of reactive oxygen species is the antioxidant glutathione. The main focus of this short review is recent results on glutathione metabolism of brain astrocytes and neurons in culture. These two types of cell prefer different extracellular precursors for glutathione. Glutathione is involved in the disposal of exogenous peroxides by astrocytes and neurons. In coculture astrocytes protect neurons against the toxicity of reactive oxygen species. One mechanism of this interaction is the supply by astrocytes of glutathione precursors to neurons.

- Enhanced PDF
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Abbreviations
CysGly cysteinylglycine
GPxglutathione peroxidase
GRglutathione reductase
GSHglutathione
GSSG glutathione disulfide
γGluCys γ-glutamylcysteine
γGT γ-glutamyl transpeptidase
ROS reactive oxygen species

Reactive oxygen species (ROS) are generated continuously during oxidative metabolism. ROS include inorganic molecules, such as the superoxide radical anion, hydrogen peroxide (H_2O_2) and hydroxyl radicals, as well as organic molecules such as alkoxyl and peroxyl radicals. In order to avoid damage caused by ROS, such as DNA strand breaks, lipid peroxidation and protein modification, mechanisms have been developed during evolution which dispose of, or prevent the generation, of ROS. For example, the removal of H_2O_2 and superoxide prevents the generation of highly reactive hydroxyl radicals, which are formed by the iron-catalyzed Fenton reaction or by the Haber–Weiss reaction [1]. Increased production of ROS and/or a decrease in the antioxidative capacity of cells causes oxidative stress which can compromize essential cellular functions.

Compared with other organs, the brain appears to be especially endangered with regard to the generation and detoxification of ROS. The cells of the human brain utilize 20% of the oxygen consumed by the body although this organ comprises only 2% of the body weight [2]. This indicates the generation of a large quantity of ROS during oxidative phosphorylation in brain. In

addition, a high iron content has been reported for some brain areas [3], which is able to catalyze the generation of ROS. In contrast, the brain might be especially vulnerable to ROS, because it is rich in lipids with unsaturated fatty acids, the targets of lipid peroxidation. In addition, the brain contains only low to moderate superoxide dismutase, catalase and glutathione peroxidase (GPx) activity compared with kidney or liver [4]. Such disadvantages of the brain have to be considered in view of the fact that a loss of neurons in adult brain cannot be compensated for by the generation of new neurons. Nevertheless, the brain is able to function during a long human life, indicating the presence of an effective antioxidant system in brain. However, the balance between the generation of ROS and antioxidative processes may become disturbed as reported for several neurological disorders. The best evidence of an altered glutathione metabolism as an important factor contributing to the pathogenesis of a neurodegenerative disease has been found in Parkinson's disease. The involvement of a compromised glutathione system in a neurological disorders is reviewed in this issue by Schulz *et al*. [5]. In this context, insufficient mitochondrial functions are considered to play an important role [6]. The knowledge on glutathione and nitric oxide in respect to mitochondrial function has recently been summarized [7].

Metabolism and functions of glutathione

The tripeptide glutathione (GSH; γ-l-glutamyl-l-cysteinylglycine) is the most abundant thiol present in mammalian cells with concentrations of up to 12 mm[4]. GSH is synthesized *in vivo* by the consecutive action of two enzymes (Fig. 1). γ-Glutamylcysteine (γGluCys) synthetase uses glutamate and cysteine as substrates and forms the dipeptide γGluCys, which is combined with glycine in a reaction catalyzed by glutathione synthetase to generate GSH. ATP is a cosubstrate for both enzymes. The balance of cellular synthesis and consumption of GSH is regulated by feedback inhibition of the γGluCys synthetase reaction by the endproduct GSH [8].

Glutathione metabolism in brain

Metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species

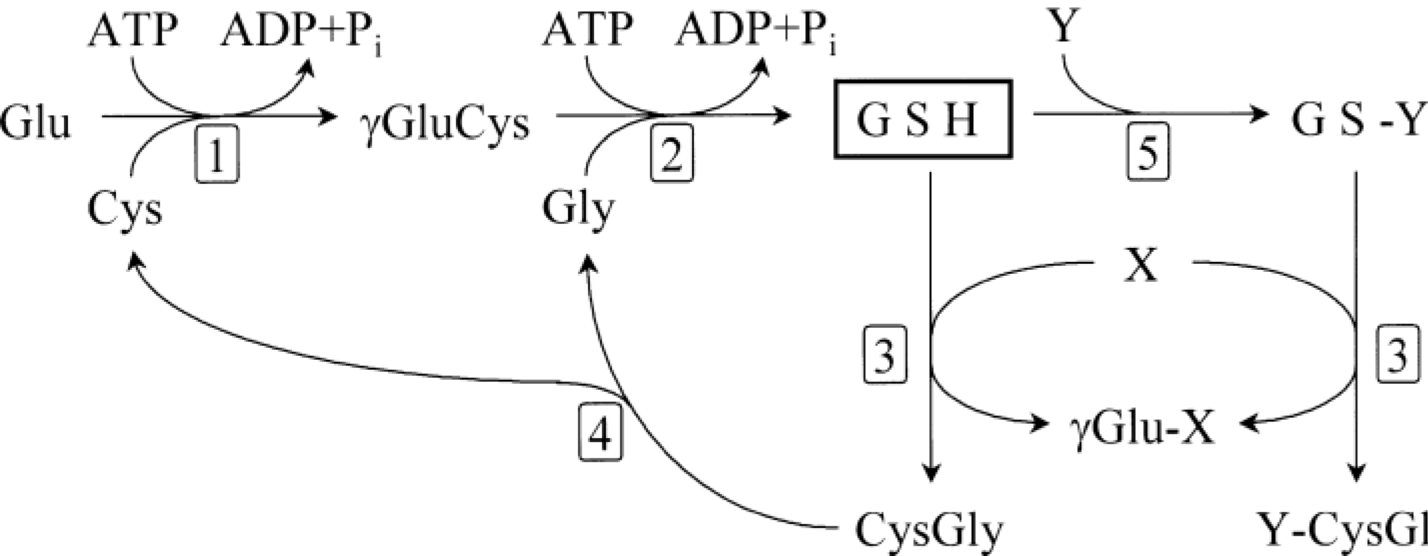

Figure 1.

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Metabolism of glutathione. GSH is synthesized by the two consecutive ATP-consuming reactions of γ-glutamylcysteine synthetase (1) and glutathione synthetase (2). GSH is a substrate of the ectoenzyme γGT (3). X represents an acceptor of the γ-glutamyl moiety transferred from GSH by γGT. The dipepetide CysGly is generated in equimolar concentrations to that of GSH used in the γGT reaction and is hydrolyzed by the reaction catalyzed by a dipeptidase (4). Intracellular GSH is conjugated by glutathione-*S*-transferase(s) (5) to xenobiotics or endogenous compounds (represented by Y). These conjugates are substrates of γGT.

GSH has important functions as an antioxidant, is a transport and storage form of cysteine, is a reaction partner for the detoxification of xenobiotica, and is a cofactor in isomerization reactions [4,9]. In addition, GSH maintains the thiol redox potential in cells keeping sulfhydryl groups of cytosolic proteins in the reduced form. Recent results suggest that GSH also plays a role in the regulation of apoptosis [10].

The glutathione system is especially important for cellular defense against ROS. GSH reacts directly with radicals in nonenzymatic reactions and is the electron donor in the reduction of peroxides catalyzed by GPx (Fig. 2). The product of the oxidation of GSH is glutathione disulfide (GSSG). GSH is regenerated from GSSG within cells in a reaction catalyzed by the flavoenzyme glutathione reductase (GR). This enzyme regenerates GSH by transferring reduction equivalent from NADPH to GSSG (Fig. 2).

Metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species

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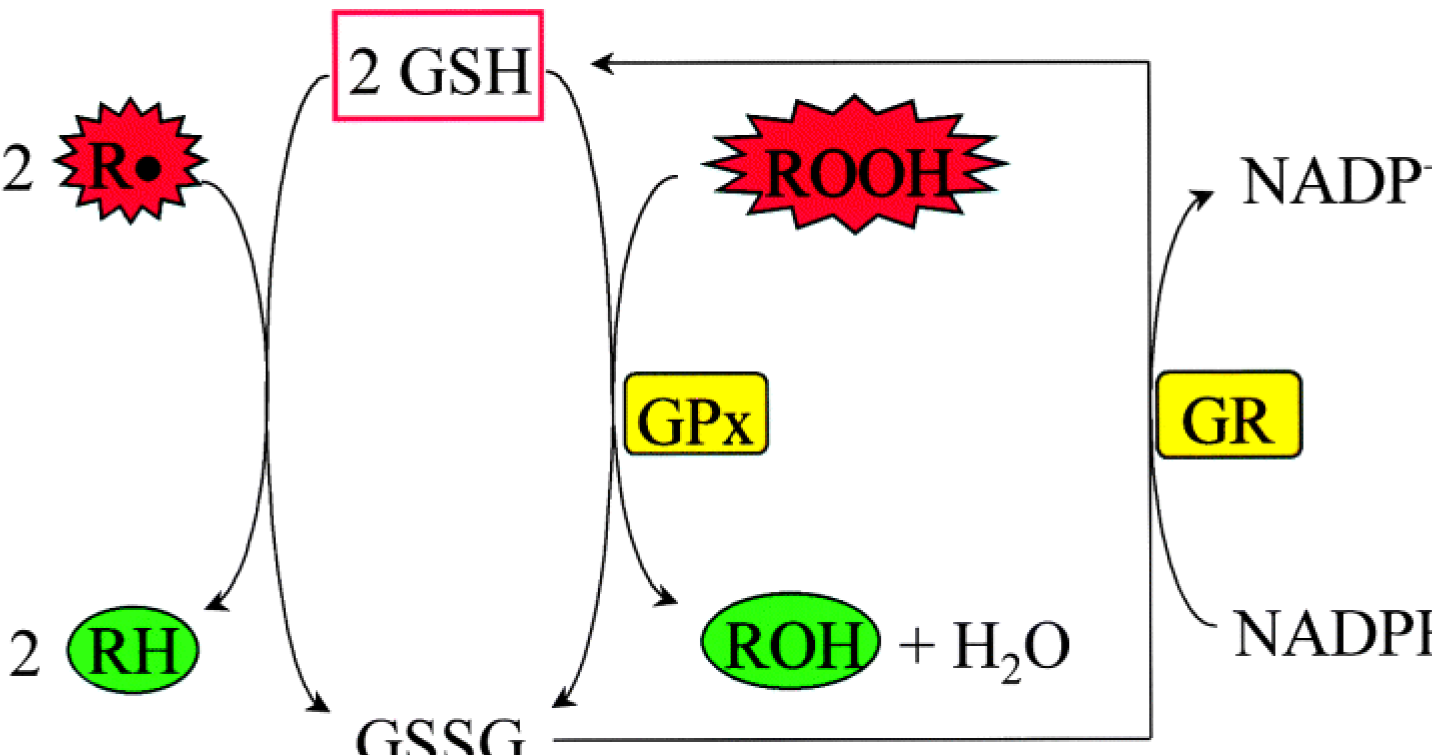

Figure 2.

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Function of GSH as an antioxidant. GSH reacts nonenzymatically with radicals (R˙) and is the electron donor for the reduction of peroxides (ROOH) in the reaction catalyzed by GPx. GSH is regenerated from GSSG by GR which uses NADPH as cofactor.

During the course of the reactions catalyzed by GPx and GR, glutathione is recycled (Fig. 2). In contrast, GSH is consumed during the generation of glutathione-*S*-conjugates by glutathione-*S*-transferases [11] or by the release of glutathione from cells [12,13]. Both processes lower the level of total intracellular glutathione. Therefore, in order to maintain a constant intracellular GSH concentration the GSH consumed has to be replaced by resynthesis from its constituent amino acids. Extracellular GSH and glutathione conjugates are substrates for the ectoenzyme γ-

glutamyl transpeptidase (γGT). This enzyme catalyzes the transfer of a γ-glutamyl moiety from GSH or a glutathione conjugate onto an acceptor molecule (Fig. 1). Products are a γ-glutamyl compound and the dipeptide cysteinylglycine (CysGly) or the CysGly conjugate [14]. Peptidases hydrolyze CysGly to cysteine and glycine. These amino acids can subsequently serve again as substrates for cellular GSH synthesis (Fig. 1).

Glutathione metabolism of astrocytes and neurons

The available data on the glutathione content of various brain areas and the localization in brain of GSH and enzymes of glutathione metabolism have recently been reviewed [4,15]. Astrocytes appear to contain higher GSH levels than neurons both *in vivo* and in culture [4,15]. In recent years, glutathione metabolism of brain cells has been studied predominantly in primary cultures enriched for one type of brain cell. From experiments performed on such cultures ample information is available regarding glutathione metabolism in astrocytes and neurons. In contrast, little is known about GSH metabolism in oligodendroglial and microglial cells [15].

The GSH content of cultured astroglial cells can be modulated by a variety of treatments [15]. For example, GSH levels decrease as soon as the synthesis of GSH is inhibited by buthionine sulfoximine or if cells are incubated in the presence of reagents such as dimethyl maleate or ethacrynic acid, which react with the thiol group of GSH. In contrast, GSH levels increase after application of GSH precursors [15]. Depletion of GSH by amino acid deprivation and the subsequent refeeding of putative GSH precursors revealed that a variety of exogenously applied amino acids, sulfur-containing compounds and peptides can be used by cultured astrocytes as precursors for GSH synthesis [16]. The best exogenous precursor of the cysteine necessary for GSH synthesis in astroglial cells is the amino acid cystine [17]. This amino acid is transported in a sodium-independent manner across the astroglial cell membrane in exchange for glutamate via the transport system X^{-}_{C}[18].

Astroglial cultures release GSH [19–21] which is used as a substrate for the ectoenzyme γGT. Within 1 h astroglial cultures release ≈10% of their intracellular glutathione [21]. Simultaneously, GSH is resynthesized in order to compensate for the GSH released and to maintain a constant cellular concentration of GSH. This release of GSH from astroglial cells is quantitatively the most important process consuming intracellular GSH. The rate of release of GSH from astroglial cells depends on the intracellular GSH concentration and follows apparent Michaelis–Menten kinetics [20]. Extracellular GSH serves as substrate for the astroglial ectoenzym γGT [21]. The dipeptide CysGly, the product of the γGT reaction, is reused by cultured astroglial cells for GSH synthesis. The peptide transporter PepT2 is expressed in these cultures and is responsible for the uptake of CysGly [22]. After intracellular hydrolysis of CysGly, the cysteine and glycine generated serve as substrates for astroglial glutathione synthesis [23].

The GSH levels reported for cultured neurons vary greatly which has been attributed to different preparation techniques, to species differences or to different culture conditions [15]. With regard to the culture conditions, the content of cysteine or cysteine precursors in the medium in particular determines the GSH level in neurons, because neurons are not able to use the cystine present in most culture media and rely on the availability of cysteine for their glutathione synthesis [24,25]. In contrast to cysteine, the availability of the glutamate precursor glutamine or of glycine does not limit neuronal glutathione synthesis [26]. In addition to cysteine, brain neurons are able to use the cysteine donors CysGly, γGluCys and *N*-acetylcysteine as precursors for GSH [26,27]. Other compounds and treatments which modulate GSH levels in cultured neurons have recently been summarized [15].

Among exogenous precursors of neuronal GSH, the dipeptide CysGly may be the most important because it is generated from extracellular GSH in the γGT reaction. CysGly in micromolar concentrations is efficiently utilized by neurons [26]. The concentration of CysGly leading to a half-maximal GSH level is lower in neurons [26] than in astroglial cells [23], indicating that neurons are more efficient in using this peptide than astroglial cells. To date, the mechanism by which CysGly is utilized by neurons has not been elucidated completely. This peptide might be taken up into neurons by a peptide transporter, as described for astroglial cells [23]. Alternatively, the dipeptide might be hydrolyzed by a neuronal ectopeptidase generating amino acids, which are subsequently taken up as precursors for GSH synthesis. Evidence obtained recently in our laboratory strongly suggests the involvement of a neuronal ectopepetidase in the utilization of CysGly by neurons (R. Dringen, unpublished results). The cysteine and glycine liberated by the hydrolysis of CysGly serve as precursors for neuronal GSH [26]. These amino acids are taken up into brain cells via sodium-dependent transport processes [18,28,29].

Glutathione and the disposal of peroxides by astrocytes and neurons

Cultured astroglial cells dispose of exogenous H_2O_2[30,31] and organic hydroperoxides such as tertiary butyl hydroperoxide or cumene hydroperoxide [32,33] very efficiently. These peroxides are substrates of GPx. Indeed, rapid oxidation of GSH was found after application of peroxides to astroglial cultures [31–33]. Inhibition of catalase, the second cellular enzyme involved in H_2O_2 disposal, reduced at best marginally the clearance rate for H_2O_2 as long as the astroglial glutathione system was not compromised. In contrast, inhibition of both catalase and GPx strongly reduced the capability of astroglial cells to dispose of H_2O_2[31]. These findings demonstrate that the glutathione system of astroglial cultures can substitute for the function of catalase in H_2O_2 clearance. Catalase does not accept organic hydroperoxides as substrates. Therefore, the glutathione system is responsible and sufficient for the rapid disposal of organic hydroperoxides by astroglial cultures [32,33].

Cultured neurons have also been reported to dispose of exogenous H_2O_2. Evidence has been presented that the neuronal defense against H_2O_2 is mediated primarily by the glutathione system [30]. Indeed, application of H_2O_2 to neurons causes rapid oxidation of GSH. Removal of the peroxide is followed by an almost complete regeneration of the original GSH to GSSG ratio within minutes [34]. Apparently, astroglial cultures have a higher capacity than neurons to detoxify H_2O_2[30,34]. However, it must be stressed that for such a comparison the differences in cell numbers between confluent astroglial cultures and cultured postmitotic neurons have to be considered. If the differences in protein content of the cultures are taken into consideration, the rate of H_2O_2 clearance by the cells in primary neuronal and astroglial cultures is almost identical [34]. This indicates that, at least in culture, both types of brain cell are equally able to detoxify exogenous H_2O_2. However, for the rapid clearance of H_2O_2 by neurons both GPx and catalase are essential and, in contrast to the situation in astroglial cultures [31], the glutathione system in neurons cannot functionally compensate for loss of the catalase reaction [34]. The lower efficiency of the neuronal glutathione system of peroxide detoxification compared with that of astroglial cells is confirmed by the reduced ability of cultured neurons to dispose of the organic peroxide cumene hydroperoxide from the medium [34].

Interaction between astrocytes and neurons in glutathione metabolism and the defense against ros

In vivo the different types of brain cell are in close contact with each other. Evidence is growing that, especially between astrocytes and neurons, an intensive metabolic exchange occurs. Such interactions also appear to be important regarding cerebral glutathione homeostasis and the protection of the brain against oxidative stress [15].

In coculture, astrocytes support other brain cell types in the defense against ROS. In the presence of astroglial cells neurons are protected against the ROS-induced toxicity of various compounds and treatments (Table 1). Because H_2O_2 is the peroxide generated in the highest quantity in the brain, the protection by astrocytes of neurons against the toxicity caused by H_2O_2 appears to be particularly important [30,35]. In coculture, neurons are protected against H_2O_2 toxicity even at a cellular ratio of 1 astroglial cell to 20 neurons [30]. Neurons in culture become damaged by extracellular ROS [43] which are detoxified in the presence of astroglial cells. GSH is important for this function, because the protective effect of astroglial cells is diminished when these cells contain low levels of GSH [44].

Table 1. **Protection by cocultured astroglial cells of neurons against the toxic effects of various compounds.**l-Dopa, 2,4-dihydroxyphenylalanine.

Neurons protected	Toxic compound/treatment	References
Striatal neurons	H_2O_2	[30]
Mesencephalic neurons	H_2O_2	[35]

Neurons protected	Toxic compound/treatment	References
	l-Dopa	[36,37]
	6-hydroxydopamine	[38,39]
Cortical neurons	γ-radiation	[40]
	NO, superoxide, $FeSO_4$	[41]
Cerebellar neurons	Dopamine	[42]

For the synthesis of GSH, a metabolic interaction between neurons and astroglial cells takes place. Only the availability of cysteine determines the level of neuronal GSH [26]. If neurons are cultured in the presence of astroglial cells, the GSH content of the neurons increases strongly indicating that, in the presence of astroglial cells, a cysteine precursor is provided from the astroglial cells to the neurons improving neuronal GSH synthesis. The dipeptide CysGly, which is generated from extracellular GSH by the γGT reaction [21], is utilized efficiently in micromolar concentrations as a precursor for neuronal GSH [26]. Inhibition of γGT prevented totally the astroglia-induced effect on the GSH content in neurons [26] demonstrating that CysGly is most probably the GSH precursor provided by astroglial cells to neurons (Fig. 3).

Glutathione metabolism in brain

Metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species

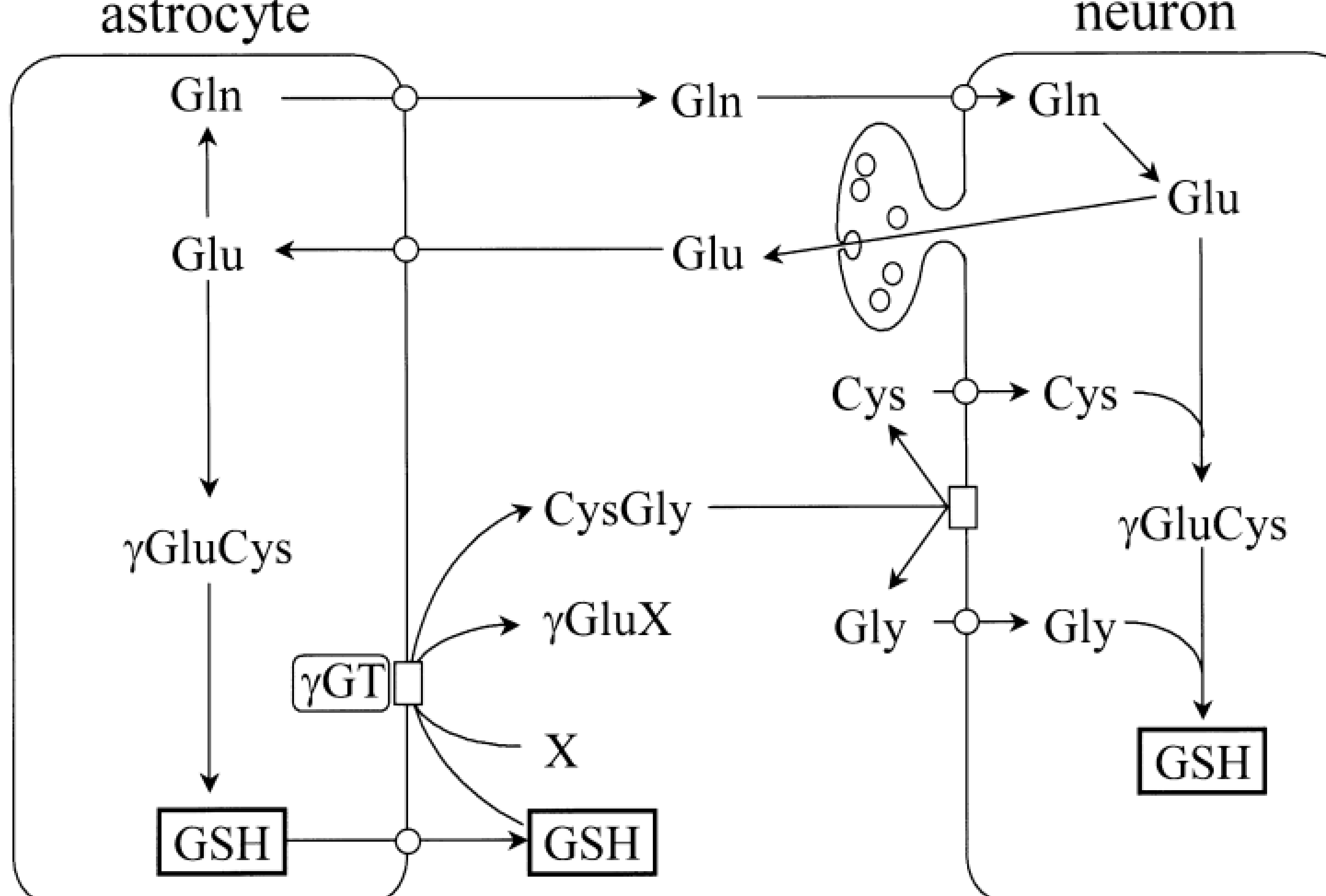

Figure 3.

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Scheme of the proposed metabolic interaction between astrocytes and neurons in GSH metabolism. The GSH released from astroglial cells is a substrate for the astroglial ectoenzyme γGT. X represents an acceptor of the γ-glutamyl moiety transferred by γGT from GSH. CysGly, generated by the γGT reaction, serves as a precursor for neuronal GSH. Most probably, the hydrolysis of CysGly for neuronal utilization occurs via a neuronal ectopeptidase. In addition, glutamine is released from astrocytes and used by neurons as a precursor for the glutamate necessary as neurotransmitter and for GSH synthesis.

Metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species

Figure 3 shows our hypothesis for the metabolic interaction between astrocytes and neurons regarding glutathione metabolism. With the release of glutamine by astroglial cells [45] and the extracellular generation of CysGly from GSH, astroglial cells provide neurons with all three constituent amino acids of GSH. The hypothesis presented here for the metabolic interaction involved in GSH metabolism between astrocytes and neurons (Fig. 3) is supported by recent results obtained on brain slices [46] and in a microdialysis study [47]. Following the onset of hypoxia, the concentration of cysteine in the superfusion solution of brain slices increased strongly, an effect which was prevented almost completely in the presence of the γGT-inhibitor acivicin [46]. After microinfusion of 1-methyl-4-phenylpyridinium into rat brain a >1000-fold transient increase in the concentration of GSH in the microdialysates was determined, which was followed by an increase in the extracellular cysteine concentration [47]. The rate of disappearance of GSH and the subsequent increase in cysteine concentration was strongly affected by inhibition of γGT [47]. These data demonstrate that the cysteine found in these experimental systems has most likely been generated from extracellular GSH by the consecutive reactions of γGT and a dipeptidase.

Conclusions

Coculture experiments have demonstrated convincingly that brain astrocytes and neurons strongly influence each other with regard to GSH metabolism and defense against ROS. The importance of astroglial cells for the defense of the brain against ROS and especially the function of astroglial GSH metabolism has become evident at least for cell culture models. Such results suggest that *in vivo* a compromised astroglial glutathione system may contribute to a lower defense capacity of the brain against ROS and subsequently to increased susceptibility to ROS of astrocytes themselves and of neighboring cells.

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Mol Aspects Med. Author manuscript; available in PMC 2010 February 1.

Published in final edited form as:
Mol Aspects Med. 2009 ; 30(1-2): 1–12. doi:10.1016/j.mam.2008.08.006.

Glutathione: Overview of its protective roles, measurement, and biosynthesis

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Abstract

This review is the introduction to a special issue concerning, glutathione (GSH), the most abundant low molecular weight thiol compound synthesized in cells. GSH plays critical roles in protecting cells from oxidative damage and the toxicity of xenobiotic electrophiles, and maintaining redox homeostasis. Here, the functions and GSH and the sources of oxidants and electrophiles, the elimination of oxidants by reduction and electrophiles by conjugation with GSH are briefly described. Methods of assessing GSH status in the cells are also described. GSH synthesis and its regulation are addressed along with therapeutic approaches for manipulating GSH content that have been proposed. The purpose here is to provide a brief overview of some of the important aspects of glutathione metabolism as part of this special issue that will provide a more comprehensive review of the state of knowledge regarding this essential molecule.

Keywords

Glutathione; Glutamate cysteine ligase; Hydroperoxide; Xenobiotic; Methods

1. Introduction

The tripeptide, γ-L-glutamyl-L-cysteinyl-glycine known as glutathione (GSH) (Fig. 1), is the most important low molecular weight antioxidant synthesized in cells. It is synthesized by the sequential addition of cysteine to glutamate followed by the addition of glycine. The sulfhydryl group (−SH) of the cysteine is involved in reduction and conjugation reactions that are usually considered as the most important functions of GSH. These reactions provide the means for removal of peroxides and many xenobiotic compounds; however, GSH is also involved in regulation of the cell cycle (Meister 1992).

2. Sources of oxidants

GSH plays a major role in removal of many reactive species. But, before addressing those aspects, it is important to understand from where these reactive species come and their pathological consequences that GSH helps avoid. Quinones are a class of redox cycling molecules that includes some drugs and xenobiotic compounds. Redox cycling in this context refers to the ability to cycle between oxidized and reduced forms and in the process, produce reactive oxygen species, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2). In this reaction (Fig. 2), the quinone is reduced by one electron transport reaction to produce a semiquinone, which is a free radical that can react with oxygen to produce O_2^-.

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There are other places in the cells where reactive oxygen species can be generated. In phagocytes, a major part of the mechanism of killing microorganisms involves production of reactive oxygen species (Forman and Thomas, 1986). The first enzyme involved is NADPH oxidase (NOX) that produces O_2^-. That enzyme is now known to be a member of a class of enzymes found in almost all cells (Vignais 2002). Once O_2^- is made, it can be dismuted into H_2O_2 both by a relatively fast non-enzymatic reaction and by very fast reaction catalyzed by one of the superoxide dismutases (SOD). Some phagocytes have the capacity to secrete enzymes called myeleoperoxidases that can catalyze a reaction of H_2O_2 and halides (chloride or bromide) to produce hypochlorous acid (HOCl) or hypobromous acid (HOBr) (Bakkenist et al., 1980). These hypohalous acids kill bacteria but can also damage normal tissue and thereby contribute to an inflammatory reaction.

The H_2O_2 formed can also be potentially hazardous if there are reduced metals present in the cells. H_2O_2 can react with ferrous iron (Fe^{2+}) and produce the hydroxyl radical (OH). This radical has capability to oxidize Teflon or fluorine and any organic molecule at near diffusion limited rates. In other words, OH can react with any molecule next to where it is produced. O_2^- can reduce ferric iron (Fe^{3+}) to Fe^{2+}, which suggests that it can play two roles in producing OH; however, reduction of Fe^{3+}can also occur with other reductants such as ascorbic acid (vitamin C).

One of the dangers of producing OH is when it is produced near a membrane. Lipids can be oxidized by OH and start a free radical chain reaction that will damage the membrane. In the initiation of lipid peroxidation by OH, the reaction with a reduced molecule of the lipid produces a lipid radical (L) and water. The L can react with oxygen to produce hydroperoxide radical (LOO), which then reacts with another lipid molecule, generating a lipid peroxide (LOOH) and another lipid radical L that can continue a chain reaction. One of the dangers from lipid peroxidation besides membrane damage is the production of byproducts such as 4-hydroxy-2-nonenal (HNE). Arachidonic acid is a polyunsaturated fatty acid found in membranes of all cells. When it becomes oxidized, it can break down yielding a large variety of compounds including a, β-unsaturated aldehydes (Poli et al., 1987) These are toxic compounds because they can react with proteins in the cells, particularly at cysteine, lysine or histidine by either Michael addition to the carbon–carbon double bond or by Schiff base formation at the carbon–oxygen double bond (Esterbauer et al., 1991; Eckl, 2003; Schaur, 2003). These reactions can inactivate the function of proteins. For example, reaction with an active site cysteine can destroy the activity of an enzyme.

The final component of oxidative damage considered here is peroxynitrite ($ONOO^-$). This ion is made in a reaction between nitrogen oxide (NO) and O_2^-. These two free radicals react at the fastest rate of any reaction known to occur in biology and is the only reaction that is faster than the dismutation reaction of O_2^- via superoxide dismutases. In its basic form, $ONOO^-$ does not react with organic molecule, it breaks down to form nitrite (NO_2^-) and nitrate (NO_3^-) But when peroxynitrite is protonated it becomes the highly reactive, peroxynitrous acid (ONOOH) that has the reactivity of nitrogen dioxide (NO_2), a very toxic free radical component in smog and cigarette smoke, and OH.

3. Protective functions of glutathione

3.1. Reduction

GSH is found in the cytosol of cells where it is in the range of 1–10 mM (Meister 1988). In most cells the GSH concentration is about 1–2 mM, while in hepatocytes, which export GSH, the concentration can reach about 10 mM. So why do we need GSH outside of the cells? In plasma GSH is in the micromolar range; however, in some extracellular spaces such as the lining fluid of the lung, a thin layer of fluid covering the air spaces where gas exchange occurs,

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there is high concentration of GSH that is secreted by epithelial cells (Sutherland et al., 1985; Cantin et al., 1987). In people who smoke or inhale particles or other oxidants, there is potential inflammation that involves invasion of neutrophils from the blood through the endothelial and epithelial cells into the air spaces. As these neutrophils squeeze between the cells, they release HOCl, which can react with GSH secreted from the epithelial cells that normally protects the epithelial cells (Venglarik et al., 2003).

In cystic fibrosis patients, who secrete lower GSH than normal individuals into the lining fluid covering their alveoli, and in smokers, who have exposed their lungs to many oxidants including nitrogen dioxide and H_2O_2, there is both chronic inflammation and lower than normal GSH (Roum et al., 1993). In that case, HOCl can oxidize proteins in the lining fluid or on the surface of the epithelial cells. It can also react with lipid to produce even more dangerous compounds than are produced by lipid peroxidation itself (Pullar et al., 2000). Fig. 3 shows how GSH reacts with HOCl and removes it (Winterbourn and Brennan, 1997). While many studies of GSH in inflammation have been done of the lungs, these reactions can occur in any organ.

Secretion of GSH to the air space in cystic fibrosis is depressed because of a mutation of a protein called cystic fibrosis transport receptor (CFTR) (Roum et al., 1993). The CFT1 cell line, which is derived from a cystic fibrosis patient, has lower GSH secretion to the apical (air space) side. If the wild type CFTR is transfected into the cells, the rate of GSH secretion is increased to the level seen in normal cells (Gao et al., 1999). The generation of HOCl in the surface fluid covering normal epithelial cells to mimic the action of stimulated neutrophils can decrease in the electrical resistance of that epithelial cell layer; however, the presence of GSH at a concentration similar to normal lining fluid protects against the loss of electrical resistance (Venglarik et al., 2003). Similar events occur during inflammation and are exaggerated in cystic fibrosis patients. There is some evidence that other lung diseases, such as idiopathic pulmonary fibrosis, also have a lower GSH concentration (Cantin et al., 1989). Further studies on the potential contribution of GSH deficiency to these pathologies are needed. Understanding the transport of GSH across the plasma membrane is an important issue that is essential to treatment of diseases involving oxidative stress (see reviews by Ballatori et al., 2008 and by Yuan and Kaplowitz, 2008 in this issue).

Compared to the extracellular environment, what happens inside of the cells is quite different. Glutathione plays major roles in the different cellular compartments. In mitochondria it plays a key role in regulating apoptosis versus necrosis (see review by Yuan and Kaplowitz 2008 in this issue). In the nucleus, GSH is a key regulator of cellular division (see review by Pallardó et al., 2008 in this issue.) While lungs are clearly adversely affected by lowered intracellular and extracellular GSH, the majority of studies on the pathologies involving GSH transport and metabolism have been done in liver. Reviews of the involvement of altered intracellular GSH in lung diseases (Biswas and Rahman, 2008), liver diseases (Yuan and Kaplowitz, 2008) and viral diseases (Fraternale et al., 2008) can be found in this issue.

Most of the GSH in antioxidant defense in cells is utilized by three members of glutathione peroxidase (GPx) family (Brigelius-Flohe, 1999) and by one of the peroxiredoxins (Prdx 6). These enzymes catalyze the reduction of H_2O_2 by GSH into H_2O and GSSG. Prdx 6 also requires GSH S transferase Pi in order to be active (Ralat et al., 2006). Phospholipid hydroperoxide glutathione peroxidase (PHGPx or GPx IV) can reduce lipid peroxides to lipid alcohols (Imai and Nakagawa, 2003). GSSG is potentially toxic to the cells but cells normally contain high glutathione reductase activity, which maintain most of the GSH in the reduced form. Some GSSG is also secreted from cells. During oxidative stress, GSSG could react by disulfide exchange with a protein thiol to produce a protein mixed disulfide (PSSG), which can further exchange with another protein thiol to a protein disulfide (Huang and Huang,

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2002). These reactions are actually quite slow unless catalyzed by an enzyme such as protein disulfide isomerase (PDI), an important enzyme that is particularly abundant in the endoplasm reticulum where protein folding occurs. In fact, the cysternae of the endoplasm reticulum is the only part of the cell with a relatively high ratio of GSSG/GSH. In the cytosol formation of PSSG is transient except during oxidative stress.

Formation of PSSG with some enzymes may play a role in signal transduction although the exact mechanism of their formation is uncertain. So how might PSSG form during normal metabolism in the cells? While protein disulfide exchange with a thiol can be catalyzed by PDI, some proteins contain a microenvironment in which thiolate ($-S^-$), which is far more reactive than is a thiol in both reaction with H_2O_2 or disulfide exchange, is formed. This requires that the microenvironment be composed in part by basic amino acids in proximity to the cysteine to allow dissociation of the thiol, which normally has a pK_a of around 8.3. GSH peroxidase catalyzes the production of GSSG, which could be potentially exchanged with a thiolate to form mixed disulfide. But in the cytosol, even during oxidative stress, the ratio of GSH/GSSG remains very high, which makes that exchange reaction unfavorable. The enzyme PDI can enhance the rate of that reaction but, like any catalyst, cannot change the equilibrium. Instead, it has been proposed that during physiological signaling when the H_2O_2 is used as the second messenger, some of protein thiolates could potentially react and form sulfenic acid (PSOH) (Fig. 4); however, for most thiolates including that formed by glutathione, the rate of the non-enzymatic reaction is too slow to account for the inactivation of the enzymes (Forman, 2007). We do know that in the active site of peroxiredoxins, where the reaction of H_2O_2 with a thiolate can occur up to six orders of magnitude faster than with glutathione in its thiolate form, the reaction can occur. Regardless, once formed, a protein sulfenate would rapidly react with GSH to produce the mixed disulfide, and this could be the mechanism through which PSSG formed for some proteins in the cytosol during oxidative stress when H_2O_2 is high enough to overcome a slow rate constant.

3.2. Conjugation

The elimination of many xenobiotic compounds can be accomplished through conjugation with GSH followed by secretion of the adduct from the cell (Boyland and Chasseaud 1969). Although the quinone, menadione, can react with GSH to form an adduct non-enzymatically, an enzymatic catalyzed Michael addition by a glutathione-S transferase (GST) is much faster. The glutathione adduct can then be secreted from cells through a membrane transporter such as the multidrug resistant proteins. The product of the addition of GSH can also rearrange into a quinol that are usually considered to be less toxic than the quinone (see above).

GSH is also used in the elimination of electrophiles such as HNE. Almost all these reactions are catalyzed by GSTs, and there is a specific one in human cells that can cause the conjugation of GSH to HNE at about 100 times faster rate than the non-enzymatic reaction. The conjugate, which is a Michael adduct (because the reaction is a Michael addition), can rearrange to form a cyclic hemiacetal (Fig. 5) (Alary et al., 2003). Both of the compounds however, can be excreted from the cells. This is the major route of elimination of HNE and other electrophiles that conjugate with GSH.

3.3. Interaction with other non-enzymatic antioxidants

While GSH is the most important small molecular weight antioxidant produced in the cells, there are other small molecular antioxidants obtained from the diet such as vitamins E (α-tocopherol) and C (ascorbic acid). Vitamin E can reduce lipid hydroxyl radicals and lipid peroxides that are produced from polyunsaturated fatty acids. The oxidized vitamin E is then reduced by vitamin C in a non-enzymatic but rapid reaction. The oxidized vitamin C can then be restored to the reduced form by enzymatic reactions, one of which uses GSH as substrate.

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4. Measurement of glutathione

One of the important issues in determining the mechanisms of both oxidative stress and redox signaling is the measurement of the different forms of thiols in cells. The predominant forms are the reduced form of GSH and GSSG. Nitrosoglutathione (GSNO) and protein nitrosothiols (PSNO) are also formed in cells and play a role in NO signaling independent of the cyclic GMP pathway. Cysteine is a precursor amino acid of GSH and cystine is the disulfide form of cysteine. Protein thiols exist as cysteine, mixed disulfides between cysteine and GSH or other thiols, and disulfides between two protein cysteines that may be in the same or different protein molecules. It is important to recognize that an increase in the oxidized forms of these thiols in the cytosol will be transient even during oxidative stress. Therefore it can be very difficult to measure thiol oxidation, particularly that occurring in signal transduction.

GSH reacts with dithionitrobenzoic acid (DTNB) (Akerboom and Sies, 1981) and by reducing GSSG total GSH (GSH + GSSG) can be measured. DTNB reacts with GSH to produce a conjugate and TNB anion that can be detected by fluorescence or absorbance (Fig. 6a). To measure total GSH, a recycling assay is used in which GSH reacts with the conjugate producing GSSG and another molecule of TNB, which can be increases fluorescence or absorbance (Fig. 6b). The enzyme glutathione reductase then reduces the GSSG releasing the GSH that can react with another molecule of DTNB. Therefore, instead of a single determination of how much DTNB reacts with GSH, the rate of TNB production is measured, as that is proportional to the initial amount of GSH. To measure GSSG however, one must first modify the GSH present at the beginning so it is removed from the recycling assay. Modification of GSH is done with N-ethylmaleimide (NEM) or vinylpyridine. To measure protein mixed disulfides, the GSH can be released from the protein mixed disulfide with sodium borohydride ($NaBH_4$), and the GSH is then measured in the recycling assay.

A more commonly used procedure for measuring GSH and GSSG now is high performance liquid chromatography (HPLC) (Fariss and Reed, 1987). In this assay, thiol compounds are first modified by the addition of iodoacetate (Fig. 6c). The amino groups on the compound then are modified by 1-fluoro-2, 4-dinitrobenzene. This then allows separation of many compounds that can be identified by their movement on HPLC.

On method that has been developed to measure nitrosoglutathione involves the production of GSH from it followed by reaction with orthophthaldehyde (OPT) to produce a fluorescent compound (Fig. 6d) (Tsikas et al., 1999) while another method uses a biotinylated fluorescent label in a method called the biotin-switch (Gladwin et al., 2006). First however, as with the measurement of GSSG above, it is necessary to first remove any GSH in the original sample with methyl methanethiosulfonate before reducing GSNO to release GSH. Various reagents have been proposed as best for differentially reducing GSNO as well as PSNO especially as the presence of GSSG or protein mixed disulfides can also yield GSH upon reduction (Gladwin et al., 2006). After reaction with OPT the products are separated by HPLC with a fluorescence detector. There are other methods for measuring GSNO such as using ^{15}N labeling (Kluge et al., 1997), but this is not commonly used and requires mass spectrometry.

5. Glutathione synthesis

The first step in de novo GSH synthesis involves the combination of cysteine with glutamate to produce γ-glutamylcysteine. This reaction is catalyzed by the enzyme glutamate cysteine ligase (GCL), which is also called γ-glutamylcysteine synthetase (Fig. 7). This enzyme requires coupled ATP hydrolysis to form an amide bond between the γ-carboxyl group of glutamate and the amino group of cysteine (Huang et al., 1993). The next step involves the enzyme glutathione synthetase, responsible for adding glycine to the dipeptide to produce GSH (γ-glutamylcysteinylglycine) and also requires coupled ATP hydrolysis (Meister, 1974).

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GSH can be transported out of cells. This mechanism is physiologically important as hepatocytes supply GSH found in the plasma, which is used as a source of cysteine for GSH synthesis in other cells (Anderson et al., 1980). In fact, GSH in the plasma is maintained at very low concentration because of the metabolism of GSH by many other cells (Sies and Graf, 1985; Hirota et al., 1986). This process requires two enzymes commonly found on the surfaces of cells. The enzyme γ-glutamyl transpeptidase transfers a glutamate to other amino acids releasing cysteinylglycine, which in turn can be broken down by a dipeptidase to produce cysteine and glycine (Kozak and Tate 1982; Hirota et al., 1986). Cysteine and glycine as well as γ-glutamyl amino acids are moved into cells by specific amino acid transporters and used for GSH biosynthesis (Meister, 1991).

5.1. Regulation of glutamate cysteine ligase activity

GCL is regulated at both the level of its enzymatic activity and the expression of its two subunits. One subunit is the relatively heavy (− 73 kDa) subunit, which has competent but low catalytic activity for production of γ-glutamylcysteine. The catalytic subunit, designated as GCLC, can be feedback inhibited by GSH (Huang et al., 1993). The lower molecular weight (− 28 kDa) subunit regulates the activity of the enzyme by reducing the inhibition by GSH (Huang et al., 1993; Choi et al., 2000) and with purified enzyme has been shown to also decrease the K_M for glutamate (Huang et al., 1993). This subunit, which is designated as GCLM for its modulatory activity can affect the steady state level of GSH found in cells when GCLM/GCLC expression is altered (Richman and Meister, 1975; Choi et al., 2000; Krzywanski et al., 2004). Thus, increased expression of GCLC will tend to elevate GSH while increasing GCLM/GCLC will further increase GSH. An example of when lowering GCLM/GCLC causes decreased GSH is the expression of the HIV-Tat protein, which suppresses GCLM expression (Choi et al., 2000). Finally, the kinetics of GCL seems to be regulated by phosphorylation of both subunits as well (Sun et al., 1996). The functional roles of the two GCL subunits are reviewed in this issue by Franklin et al. (2008)).

5.2. Regulation of glutamate cysteine ligase expression

The expression of GCL is also regulated at many levels. Oxidant species and electrophiles are able to increase the transcription of both the modulatory and catalytic subunits (Shi et al., 1994; Rahman et al., 1996; Tian et al., 1997) (also see review by Lu, 2008 in this issue). This occurs by the activation of signal transduction pathways involved in the control of transcription of GCLC and GCLM genes but also there is some evidence of mRNA stabilization by oxidants and electrophiles (Liu et al., 1998).

It has been known for almost twenty years that sublethal concentrations of electrophiles could increase GSH production (Ogino et al., 1989; Darley-Usmar et al., 1991); however, it was unclear whether the increase was on the kinetic or the transcriptional level or even whether GSSG reduction was increased. Using redox cycling quinones to increase production of hydrogen peroxide and by measuring transcription by nuclear run-on analysis, it was then shown that a sustained increase the amount of GSH in cells could be achieved by increasing the transcription of GCLC (Shi et al., 1994; Shi et al., 1994). Subsequently many labs showed that a variety of other agents, able to generate an oxidative stress throughH_2O_2 generation, increasing concentrations of electrophiles or nitric oxide could also induce GCLC or GCLM subunits or both (Rahman et al., 1996; Tian et al., 1997; Galloway and McLellan, 1998; Liu et al., 1998; Moellering et al., 1999; Wild and Mulcahy, 1999).

The GCLC and GCLM promoter sequences were described first from humans and then they were determined in rodents (Gipp et al., 1992; Gipp et al., 1995; Hudson and Kavanagh, 2000; Yang et al., 2001). The human and rodent promoters have some similar cis elements and appear to be regulated somewhat differently than the human genes (Iles and Liu 2005) (see

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review by Lu, 2008 in this issue). For the human GCL genes, the promoter enhancer regions of the two genes contain several elements able to respond to oxidants and electrophiles (Gipp et al., 1992; Gipp et al., 1995; Yang et al., 2001; Dickinson et al., 2002). One of the important oxidant responsive cis elements (transcription factor binding sites) regulating GCL genes is the AP-1 binding site also called the TRE element. TRE binds members of the Jun and Fos family of transcription factors (Ofir et al., 1990; Binetruy et al., 1991). Another important element in human GCL gene promoters that responds to electrophiles in cells and increases gene expression is the EpRE or electrophile response element (Rushmore et al., 1991; Jaiswal, 1994; Vasiliou et al., 1995). EpRE elements are also present in both human GCLC and GCLM promoters (Gipp et al., 1992; Gipp et al., 1995). Initially EpRE was called the antioxidant response element (ARE) because the first compound, shown to activate ARE was a so-called antioxidant that was subsequently shown to generate H_2O_2 through redox cycling (Pinkus et al., 1996). The EpRE elements bind proteins members of the Nrf family, Jun family and small Maf family (Venugopal and Jaiswal, 1998;Kong et al., 2001; Moran et al., 2002; Itoh et al., 2004). One of the transcription factors established as able to bind EpRE is Nrf2, which located in the cytosol through the inhibitory interaction with Keap1 in resting cells. Upon stimulation, Nrf2 is translocated into the nucleus after dissociation from Keap1 (Itoh et al., 1999).

While the redox and electrophilic response cis elements have been identified, less has been done to identify the signaling mechanisms that activate the transcription factors that bind to those elements. We will describe here briefly what is understood regarding the signaling by HNE. Darley–Usmar and coworkers have shown that HNE directly modifies Keap1, which allows Nrf2 to avoid degradation and migrate to the nucleus where it can bind to EpRE elements in the promoters of the human GCLC and GCLM genes (Levonen et al., 2004). But, this cannot be the whole story as there are actually multiple EpRE elements in the promoters and not all of them are involved in regulating transcription (Dickinson et al., 2004). While Nrf2 is critical, EpRE binding also involves a partner protein. For the EpRE element that regulates transcription of GCLC in human bronchial epithelial cells that partner has not yet been firmly identified.

More is understood about the TRE element. Interestingly, the TRE element in the human GCLC promoter appears to bind c-Jun dimers preferentially (Rahman et al., 1999). For HNE induction, the activation of the critical AP-1 binding elements in both human GCL genes can is achieved through the Jun N-terminal kinase (JNK) pathway (Dickinson et al., 2002). JNK phosphorylates c-Jun, which translocates into the nucleus, and binds to the TRE element. Inhibition of JNK completely eliminates GCLC and GCLM gene expression in response to HNE in human bronchial epithelial cells while inhibition of the ERK or $p38^{MAPK}$ pathways had no effect. Recently, the activation of the JNK pathway by HNE has been shown to occur upstream at the protein tyrosine phosphatase SHP-1that is inhibited by HNE, which also appears to accelerate the degradation of the enzyme (Rinna and Forman, 2008).

6. Glutathione therapeutics

As an increase in GSH appears to be a ubiquitous response to oxidants and electrophiles and some diseases appear to be exacerbated by decreasing GSH, increasing GSH by using delivery of permeable esters (Levy et al., 1993) or increasing the availability of cysteine using the non-toxic precursor N-acetylcysteine (Thor et al., 1979) have been proposed. Increasing GSH through synthesis would also seem to be useful therapeutically but as oxidants and most electrophiles would not seem appropriate, natural compounds such as curcumin, a principal ingredient of curry powder (Dickinson et al., 2003), and sulforaphane, a potent Phase II gene-inducing compound in broccoli, (Brooks et al., 2001) have been proposed but none of these natural has actually become a major therapeutic agent.

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On the other hand, compounds that decrease GSH and increase the susceptibility of tumors to chemotherapy or radiation have been used. GCL can be inhibited by a buthionine sulphoximine quite specifically making it a useful tool in studying GSH metabolism, and useful in cancer chemotherapy (Martensson et al., 1989; Anderson et al., 1997; Gartenhaus et al., 2002). An inhibitor of γ-glutamyl transpeptidase (GGT), acivicin (AT-125) (Griffith and Meister, 1980) was tried in chemotherapy before it was known to inhibit GGT; however, acivicin also inhibits enzymes in purine and pyrimidine biosynthesis, which may be its actual mode of action (Poster et al., 1981; Elliott and Weber, 1985). Thus, there is still much to be done in understanding how GSH synthesis and metabolism may be manipulated to therapeutic advantage. Further information about the use of GSH and related compounds in therapy for a variety of diseases including viral infection, cystic fibrosis and cancer, can be found in the reviews by Biswas and Rahman (2008)) and by Fraternale et al. (2008) in this issue.

Abbreviations

GSH, glutathione
−SH, sulfhydryl group
O_2^-, superoxide
H_2O_2, hydrogen peroxide
NOX, NADPH oxidase
SOD, superoxide dismutase
HOCl, hypochlorous acid
HOBr, hypobromous acid
Fe^{2+}, ferrous iron
OH, hydroxyl radical
Fe^{3+}, ferric iron
L, lipid radical
LOO, hydroperoxide radical
LOOH, lipid peroxide
HNE, 4-hydroxy-2-nonenal
$ONOO^-$, peroxynitrite
NO, nitrogen oxide
NO_2^-, nitrite
NO_3^-, nitrate
ONOOH, peroxynitrous acid
NO_2, nitrogen dioxide
CFTR, cystic fibrosis transport receptor
GPx, glutathione peroxidase
PHGPx GPx IV, phospholipid hydroperoxide glutathione peroxidase
GSSG, glutathione disulfide
Prdx, peroxiredoxin
PSSG, protein mixed disulfide
PDI, protein disulfide isomerase
$-S^-$, thiolate
PSOH, sulfenic acid
GST, glutathione-S transferase
GSNO, nitrosoglutathione
DTNB, dithionitrobenzoic acid
NEM, N-ethylmaleimide
$NaBH_4$, sodium borohydride
OPT, orthophthaldehyde
GCL, glutamate cysteine ligase

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ARE, antioxidant response element
JNK, Jun N-terminal kinase
GGT, γ-glutamyl transpeptidase.

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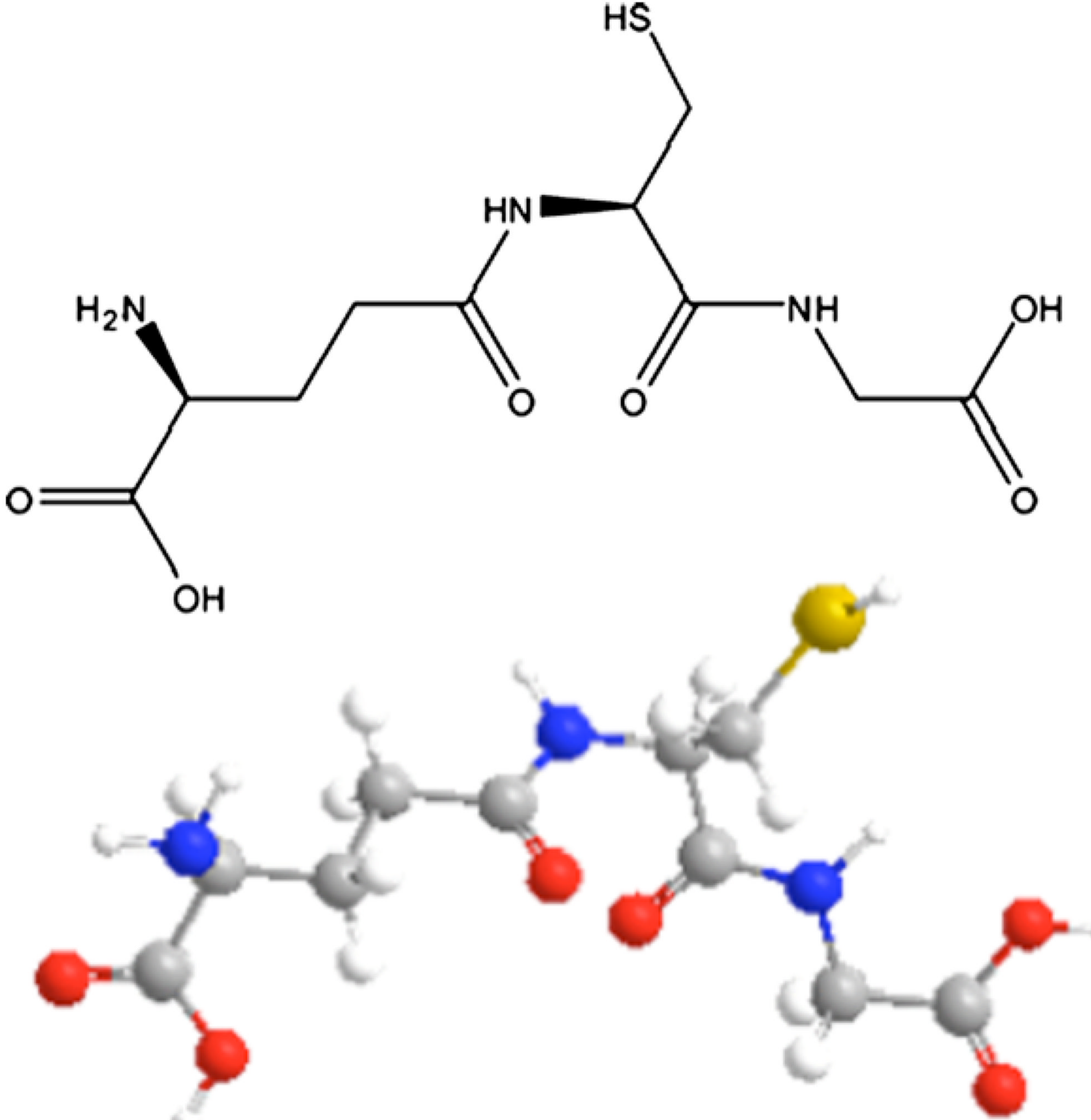

Fig. 1.
Glutathione structure. A stereochemical and ball and stick figure showing γ-glutamyl-cysteinyl-glycine are shown.

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Fig. 2.

Redox cycling of 1,4-naphthoquinones. A naphthoquinone with two variable groups (R) can be reduced by NADPH (or NADH, which is not shown) enzymatically to the semiquinone radical and then will react with oxygen to generate superoxide and restore the naphthoquinone.

$$GSH + HOCl \rightarrow GSCl + H_2O$$
$$GSCl + GSH \rightarrow GSSG + HCl$$
$$GSCl + 2HOCl \rightarrow GSO_2Cl + 2HCl$$
$$GSO_2Cl \rightarrow GSO_2NH \text{(formed with an amino group of GSH itself)} + HCl$$
$$GSO_2Cl + GSH \rightarrow GSO_2SG + HCl$$

Fig. 3.
Reactions of glutathione with hypochlorous acid. GSH and HOCl can react to produce several different products.

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$$2GSH + H_2O_2 \xrightarrow{GPx\ or\ Prdx\ 6} GSSG + 2H_2O$$

$$PSH + GSSG \xrightarrow{PDI} PSSG + GSH$$

but GSH/GSSG is very high in the cytosol

$$PS^- + H_2O_2 \rightarrow PSO^- + H_2O$$

but the rate is very slow except for peroxiredoxins

$$PSO^- + GSH \rightarrow PSSG + OH^-$$

Fig. 4.

Formation of protein mixed disulfide. Both glutathione peroxidases and peroxiredoxin 6 can catalyze the oxidation of glutathione by hydrogen peroxide to glutathione disulfide and water. GSSG can then undergo an exchange reaction with protein sulfhydryl to form PSSG, which is usually catalyzed by a protein disulfide isomerase. An alternative mechanism is the oxidation of a protein thiolate to a sulfenic acid, which then will react with GSH to form PSSG and water.

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Fig. 5.
Glutathione conjugations with 4-hydroxynonenal. Glutathione S-transferases catalyze the conjugation of GSH with HNE. This is a Michael addition that can slowly occur non-enzymatically.

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a

DTNB

TNB

b

$$DTNB + GSH \rightarrow GS - TNB + TNB$$
$$GS - TNB + GSH \rightarrow GSSG + TNB$$
$$NADPH + GSSG + H^{+} \xrightarrow{\text{GSSG Reductase}} NADP^{+} + 2GSH$$

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c

iodoacetate + RSH → RS–CH₂–COO⁻ + I⁻ + H⁺

1-fluoro-2,4-dinitrobenzene + amino acid → DNP-amino acid + F⁻ + H⁺

d

OPT + glutathione → product

Fig. 6.

Measurements of thiols. (a) Reaction of GSH with DTNB produces an adduct and TNB, which is measured spectrofluorometrically or spectrophotometrically; (b) total glutathione can be determined by recycling of GSSG produced in the reaction in (a) and measuring the rate of TNB; (c) Glutathione and related compounds are first derivatized with iodoacetate followed by a second derivatization with 1-fluoro-2,4-dinitrophenol. The second products are then separated by HPLC and measured spectrofluorometrically; (d) Reaction of glutathione with orthophthaldehyde (OPT) yields a product that can be measured spectrofluorometrically.

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Fig. 7.

Glutathione synthesis. The sequential ATP dependent formation of amide bonds between cysteine and the γ-carboxyl group of glutamate and then between glycine and cysteine are shown.

Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 986-990, February 1991
Medical Sciences

Suppression of human immunodeficiency virus expression in chronically infected monocytic cells by glutathione, glutathione ester, and *N*-acetylcysteine

(tumor necrosis factor/interleukin 6/phorbol 12-myristate 13-acetate)

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Contributed by Alton Meister, November 20, 1990

ABSTRACT **The effects of glutathione (GSH), glutathione ester (GSE), and *N*-acetyl-L-cysteine (NAC) on the induction of human immunodeficiency virus (HIV) expression were investigated in the chronically infected monocytic U1 cell line, a previously described cellular model for HIV latency. U1 cells constitutively express low levels of virus, which can be increased by phorbol 12-myristate 13-acetate (PMA), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and other inducers. GSH, GSE, and NAC suppressed in a dose-dependent fashion the induction of HIV expression mediated by PMA, TNF-α, and IL-6, in the absence of cytotoxic or cytostatic effects. Reverse transcriptase activity, inducible by PMA, TNF-α, or IL-6, was decreased by 80–90% after pretreatment with GSH, GSE, or NAC. The induction of total HIV protein synthesis was also decreased appreciably after pretreatment with GSH, GSE, or NAC. The accumulation of HIV mRNA was substantially suppressed after pretreatment with NAC but to a lesser extent after pretreatment with GSH or GSE. Although PMA induces the expression of TNF-α in U1 cells, the suppressive effect of GSH, GSE, and NAC on PMA-induced HIV expression in U1 cells was not associated with the inhibition of TNF-α expression. The present findings, which elucidate relationships between cellular GSH and HIV expression, suggest that therapy with thiols may be of value in the treatment of HIV infection.**

The early stages of human immunodeficiency virus (HIV) infection can be separated from the later progressively symptomatic stage by several years of clinical latency (1, 2). Early infection is characterized by a low frequency of infected cells and a low level of viral expression (3). The progression to acquired immunodeficiency syndrome (AIDS) is characterized by increased levels of viremia and p24 antigenemia, activation of HIV expression in infected cells, an increased number of infected cells, and severe immune dysfunction (1–3). These findings suggest that the evolution of HIV infection and the progression of immunosuppression is associated with an increased activation of latent virus. A number of factors that induce the activation of latent HIV *in vitro* have been identified by use of the chronically infected promonocytic U1 cell line, which harbors HIV proviral DNA in its genome (4). In particular, phorbol 12-myristate 13-acetate (PMA) and tumor necrosis factor α (TNF-α) are potent transcriptional activators of HIV expression in this model system (4–6). TNF-α may play a critical role in the pathogenesis of AIDS, consistent with the markedly increased level of this cytokine in HIV-infected individuals (7, 8). In addition, elevated levels of interleukin 6 (IL-6) in the plasma (9) and cerebrospinal fluid (10) of HIV-infected individuals have been described, and it has been found that IL-6 can induce the expression of HIV in chronically infected U1 cells by acting predominantly at the posttranscriptional level (11).

The possibility that oxidative phenomena and thiols may be involved in the induction of HIV expression has not been extensively examined. It has been reported that HIV-seropositive individuals have decreased levels of total acid-soluble thiols and of glutathione (GSH, L-γ-glutamyl-L-cysteinyl glycine) in their plasma, peripheral blood monocytes, and lung epithelial lining fluid (12, 13). There is evidence that GSH is an important immunomodulator and that it is required, for example, for T-cell activation (14–17). GSH, which constitutes >90% of the cellular non-protein thiols and which is present at levels of 0.5–10 mM, provides cells with their reducing environment and serves as the major cellular antioxidant (18–20).

It has recently been reported that the transcriptional activation by PMA or TNF-α of a transfected HIV long terminal repeat (LTR) construct in T lymphocytic cells is inhibited by *N*-acetyl-L-cysteine (NAC) (21). NAC, which has been used clinically to treat acetaminophen toxicity, is believed to act by increasing the intracellular level of GSH (22, 23). Cellular GSH levels may also be increased by administration of GSH monoester (GSE), a readily transported derivative of GSH, which also protects very effectively against acetaminophen toxicity. Although GSH is not transported intact into cells, it may be degraded into its constituent amino acids, which after transport are used for the synthesis of GSH. These findings suggested that cellular reducing systems may be involved in preventing HIV activation and thus in maintaining a state of viral latency. We therefore investigated the hypothesis that GSH, GSH derivatives, or NAC may inhibit the induction of virus expression in our model system of chronic HIV infection. We used the U1 cell line and the transcriptional activators PMA and TNF-α as well as the posttranscriptionally active cytokine IL-6.

MATERIALS AND METHODS

Cell Lines. The origin and characterization of the U1 cell line have been reported (4). Briefly, the U1 cell line was derived from infection of U-937 promonocytic cells with HIV and carries two copies of proviral DNA, as determined by restriction enzyme analysis. U1 cells show a minimal constitutive level of HIV expression, as determined by immu-

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Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; GSH, glutathione; GSE, glutathione monoester; NAC, *N*-acetyl-L-cysteine; RT, reverse transcriptase; LTR, long terminal repeat.

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nohistochemical staining for viral antigens and by measurement of the expression of the p24 gag antigen, reverse transcriptase (RT) activity, viral protein, and mRNA synthesis. Activation of HIV expression in U1 cells can be induced by a variety of stimuli including PMA, TNF-α, and IL-6 (4–6, 11). The cells are routinely maintained at a density of 2–5 × 10^5 per ml in RPMI 1640 (Whittaker Bioproducts), supplemented with 10% heat-inactivated fetal bovine serum (Biofluids, Rockville, MD).

Induction of HIV Expression. In order to study the effect of GSH, GSE, and NAC on PMA-, TNF-α-, or IL-6-mediated induction of HIV expression, U1 cells were pretreated with various concentrations of these agents for 6 hr at 37°C in 5% CO_2. Then, PMA (10 nM; Sigma), recombinant TNF-α (100 units/ml; Genzyme), or IL-6 (100 units/ml; Amgen Biologicals) were added to the culture and further incubated for various periods of time. To monitor HIV activity, the level of RT was measured in the supernatant and the levels of HIV mRNA and protein synthesis were determined. GSH and NAC were purchased from Sigma. GSE, a GSH derivative that is transported into cells without undergoing transmembrane degradation, was prepared as the semihydrosulfate and carefully neutralized as described (24, 25).

RT Assay. Levels of RT activity present in U1 culture supernatants were determined by the method of Willey *et al.* (26). Briefly, aliquots (10 μl) of supernatant were added to 50 μl of a mixture containing poly(A), oligo(dT) (Pharmacia), $MgCl_2$, and ^{32}P-labeled dTTP (Amersham) and were incubated at 37°C. After 2 hr of incubation, 6 μl of the reaction mixture was spotted onto DE81 filter paper (Whatman) and air-dried. The filters were then washed in 1× standard saline citrate (SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7) and 95% ethanol, dried, cut, and placed in a scintillation counter (LS 7000, Beckman) for measurement of radioactivity.

Western Blot Analysis. Cell lysates were prepared from U1 cells that had been pretreated for 6 hr with GSH, GSE, or NAC (15 mM) and then stimulated with PMA (10 nM), TNF-α (100 units/ml), or IL-6 (100 units/ml) for 40 hr. The amount of protein in each cell lysate was determined by using a Bio-Rad protein quantification kit. Samples containing equal amounts of proteins were loaded onto an SDS/10–20% gradient polyacrylamide gel (Integration Separation Sciences, Hyde Park, MA) and fractionated by electrophoresis. The proteins were then transferred for 8 hr onto nitrocellulose filters and fixed with methanol. Following saturation of specific binding sites with a 5% milk solution, filters were incubated with a 1:1000 dilution of a pool of AIDS patients' sera containing anti-HIV antibodies recognizing the major viral proteins. After three washes, filters were incubated with ^{125}I-labeled protein A (200,000 dpm/ml) for 90 min. Filters were washed, dried, and exposed to x-ray film (Eastman Kodak).

Northern Blot Analysis. Total cellular RNA was isolated from U1 cells that had been pretreated for 6 hr with GSH, GSE, or NAC (15 mM) and then stimulated with PMA (10 nM) or TNF-α (100 units/ml) for 24 hr prior to extraction of RNA with an RNA isolation kit (Stratagene). Ten micrograms of RNA was loaded onto a 1% agarose/2.2 M formaldehyde gel, fractionated electrophoretically, blotted onto a nitrocellulose filter, and hybridized as described (27). To detect HIV mRNA, a ^{32}P-labeled HIV LTR (*Hin*dIII–Ava I) was used as a probe (28). To measure the level of TNF-α mRNA, total cellular RNA was hybridized with a full-length TNF-α cDNA probe (29).

RESULTS

Suppression of RT Activity by GSH, GSE, and NAC in U1 Cells. While U1 cells express only a minimal level of RT activity under normal growing conditions, the levels of RT increase remarkably in the presence of HIV inducers. PMA (10 nM) and TNF-α (100 units/ml) induced 10- to 30-fold increases of RT activity, while IL-6 (100 units/ml) produced a 3- to 5-fold increase in RT activity in U1 cells (Fig. 1). We observed that a single pretreatment with GSH (15 mM), GSE (15 mM), or NAC (15 mM) produced a long-lasting suppression of RT activity in U1 cells stimulated with each of these viral inducers (Fig. 1). The suppression of RT activity produced by GSH, GSE, or NAC was dose-dependent (Fig. 2). At a concentration of 15 mM, GSH, GSE, or NAC suppressed RT activity usually >90% in PMA- or TNF-α-stimulated cells. Treatment with GSH, GSE, or NAC at this concentration also produced a substantial suppression of RT activity in IL-6-stimulated cells. The degree of suppression of RT activity was dependent on the duration of treatment of cells with GSH, GSE, or NAC, prior to the addition of the viral inducers. A 6-hr pretreatment with GSH, GSE, or NAC produced more suppression of RT activity than did 3 hr of pretreatment or the simultaneous addition of GSH, GSE, or NAC with the viral inducers (data not shown).

Effect of GSH, GSE, and NAC on HIV Protein Synthesis. Consistent with the suppression of RT activity detected in the culture supernatant, a 6-hr pretreatment of cells with GSH, GSE, or NAC (15 mM) inhibited the induction of total HIV protein synthesis mediated by PMA (Fig. 3). Similarly, stim-

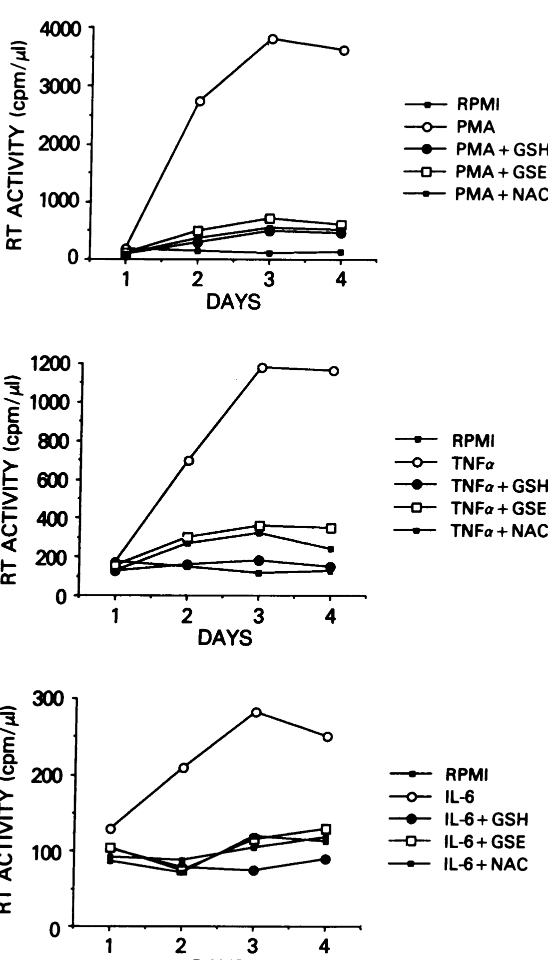
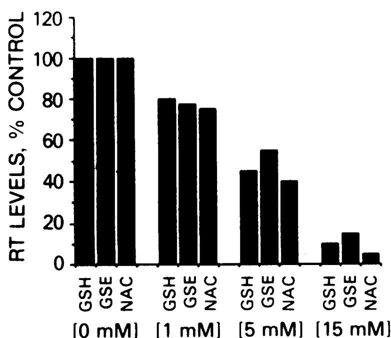
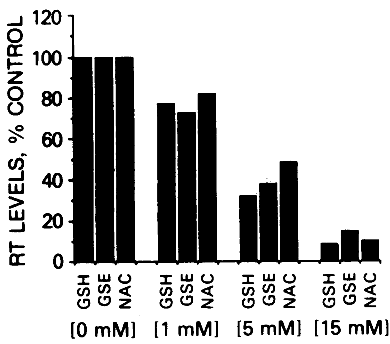


FIG. 1. Kinetics of inhibition of RT activity by GSH, GSE, and NAC in U1 cells stimulated with PMA, TNF-α, or IL-6. Cells plated at a density of 3 × 10^5 per ml in fresh growth medium (RPMI) were pretreated for 6 hr at 37°C with GSH (15 mM), GSE (15 mM), or NAC (15 mM) and then stimulated with PMA (10 nM), TNF-α (100 units/ml), or IL-6 (100 units/ml). Over a period of 4 days the supernatants were harvested at specific time intervals and RT activity was measured. A representative of three independent experiments performed in triplicate is shown. Variability between triplicate replicas was <15%.





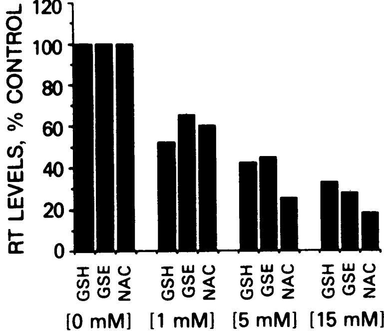


FIG. 2. Concentration-dependent suppression of RT activity by GSH, GSE, and NAC in U1 cells. Cells were pretreated for 6 hr with various concentrations of GSH, GSE, or NAC and then stimulated with PMA (10 nM), TNF-α (100 units/ml), or IL-6 (100 units/ml) for 48 hr. RT activity was determined in the culture supernatants. The average RT values determined in three independent experiments are shown as a percentage of control (given as 100%). Each experiment was performed in triplicate. Variability among triplicate replicas was <15%.

ulation of HIV protein synthesis by TNF-α and IL-6, was also inhibited by each of these agents (data not shown). We did not detect any significant qualitative or quantitative change in the pattern of total cellular protein as a result of treatment with GSH, GSE, or NAC in stimulated or unstimulated cells. At lower concentrations (1–5 mM), GSH, GSE, and NAC did not show a suppressive effect on the induction of total HIV protein synthesis (data not shown), although they did produce a decrease of RT levels (Fig. 2). Consistent with previous reports, we observed that unstimulated U1 cells did not express significant amounts of viral proteins (4, 11).

Differential Effect of GSH, GSE, and NAC on HIV mRNA Accumulation. Having determined that GSH, GSE, or NAC at 15 mM suppressed HIV protein synthesis, we investigated

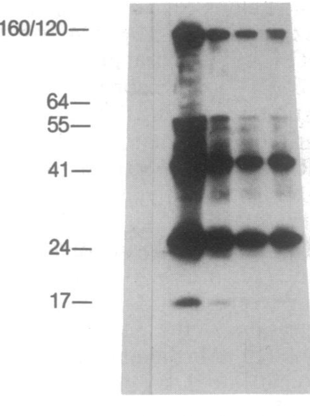


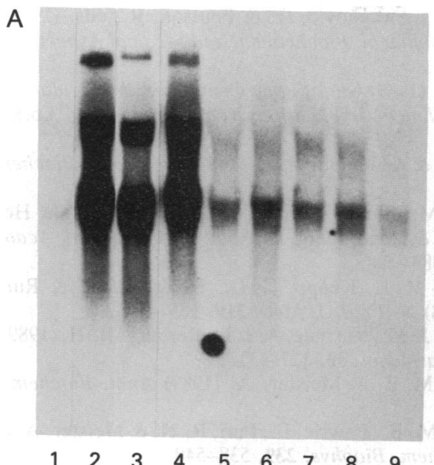
FIG. 3. Suppression of HIV protein level by GSH, GSE, and NAC (15 mM) in U1 cells stimulated with PMA. Western blot analysis of total cellular lysates (100 μg of protein per lane) from unstimulated cells (lane 1), PMA-stimulated cells (lane 2), PMA-stimulated cells pretreated with GSH (lane 3), PMA-stimulated cells pretreated with NAC (lane 4), and PMA-stimulated cells pretreated with GSE (lane 5). Molecular mass (kDa) is indicated at left. Results are representative of five independent experiments.

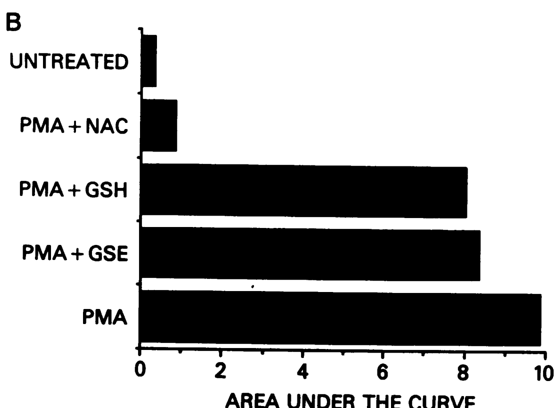
whether these three agents had any effect on mRNA accumulation in U1 cells. It had been shown that PMA and TNF-α increase HIV expression in U1 (5) and other infected cells (30–33) by inducing the cellular transcription factor NF-κB, whereas IL-6 induced HIV expression via a posttranscriptional mechanism without affecting the level of HIV mRNA in unstimulated or stimulated U1 cells (11). We found that a 6-hr pretreatment of cells with NAC (15 mM) completely blocked PMA induction of HIV mRNA. The level of mRNA in cells pretreated with NAC and stimulated with PMA was similar to the level of mRNA in unstimulated control cells (Fig. 4). Traces of mRNA from NAC-pretreated cells were detected by autoradiography only after several days of exposure. In contrast, pretreatment of U1 cells with GSH (15 mM) or GSE (15 mM) caused only a moderate reduction of mRNA accumulation in PMA-stimulated cells, despite the fact that GSH and GSE showed similar potency as compared with NAC in the suppression of RT activity and total HIV protein synthesis. Similarly, the increase of HIV mRNA induced by TNF-α was also inhibited markedly by NAC (15 mM), whereas pretreatment with GSH and GSE yielded only a 10% reduction.

GSH, GSE, and NAC Do Not Inhibit TNF-α Expression in Unstimulated or PMA-Stimulated U1 Cells. It was previously demonstrated that PMA induced HIV expression in U1 cells in part by TNF-α-dependent mechanisms (6). In addition, it was shown that the constitutive expression of HIV in certain cell lines is dependent on the endogenous and autocrine production of TNF-α (6). In this study we determined by Northern blot analysis that GSH (15 mM) or NAC (15 mM) had no suppressive effect on the PMA-induced increase of TNF-α expression (Fig. 5). This finding suggests that suppression of HIV activity by GSH and NAC is not due to the suppression of endogenous TNF-α production. In addition, these agents did not alter the expression of TNF-α in unstimulated U1 cells.

DISCUSSION

In the present study, we have used the chronically HIV-infected U1 cell line to investigate the potential antiviral effects of GSH, GSE, and NAC. Treatment of U1 cells with PMA, TNF-α, or IL-6 leads to an increased production of HIV virions and corresponding increases in the levels of RT,





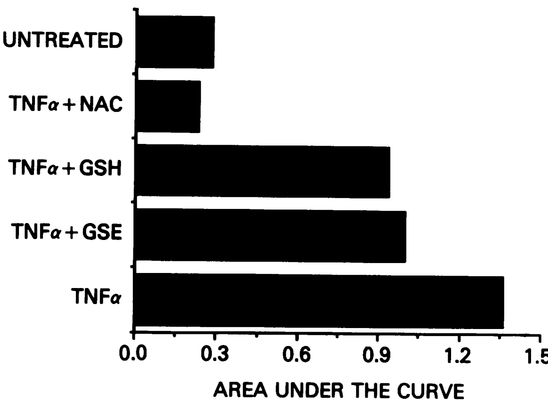


FIG. 4. Suppression of HIV mRNA accumulation by GSH, GSE, and NAC in U1 cells stimulated with PMA or TNF-α. (*A*) Northern blot analysis of total cellular RNA extracted from U1 cells pretreated for 6 hr with GSH (15 mM), GSE (15 mM), or NAC (15 mM) before stimulation with PMA or TNF-α. A ^{32}P-labeled HIV LTR was used as a probe. Lanes: 1, unstimulated cells; 2, PMA-stimulated cells; 3, PMA-stimulated cells pretreated with GSH; 4, PMA-stimulated cells pretreated with GSE; 5, PMA-stimulated cells pretreated with NAC; 6, TNF-α-stimulated cells; 7, TNF-α-stimulated cells pretreated with GSH; 8, TNF-α-stimulated cells pretreated with GSE; 9, TNF-α-stimulated cells pretreated with NAC. (*B*) Densitometric quantification of HIV mRNA from GSH-, GSE-, and NAC-pretreated U1 cells stimulated with PMA or TNF-α performed by scanning the autoradiogram from the same experiment shown in *A*. An LKB-222210 densitometer was used to perform the scanning.

cell-associated viral proteins, and mRNA (4–6, 11). In this model system, IL-6 stimulates HIV expression at a posttranscriptional level, while PMA and TNF-α stimulate HIV expression at a transcriptional level through an NF-κB-dependent mechanism (5, 30–33). Our experiments demonstrated that GSH, GSE, and NAC suppressed the induction of HIV expression in chronically infected cells stimulated with viral inducers that utilize different molecular mechanisms.

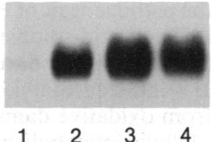


FIG. 5. Levels of TNF-α mRNA in U1 cells. Total cellular RNA was extracted from U1 cells pretreated for 6 hr with GSH or NAC (15 mM) and stimulated with PMA (10 nM) for 24 hr. Northern blot analysis was performed using a TNF-α cDNA probe. Lanes: 1, unstimulated cells; 2, PMA-stimulated cells; 3, PMA-stimulated cells pretreated with NAC; 4, PMA-stimulated cells pretreated with GSH. Actin gene expression was also unaffected in both untreated and PMA-treated U1 cells (data not shown).

We found that GSH, GSE, and NAC produced a comparable suppressive effect on RT activity in PMA-, TNF-α-, and IL-6-stimulated cells. Although GSH, GSE, and NAC produced a similar level of suppression of RT activity in the supernatant of U1 cells stimulated with these various stimuli, which corresponded to a decreased level of extracellular viral particles, these agents had a differential effect on HIV mRNA accumulation. NAC produced >90% suppression of the HIV mRNA level in both PMA- and TNF-α-stimulated U1 cells. Furthermore, consistent with previous reports (21), pretreatment with NAC decreased the transcriptional activity of the HIV LTR in U1 and U-937 cells transfected with an HIV LTR construct and stimulated with PMA or TNF-α (data not shown). In contrast, GSH and GSE reduced the HIV mRNA level only 10–15%. Although it has been assumed that NAC exerts some of its effects by increasing intracellular GSH (19, 23), our findings suggest that the mechanisms of NAC-mediated inhibition of HIV expression may be complex and involve, for example, a more efficient rate of internalization, an extracellular effect, or an effect on intracellular proteins. It was recently reported that NAC suppresses TNF-α-dependent, acute HIV infection *in vitro* and exerts a cooperative *in vitro* antiviral effect with 3′-azido-3′-deoxythymidine (AZT) (21). In addition, NAC was found to directly inhibit HIV LTR transcription (21) and to exert an inhibitory effect on NF-κB binding activity (34). Other thiols, which are not directly used for GSH synthesis, have been reported to suppress HIV replication in acute systems. Such thiols [including penicillamine (35, 36), and 2,3-dimercapto-1-propanol (37)], as well as ascorbate (vitamin C) (38) could act to spare cellular GSH; the thiols might liberate GSH moieties from mixed disulfide linkages with proteins.

Our data thus demonstrate that these three thiols affect HIV expression at multiple stages of the HIV activation process. At 15 mM, GSH, GSE, and NAC suppressed both HIV production and total viral protein synthesis. At lower concentrations (1–5 mM), however, GSH, GSE, and NAC did not affect HIV protein synthesis or mRNA accumulation but appeared to suppress predominantly posttranslational events of HIV expression, such as viral assembly or budding from the plasma membrane. Unlike interferon α-treated cells (39), RT levels measured after cell disruption were not different from those detected in the supernatants of intact cells (data not shown), and ultrastructural analysis failed to detect increased aggregation of viral particles on the plasma membrane (Jan Orenstein, personal communication), indicating that low concentrations of NAC, GSH, and GSE affected earlier posttranslational steps of viral expression than did interferon α (39).

The observation that GSH, GSE, and NAC downregulate the expression of HIV in chronically infected cells may further

our understanding of the pathogenesis of HIV infection *in vivo*. Decreased levels of GSH and acid-soluble thiols were found in the plasma, lung epithelial lining fluid, and peripheral blood monocytic cells of HIV-infected individuals (12, 13). GSH, which protects cells from oxidative damage and which plays an important role in cellular metabolism and function, also plays a critical role in immunoregulation (14–20). GSH deficiency in association with increased levels of TNF-α (40–43) and IL-6 (43, 44) may play an important role in the progression of HIV infection. A decreased level of GSH potentiates sensitivity to TNF-α (45), which has a direct stimulatory effect on HIV expression (5, 6, 11, 31–33, 43) and also increases susceptibility to oxidative effects (46, 47). An increase in the serum level of TNF-α has been found in AIDS patients (7, 8). In addition, increased levels of IL-6 have been reported in the plasma (9) and cerebrospinal fluid (10) of HIV-infected individuals. Moreover, IL-6 can activate superoxide generation in mononuclear phagocytes (48), leading to a further increase in reactive oxygen intermediates and a consequent consumption of GSH. Our observation that GSH, GSE, and NAC can suppress the induction of HIV expression *in vitro* by TNF-α and IL-6 leads to the hypothesis that these agents may have similar effects *in vivo* and therefore that the administration of these or similar agents may have therapeutic value in HIV-infected patients. Such therapy may be effective in limiting progression of the disease process, which, by a presently unknown mechanism, produces a striking decrease in the level of a major cellular antioxidant.

This research was supported in part by National Institutes of Health Grant 2 R37 DK-12034 to A.M.

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PHARMACOLOGY

ORIGINAL RESEARCH ARTICLE
published: 11 April 2014
doi: 10.3389/fphar.2014.00073

Glutathione synthesis is compromised in erythrocytes from individuals with HIV

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We demonstrated that the levels of enzymes responsible for the synthesis of glutathione (GSH) such as glutathione synthase (GSS), glutamate-cysteine ligase-catalytic subunit (GCLC), and glutathione reductase (GSR) were significantly reduced in the red blood cells (RBCs) isolated from individuals with human immunodeficiency virus (HIV) infection and this reduction correlated with decreased levels of intracellular GSH. GSH content in RBCs can be used as a marker for increased overall oxidative stress and immune dysfunctions caused by HIV infection. Our data supports our hypothesis that compromised levels of GSH in HIV infected individuals' is due to decreased levels of GSH-synthetic enzymes. The role of GSH in combating oxidative stress and improving the functions of immune cells in HIV patients' indicates the benefit of an antioxidant supplement which can reduce the cellular damage and promote the functions of immune cells.

Keywords: glutathione, HIV, GSS, GCL, GSR

INTRODUCTION

Roughly 34 million people around the world are infected with human immunodeficiency virus (HIV). Since its first reporting in 1981, the beginning of an epidemic, 60 million people have contracted HIV and an estimated 30 million have died due to HIV related causes (World Health Organization, 2010). HIV infection is associated with a wide range of different opportunistic infections that are usually the prime suspect for patients' poor survival. Among the array of opportunistic infections, one of the leading life-threatening infection common among HIV positive individuals with compromised immune system is *Mycobacterium tuberculosis*. Especially in developing countries, as many as eighty percent of people with AIDS are at risk of developing tuberculosis (TB) (World Health Organization, 2009). HIV's primary targets *in vivo* are blood monocytes, CD4 T lymphocytes, and resident macrophages. Due to HIV's high affinity for infecting and killing CD4+ T lymphocytes, cell-mediated immunity is drastically lowered. This results in greater probability for opportunistic infections, primarily *M. tuberculosis* (Levy, 1993; Pantaleo et al., 1993; Droge and Holm, 1997; Herzenberg et al., 1997).

Glutathione (GSH) is a major component involved in the control and maintenance of cellular redox state and cellular homeostasis (Griffith, 1999). In addition, GSH is also important in an array of cellular functions such as protein synthesis, transport across membranes, receptor action, and cell growth (Griffith, 1999). As a natural antioxidant, GSH scavenges peroxide species. Low levels of GSH have been shown to play a role in the apoptosis of CD4+ T cells, which is the major pathology of the HIV infection, therefore signifying the importance of GSH (Levy, 1993; Pantaleo et al., 1993; Droge and Holm, 1997; Herzenberg et al., 1997).

Glutathione is produced by almost all cell types and are present in two forms, reduced (*r*GSH) and oxidized (GSSG). *r*GSH is synthesized by two different mechanisms. *De novo* synthesis of *r*GSH occurs in a two-step process mediated by two different enzymes, glutamate-cysteine ligase (GCL) and glutathione synthase (GSS). *r*GSH is also synthesized via the reduction of GSSG by glutathione reductase (GSR; Staal, 1998). In this study, we went beyond the innate immune response components and investigated the changes in the levels of GSH in red blood cells (RBCs) isolated from individuals with HIV infection. We hypothesized that compromised levels of GSH in HIV-infected individuals is due to decreased levels of enzymes that are involved in the synthesis of GSH. Since RBCs are systemically present in abundance, we tested our hypothesis by determining the extent to which the levels of GSH-synthetic enzymes are compromised in RBCs derived from individuals with HIV infection and correlating decreased levels of GSH-synthetic enzymes with deficiency in the levels of GSH.

MATERIALS AND METHODS

SUBJECTS

The protocol was approved by Institutional Review Board with the requirement that each volunteer recruited would need to be given a consent form that described the basis and the procedures of the study. A signed informed consent from each volunteer that agreed to participate was obtained. A total of 16 volunteers (eight healthy subjects and eight individuals with HIV infection) were recruited for the study. Individuals with HIV infection were recruited from the Foothills AIDS project. Healthy subjects without HIV infection

or a history of TB were recruited from the staff of Western University of Health Sciences. All HIV-infected volunteers had been diagnosed with HIV-1, were taking some form of anti-retroviral treatment, and had CD4+ T-cell counts between 271 and 1415 cells per mm^3. Thirty five milliliters (mL) of blood was drawn once from both healthy volunteers and individuals with HIV infection.

ERYTHROCYTE ISOLATION

Red blood cells were isolated from whole blood by density gradient centrifugation with FICOLL-Paque (GE Healthcare, 17-440-02). RBCs that aggregated as the bottom layer were collected and stored at −20°C in a cell lysis/protein storage buffer [20 mM Tris, 100 mM NaCl (Amresco, N653), 1X protease inhibitor cocktail (Amresco, M221)] for western blot analysis.

GEL ELECTROPHORESIS AND WESTERN BLOT ANALYSIS IN RBC LYSATES FROM HEALTHY AND HIV$^+$ SUBJECTS

Total protein content was determined using Coomassie blue colorimetric assay (Thermo Scientific, PI-23200). 200 μg of total RBC proteins per sample were separated via denaturing polyacrylamide electrophoresis (12%). Separated proteins were transferred to a Polyvinylidene fluoride membrane (GE, PV4HY00010) by electroblotting. Membranes were blocked for 1 h at room temperature in tris buffered saline with tween 20 (TBST) and 5% non-fat dry milk followed by three washes (15 min for each wash) in TBST with mild shaking. The membranes were then incubated with a primary antibody overnight at 4°C in TBST with gentle shaking. The primary antibodies used were mouse anti-human GSS (1:1000, Abcam, ab5513), mouse anti-human GSR (1:500, Abcam, ab55075), and rabbit anti-human glutamate-cysteine ligase-catalytic subunit (GCLC; 1:250, Abcam, ab40929). Following overnight incubation with the primary antibodies, membranes were washed five times for 15 min in TBST with mild shaking. Washed membranes were incubated with a secondary antibody conjugated with horse radish peroxidase, anti-mouse (1:1000, Abcam, ab7064), or anti-rabbit (1:1000, Abcam, ab72465) in TBST for an hour at room temperature. Membranes were washed again five times for 15 min in TBST with mild shaking. Chemiluminescent substrate was applied to the membranes which were then exposed to an x-ray film (Genemate, F-9024) and developed in a dark room. Digital images of the immunoblots were captured using a Versadoc gel imaging system (Bio-rad, 4000 MP). Densitometric analysis of the images was performed using ImageJ, a free software program available from the National Institutes of Health (http://rsbweb.nih.gov/ij/).

ASSAY OF GSH LEVELS IN RBCs FROM HEALTHY AND HIV$^+$ SUBJECTS

Glutathione concentrations were measured in RBCs isolated from healthy and HIV$^+$ individuals by spectrophotometry using a colorimetric assay kit (Arbor Assays, K006-H1). RBCs were suspended in an ice cold 5% 5-sulfosalicylic acid dihydrate solution (MP Biomedicals, 160001-4924H). Supernatants collected after centrifugation were analyzed for the total GSH as per the manufacturer's instructions. All GSH measurements were normalized with total protein concentrations.

STATISTICAL ANALYSIS

Statistical analysis of the data was carried out using GraphPad Prism 6. The data was analyzed by comparing the means of $n = 8$ individuals (unless otherwise specified) using unpaired student's t-tests. $P \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Glutathione is a tripeptide made of glutamine, cysteine, and glycine. In the *de novo* synthesis of GSH, glutamine is linked to cysteine by GCL to form -glutamylcysteine (Griffith, 1999). Then GSS links the dipeptide -glutamylcysteine to glycine to form the final GSH molecule (Griffith, 1999). The GSH redox system plays a major role in ridding the body of oxidative stress and restoring homeostasis (Griffith, 1999). To elicit antioxidant effects, GSH is converted to oxidized glutathione (GSSG) by glutathione peroxidase (GPx). GSSG can be converted back to GSH by GSR (Staal, 1998). It is important to note that only free GSH has antioxidant effects. On the other hand, GSSG lacks antioxidant functions and is a byproduct of the scavenging activity of GSH (Staal, 1998; Griffith, 1999). GSH/GSSG ratio should be maintained to optimize the GSH redox system. GCL, the rate-limiting enzyme of GSH synthesis, is composed of a catalytic subunit (GCLC) and a modulating subunit (GCLM). GCLC is the component that performs the amino acid linkage between glutamine and cysteine, whereas GCLM modulates the activity of GCLC (Huang et al., 1993).

It has previously been reported that GSH levels in the plasma, erythrocytes, and peripheral blood mononuclear cells (PBMC) of HIV$^+$ individuals are compromised (Sbrana et al., 2004; Venketaraman et al., 2006; Guerra et al., 2011, 2012; Morris et al., 2012, 2013). The goal of our study is to characterize the causes for diminished levels of GSH in HIV infected individuals by determining the extent to which the levels of GCLC, GSS, and GSR are decreased in RBCs isolated from individuals with HIV infection compared to healthy subjects. Measurement of GSS and GCLC revealed a significant decrease in the levels of these enzymes present in RBCs of HIV-infected individuals compared to healthy subjects (**Figures 1** and **2**). Both GSS and GCLC are crucial enzymes that are involved in the catalytic rate limiting step and second step reaction, respectively, in the biosynthesis of GSH (Staal, 1998; Griffith, 1999; Morris et al., 2012, 2013). We also observed a significant decrease in the expression of GSR in RBCs isolated from HIV positive subjects (**Figure 3**). This explains the reason for decreased levels of GSH and the consequences related to the GSH deficiency such as loss of immune function observed in HIV patients (Venketaraman et al., 2006; Guerra et al., 2011, 2012; Morris et al., 2012, 2013). Reduced expressions of GSH synthesis enzymes in RBCs from individuals with HIV infection was accompanied by decreased levels of total GSH (**Figure 4**).

HIV$^+$ individuals were also found to have increased levels of TGF-β in their plasma and macrophage supernatants (Morris et al., 2012). Moreover, TGF-β is known to block the production of GCLC which leads to decreased GSH synthesis (Morris et al., 2012). HIV-1 transactivator protein (TAT) decreases the amount of GSH present in mice through the modulation of GSH biosynthetic enzymes (Choi et al., 2000).

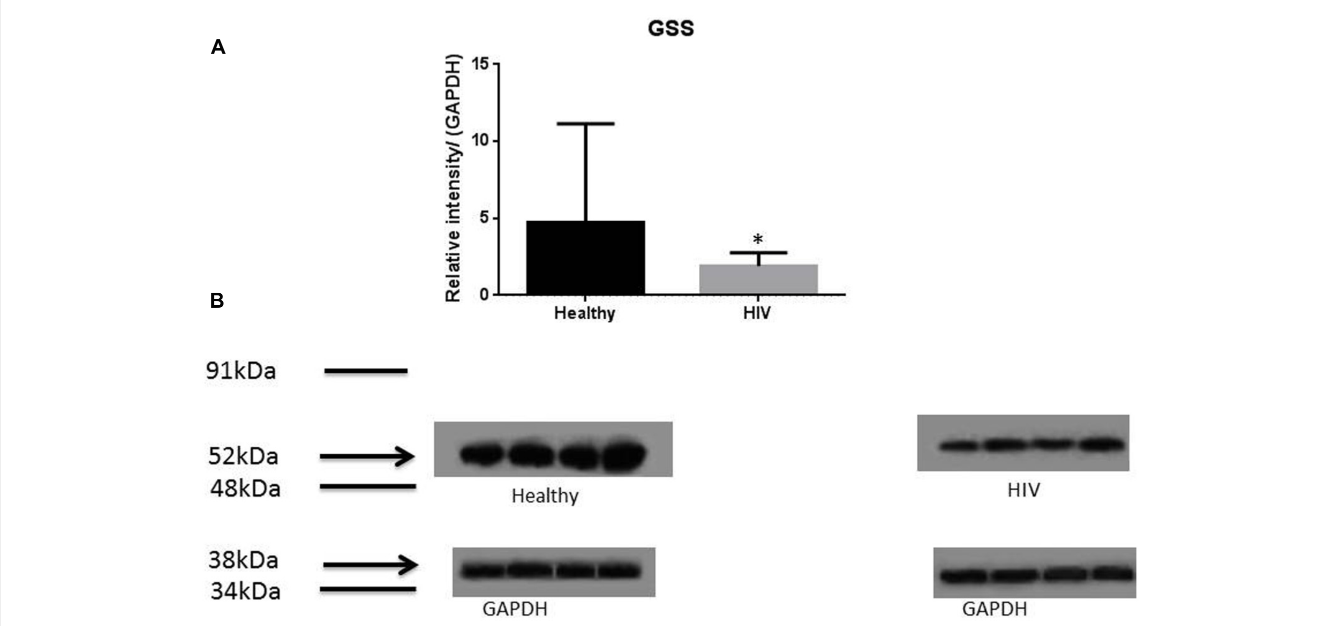

FIGURE 1 | Quantifying of GSS enzyme levels in Healthy and HIV-infected subjects. Red blood cell samples were separated from blood of healthy volunteers and HIV-infected individuals were used for measurement of GSS, the enzyme that is involved in the second step of GSH synthesis. Electrophoresis and Western blot were used. There was a significant decrease of GSS in the RBC of HIV-infected individuals compared to healthy individuals. Data in **(A)** represents significant difference in the levels of GSS between RBCs isolated from eight healthy individuals and eight individuals with HIV infection (*$p \leq 0.05$). **(B)**: Illustrates representative images of GSS and their corresponding GAPDH bands from four healthy subjects and four individuals with HIV infection.

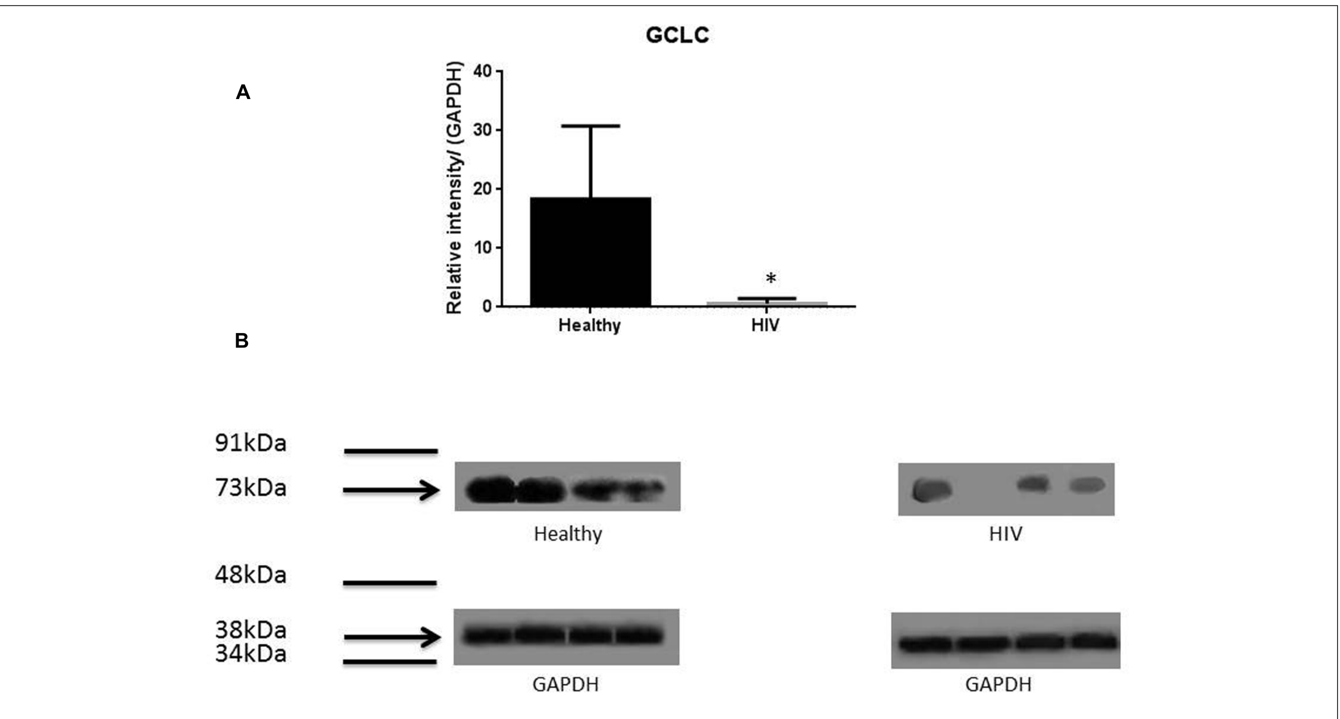

FIGURE 2 | Quantifying of GCLC enzyme levels in Healthy and HIV-infected subjects. Red blood cell samples were separated from blood of healthy volunteers and HIV-infected individuals were used for measurement of the catalytic subunit of GCLC that is responsible for the linkage between glutamine and cysteine in the first step of GSH synthesis. Electrophoresis and Western blot were used. GCLC levels were significantly lower in the RBC of HIV-infected individuals compared to healthy subjects. Data in **(A)** represents significant difference in the levels of GCLC between RBCs isolated from eight healthy individuals and eight individuals with HIV infection (*$p \leq 0.05$). **(B)**: Illustrates representative images of GCLC and their corresponding GAPDH bands from four healthy subjects and four individuals with HIV infection.

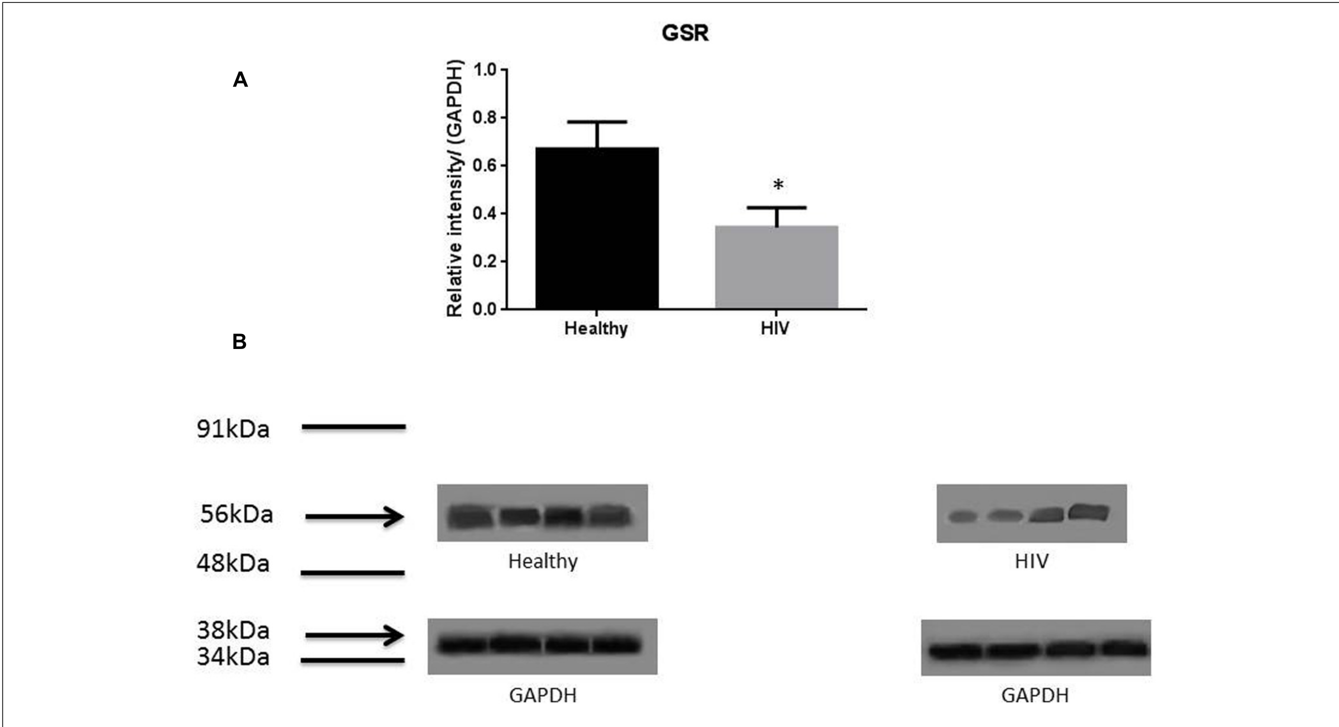

FIGURE 3 | Quantifying of GSR enzyme levels in Healthy and HIV-infected subjects. Red blood cell samples were separated from blood of healthy volunteers and HIV-infected individuals were used for measurement of GSR. GSR converts oxidized glutathione (GSSG) to glutathione (GSH). The GSR levels in the RBC of HIV-infected individuals are lower than healthy individuals. Electrophoresis and Western blot were used. GSR levels in HIV-infected individuals are lower compared to healthy. Data in **(A)** represents significant difference in the levels of GSR between RBC isolated from five healthy individuals and five individuals with HIV infection (*$p \leq 0.05$). **(B)**: Illustrates representative images of GSR and their corresponding GAPDH bands from four healthy subjects and four individuals with HIV infection.

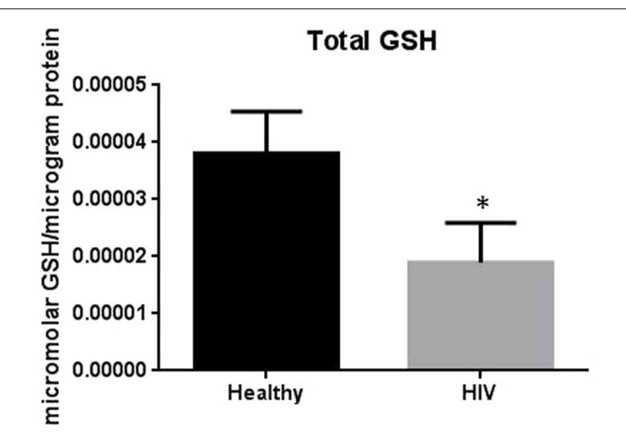

FIGURE 4 | Assay of GSH levels in RBCs from healthy subjects and individuals with HIV infection. Analysis of total GSH which includes both free GSH and GSSG indicated significantly higher levels of GSH in healthy individuals compared to HIV positive subjects. Data in **Figure 4** represents significant difference in the levels of total GSH between RBCs isolated from five healthy individuals and five individuals with HIV infection (*p=0.05).

TAT also increases free radical production. Therefore, marked increase in oxidative stress along with increased levels of TGF-β lead to the compromised levels of GSH synthesis enzymes. The master transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) regulates the expression of antioxidant and phase II-metabolizing enzymes by activating the antioxidant response element (ARE) and thereby protects cells and tissues from oxidative stress. The Nrf2 gene binding to the ARE results in the upregulation of GSH synthesis enzymes such as GCLC, GCLM, and GSR. New findings argue that HIV-1-related proteins downregulate Nrf2 expression and/or activity within the alveolar epithelium, which in turn impairs antioxidant defenses and barrier function, thereby rendering the lung susceptible to oxidative stress and injury (Fan et al., 2013). Furthermore, this study suggests that activating the Nrf2/ARE pathway with the dietary supplement sulforaphane could augment antioxidant defenses and lung health inHIV-1-infected individuals (Fan et al., 2013).

We have previously reported that the virulent laboratory strain of *M. tuberculosis* H37Rv is sensitive to GSH at physiological concentrations (5 mM) when grown *in vitro* (Venketaraman et al., 2005). Thus, GSH has direct antimycobacterial activity, functioning as an effector molecule in innate defense against *M. tuberculosis* infection (Venketaraman et al., 2005; Dayaram et al., 2006). We recently reported that GSH is integral in facilitating the control of intracellular growth of *M. tuberculosis* in human macrophages (Venketaraman et al., 2005; Dayaram et al., 2006; Morris et al., 2012, 2013). These results further confirm that GSH has direct antimycobacterial activity and unfolds a novel and potentially important innate defense mechanism adopted by

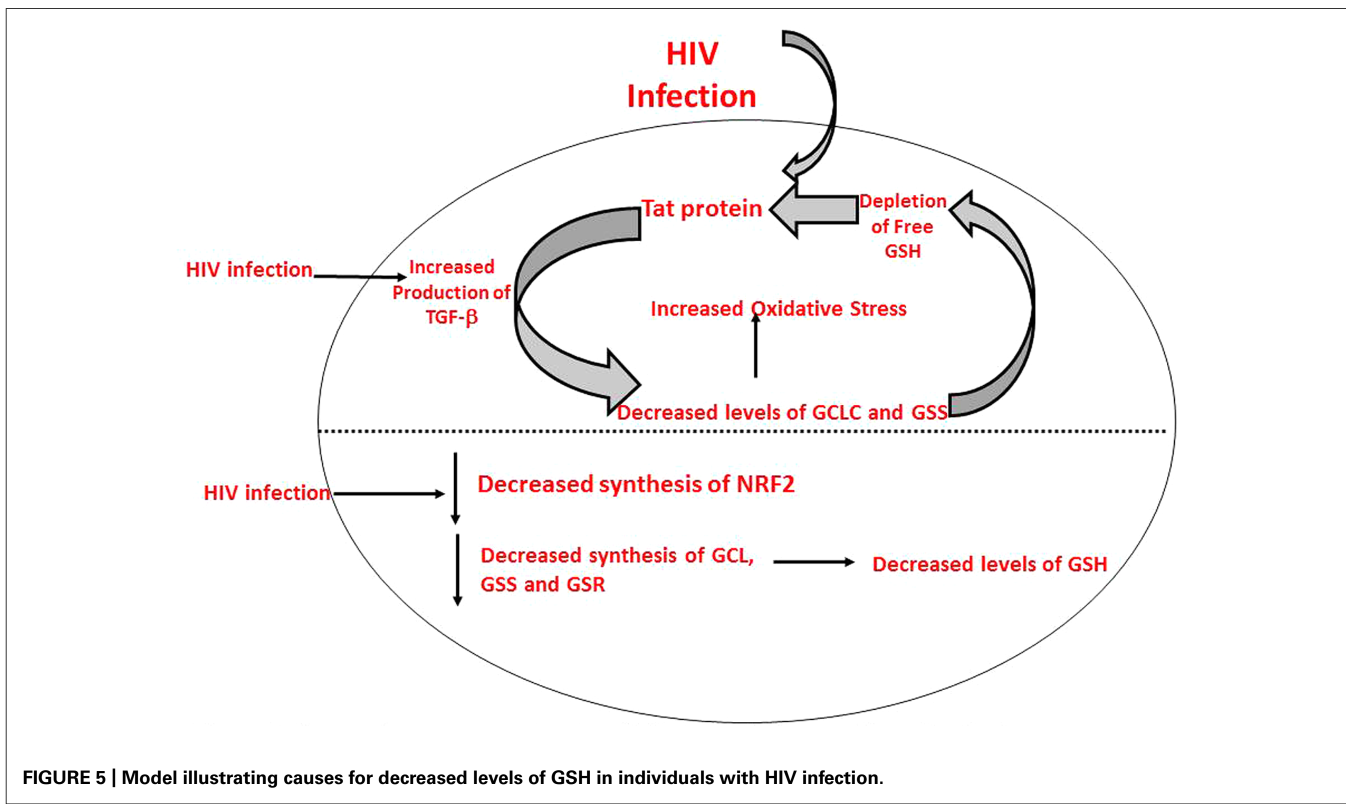

FIGURE 5 | Model illustrating causes for decreased levels of GSH in individuals with HIV infection.

human macrophages to control *M. tuberculosis* infection. We also demonstrated that GSH in combination with cytokines such as IL-2 and IL-12 enhances the functional activity of natural killer (NK) cells to inhibit the growth of *M. tuberculosis* inside human monocytes (Millman et al., 2008; Guerra et al., 2012). Importantly, data from our most recent studies indicate that GSH activates the functions of T lymphocytes to control *M. tuberculosis* infection inside human monocytes (Guerra et al., 2011). These results indicate that GSH inhibits the growth of *M. tuberculosis* by both direct antimycobacterial effects as well as by activating the functions of immune cells (Venketaraman et al., 2005; Dayaram et al., 2006; Millman et al., 2008; Guerra et al., 2011, 2012; Morris et al., 2012, 2013). We also reported that the GSH concentrations were significantly lower in macrophages, NK, and T cells isolated from individuals with HIV infection compared to healthy subjects (Venketaraman et al., 2006; Guerra et al., 2011, 2012; Morris et al., 2012, 2013). Decreased levels of GSH in macrophages, NK ,and T cells derived from individuals with HIV infection was accompanied by diminished control of intracellular *M. tuberculosis* infection (Venketaraman et al., 2006; Guerra et al., 2011, 2012; Morris et al., 2012, 2013). Our group is a pioneer in reporting that GSH levels were decreased in macrophages, T cells, and NK cells from individuals with HIV infection and correlating decreased GSH levels with impaired innate and adaptive immune responses against *M. tuberculosis* infection (Venketaraman et al., 2006; Guerra et al., 2011, 2012; Morris et al., 2012, 2013).

In this study we investigated the cause for decreased levels of GSH in individuals with HIV infection by quantifying the levels of GSS, GCLC, and GSR in the RBCs derived from healthy subjects and individuals with HIV infection. The results of the Western Blot indicate that there is a significant difference in the levels of GSS, GCLC, and GSR between HIV-infected individuals and healthy individuals, which supports our hypothesis that individuals with HIV infection have lower concentrations of enzymes that are responsible for both *de novo* synthesis of GSH and conversion of GSSG to GSH (**Figures 1–3**). In addition, our results indicate that there is a significant decrease in the levels of total GSH in the RBCs derived from HIV-infected individuals (**Figure 4**). Overall, these significant findings indicating lower levels of GSS, GCLC, and GSR in HIV-infected individuals support our hypothesis and contribute to previous findings that there are lower levels of GSH in HIV-infected individuals than healthy individuals (**Figure 5**). Observations from the current study combined with our previous findings strongly suggest that liposomal formulations of GSH can be used as a possible supplement to current HIV treatments since they can provide complete *r*GSH molecules, bypassing the cellular machinery for GSH production. Liposomal formulations containing GSH can be more effective in supplementing the intracellular *r*GSH and restoring the immune cell functions including the antimycobacterial activity in macrophages from HIV patients at concentrations lower than NAC (Venketaraman et al., 2006; Guerra et al., 2011, 2012; Morris et al., 2012, 2013).

ACKNOWLEDGMENTS

We would like to acknowledge Dr. Cesar Ochoa for helping us with the blood draw for the study. We would like to thank all of

our volunteers for without them this study would not have been possible.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 March 2014; paper pending published: 19 March 2014; accepted: 28 March 2014; published online: 11 April 2014.

Citation: Morris D, Ly J, Chi P-T, Daliva J, Nguyen T, Soofer C, Chen YC, Lagman M and Venketaraman V (2014) Glutathione synthesis is compromised in erythrocytes from individuals with HIV. Front. Pharmacol. ***5****:73. doi: 10.3389/fphar.2014.00073*

This article was submitted to Experimental Pharmacology and Drug Discovery, a section of the journal Frontiers in Pharmacology.

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Lyn Patrick, ND

Abstract

Mercury exposure is the second-most common cause of toxic metal poisoning. Public health concern over mercury exposure, due to contamination of fish with methylmercury and the elemental mercury content of dental amalgams, has long been a topic of political and medical debate. Although the toxicology of mercury is complex, there is evidence for antioxidant protection in the prevention of neurological and renal damage caused by mercury toxicity. Alpha-lipoic acid, a coenzyme of pyruvate and alpha-ketoglutarate dehydrogenase, has been used in Germany as an antioxidant and approved treatment for diabetic polyneuropathy for 40 years. Research has attempted to identify the role of antioxidants, glutathione and alpha-lipoic acid specifically, in both mitigation of heavy metal toxicity and direct chelation of heavy metals. This review of the literature will assess the role of glutathione and alpha-lipoic acid in the treatment of mercury toxicity.

(*Altern Med Rev* 2002;7(6):456-471)

Mercury: Sources of Exposure

According to the Agency for Toxic Substances and Disease Registry (ATSDR) of the U.S. Department of Health and Human Services, mercury is listed as the third-most frequently found (lead and arsenic are first and second), and the most toxic substance in the United States.[1] This figure originates from the U.S. Government's Priority List of Hazardous Substances. This list includes, in order of priority, substances that have been found at hazardous waste sites on the National Priorities List (Superfund sites) that "pose the most significant potential threat to human health due to their known or suspected toxicity and the frequency of exposure." Of 1,467 hazardous waste sites listed on the National Priorities List in 1998, toxic levels of mercury were identified in 714. Mercury toxicity is also considered the second-most common cause of acute heavy metal poisoning, with 3,596 cases reported in 1997 by the American Association of Poison Control Centers.[2]

Annual worldwide emissions of mercury into the atmosphere have been estimated at 2,200 metric tons.[3] One-third of these emissions are estimated to originate from natural sources (volcanic eruptions and decay of mercury-containing sediment) and two-thirds from man-made sources. Twenty-five percent of total worldwide emissions come from fossil fuel combustion. In the United States, 26 percent (64.7 tons/year) of atmospheric mercury emissions come from medical waste incineration, such as cremation.[4]

There are currently 1,782 advisories (one per body of water) issued by the U.S. Environmental Protection Agency (EPA) in 41 states in the United States restricting the consumption of any locally caught fish or shellfish due to their mercury content. Sixteen states have issued statewide or statewide-coastal advisories recommending restricting the consumption of fish caught in the state or along the coastline due to methylmercury contamination.[4] The Environmental

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Working Group, in a presentation to the Food Advisory Committee of the U.S. Food and Drug Adminstration (FDA), recently presented data warning of the consequences for fetuses of women who follow the current FDA's fish consumption advisory and eat 12 ounces of "safe" fish per week. The Environmental Working Group estimates that more than 25 percent of children *in utero* in the United States would be exposed to levels of mercury above the EPA safe reference dose (0.1 μg methylmercury/kg body weight/day) for at least 30 days during gestation and would have an increased risk for neurological damage.[5]

The ATSDR considers anyone who lives in close proximity to a former mercury mining site, recycling facility, municipal or medical incinerator, or coal-fired electric generating plant to be at risk for mercury toxicity. Anyone who routinely consumes contaminated fish, subsistence hunters who consume meat or organ tissues of marine mammals or feral wildlife, individuals with a "large number" of dental amalgams, pregnant or nursing women (and their developing fetuses and breast-fed babies), those who use consumer products containing mercury (skin-lightening creams or antiseptic facial products, mercury-containing diuretics or laxatives, and teething powders), or those living or working in buildings painted with mercury-containing latex paint are also considered at significant risk. Mercury-containing latex paint was removed from paint manufacturing in 1991 but may still be available in the reserve inventories of contractors and warehouses.[4]

Mercury is found in the environment in three basic states: elemental mercury or mercury vapor, inorganic mercury, and organic mercury (ethyl-, methyl-, alkyl-, or phenylmercury). Each form has an individual toxicological profile and metabolic fate. The most frequent source of mercury exposure is open to debate. On an individual exposure basis, the estimated intake and retention of elemental mercury vapor (from dental amalgams and atmospheric pollution) in non-occupationally exposed individuals has a much broader range (3.9-21.0 μg/day) than either inorganic (4.3 μg/day) or methylmercury (1-6 μg/day) exposure.[6]

Elemental Mercury

Elemental mercury, found in thermometers, thermostats, dental amalgams, and mercury added to latex paint, eventually enters a vaporized state. Eighty percent of inhaled elementary mercury vapor is absorbed and can cross the blood-brain barrier or reach the placenta.[2] Mercury vapor in the gastrointestinal tract is converted to mercuric sulfide and excreted in the feces.[6] Mercury vapor in the kidneys, however, the main repository for elemental mercury, is carried to all parts of the central nervous system as a lipid-soluble gas. Mercury vapor can also be oxidized to inorganic mercury by catalase and can attach to the thiol groups in most proteins – enzymes, glutathione, or almost any structural protein.[7] Elemental mercury can also be methylated by microorganisms in soil and water and potentially the human gastrointestinal tract,[8] where it can then be transformed into organic methylmercury, the form found in fish, fungicides, and pesticides. Elemental mercury and its metabolites have the toxic effect of denaturing biological proteins, inhibiting enzymes, and interrupting membrane transport and the uptake and release of neurotransmitters.[7] Chronic exposure most commonly manifests as a triad of increased excitability and irritability, tremors, and gingivitis.[2] Less commonly, chronic exposure causes central and peripheral nervous system damage, manifesting as a characteristic fine tremor of the extremities and facial muscles, emotional lability, and irritability. Rarely, significant exposure can cause acrodynia or "pink disease," involving a pink rash on the extremities, pruritis, paresthesias, and pain.[9]

Inorganic Mercury

Inorganic mercury (mercury salts) is found in cosmetic products, laxatives, teething powders, diuretics, and antiseptics.[2] Inorganic mercury can be formed from the metabolism of elemental mercury vapor or methylmercury.[7] Although inorganic mercury does not normally reach the placenta or cross the blood-brain barrier, it has been found in the neonatal brain due to the absence of a fully formed blood-brain barrier.[6] Inorganic mercury is complexed with glutathione in

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the liver and secreted in the bile as a cysteine-mercury or glutathione-mercury complex. Chronic exposure to inorganic mercury salts primarily affects the renal cortex[10] and may manifest as renal failure (dysuria, proteinuria, hematuria, oliguria, and uremia) or gastrointestinal problems (colitis, gingivitis, stomatitis, and excessive salivation). Irritability and occasionally acrodynia can occur.[2]

Organic Mercury

Considered the most toxic and most frequent form of mercury exposure, organic mercury is found in fish, poultry that has been fed fishmeal, pesticides, fungicides, insecticides, and thimerosal-containing vaccines. Thimerosal, which is 49.6-percent ethylmercury (a form of organic mercury), has been used as a preservative in vaccinations since the 1930s. It is currently mixed with DTaP, HIB, and hepatitis B vaccines or is used in the manufacturing process for vaccines, with resultant trace amounts being present in the final product. Based on existing Centers for Disease Control (CDC) recommendations for vaccinations, a typical six-month-old child, if receiving all thimerosal-containing vaccines, could potentially be injected with as much as 187.5-200 μg of methylmercury; the equivalent of more than 1.0 μg per day. This amount exceeds the reference limits for exposure to mercury set by the EPA of 0.1 μg/kg/day.[11] In the United States, at the FDA's request, all vaccines are currently being produced as thimerosal-free or thimerosal-reduced (> 95-percent reduction) vaccines. Thimerosal-preserved vaccines are still available and used in clinical practice.

Methylmercury is almost completely absorbed (95-100 percent) in the human gastrointestinal tract,[2,7] 90 percent of which is eventually eliminated through the feces. Methylmercury is present in the body as a water-soluble complex, mainly with the sulfur atom of thiol ligands,[7] and crosses the blood-brain barrier complexed with L-cysteine in a molecule resembling methionine. Methylmercury is absorbed into the placenta and stored in the fetal brain in concentrations that exceed maternal blood levels.[12] After being released from cells in a complex with reduced glutathione, methylmercury is degraded in the bile duct to an L-cysteine complex. Only 10 percent of methylmercury is eliminated through the kidneys. The rest either undergoes enterohepatic recycling or demethylation by microflora in the intestine and immune system and eventual elimination through the feces.

Most methyl mercury in animal exposure studies is degraded to, and eliminated as, inorganic mercury at the rate of one percent per day.[7] At least one study has demonstrated the capacity of two common forms of gastrointestinal yeast to convert inorganic mercury to methylmercury.[8] Demethylation by intestinal microflora is a crucial step in the elimination of methylmercury from the body, but research has not yet identified the mechanisms or the microbes responsible for this detoxification system.[7] Enterohepatic reabsorption is also a significant event in the metabolism of methylmercury; more than 70 percent is reabsorbed from the gut and returned to the liver.[7,13]

Inorganic mercury has been found as the major form of mercury in brain tissue in humans fatally exposed to methylmercury.[14] The conversion of methylmercury to inorganic mercury is thought to take place in phagocytic cells in the liver or in the astroglial cells of the brain.[7]

The majority of toxicity due to methylmercury exposure involves the central nervous system. Methylmercury can cause demyelination, autonomic dysfunction, sensory nerve conduction delay, abnormal neuronal migration, and abnormal central nervous system cell division. Chronic toxicity symptoms include paresthesia, peripheral neuropathy, cerebellar ataxia, akathisia, spasticity, memory loss, dementia, constricted vision, dysarthria, impaired hearing, smell and taste, tremors, and depression.[2,7]

Methylmercury exposure also appears to increase risk for cardiovascular disease. In a long-term prospective study, both intake of nonfatty freshwater fish and hair mercury content demonstrated a statistically significant correlation with increased risk for acute myocardial infarction.[15] Men with the highest hair mercury had a 2.9-fold increased risk for cardiovascular death. An examination of the same cohort found a significant correlation between hair mercury and increased risk

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Table 1. Mercury Species – Sources, Routes of Absorption, Distribution, and Excretion[18]

	Methylmercury	Elemental Mercury	Inorganic Mercury
Sources	Fish, poultry, pesticides	Dental amalgams, fossil fuels, old latex paint, thermometers, incinerators, occupational	Demethylation of methylmercury by intestinal microflora; biological oxidation of elemental mercury
Absorption	95-100 percent in intestinal tract; 100 percent of inhaled vapor	75-85 percent of vapor absorbed	7-15 percent of ingested dose absorbed; 2-3 percent of dermal dose absorbed in animals
Distribution	Lipophilic, distributed throughout body; readily crosses blood-brain barrier and placental barrier; accumulates in brain, kidney	Lipophilic, distributed throughout body; crosses blood-brain and placental barriers; accumulates in brain, kidney	Does not cross blood-brain or placental barrier; found in brain of neonates; accumulates in kidney
Metabolism	Cysteine complex necessary for intracellular absorption; slowly demethylated to inorganic mercury in brain by tissue macrophages, fetal liver, and free radicals	Oxidized intracellularly to inorganic mercury by catalase and hydrogen peroxide	Methylated by intestinal microflora; binds and induces metallothionein biosynthesis
Excretion	90 percent in bile,feces; 10 percent in urine	Urine, feces, sweat and saliva	Urine, bile, feces, sweat, saliva
Cause of Toxicity	Demethylation to inorganic (divalent) mercury; free radical generation; binding to thiols in enzymes and structural proteins	Oxidation to inorganic (divalent) mercury	Binding to thiols in enzymes and structural proteins

for progression of carotid atherosclerosis.[16] Prenatal exposure to methylmercury has been correlated with significant blood pressure elevations in seven-year-old children as a result of maternal fish intake.[17]

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Ethylmercury (fungicides, thimerosal in vaccines, and gamma-globulin) also causes renal and central nervous system toxicity and is deposited in the liver, kidneys, skin, brain, spleen, and plasma.[7] Ethylmercury, like methylmercury, is metabolized to the inorganic form and accounts for 50 percent of the mercury eliminated in urine. Ethylmercury may actually be converted to inorganic mercury in the tissues in greater amounts and more rapidly than methylmercury.[7] As with methylmercury, the feces are the main natural route of elimination. Table 1 summarizes the forms of mercury and their pharmacokinetics.

Mechanisms of Mercury Toxicity

Mercury can cause biochemical damage to tissues and genes through diverse mechanisms, such as interrupting intracellular calcium homeostasis, disrupting membrane potential, altering protein synthesis, and interrupting excitatory amino acid pathways in the central nervous system.[19] Mitochondrial damage, lipid peroxidation, microtubule destruction,[20] and the neurotoxic accumulation of serotonin, aspartate, and glutamate are all mechanisms of methylmercury neurotoxicity.[19]

Over time, both methylmercury and elemental mercury vapor in the brain are transformed to inorganic mercury, and become firmly bound to sulfhydryl-containing macromolecules.[21] Both methylmercury and inorganic mercury bind to various molecular weight thiol-containing proteins (glutathione, cysteine, albumin, etc.). The binding and dissociation of these mercury-thiol complexes are believed to control the movement of mercury and its toxic effects in the body.[7]

Mitochondrial damage from oxidative stress may be the earliest sign of neurotoxicity with methylmercury. A study in neural tissue indicates the electron transport chain appears to be the site where free radicals are generated, leading to oxidative damage induced by methylmercury.[19]

Mercury-Thiol Binding

Because the stability constants (energy necessary to form and break bonds) for mercury and thiol complexes (glutathione, albumin, cysteine, etc.) are so high, mercury will bind to any free thiol available and the thiol in the highest concentration will be the most frequently-bound.[22] The reaction rate is almost instantaneous.[7] Although the mercury-sulfhydryl bond is stable, it is labile in the presence of other free sulfhydryl groups; therefore, methylmercury will be redistributed to other competing sulfhydryl-containing ligands.[23] This is the basis for chelation of heavy metals with sulfhydryl compounds like DMPS and DMSA – providing free sulfhydryl groups in high concentrations to encourage the metal to move from one sulfhydryl-containing ligand to another.

The endogenous thiol-containing molecules – glutathione, cysteine, homocysteine, metallothionein, and albumin – all contain reduced sulfur atoms that bind to mercuric ions and determine the biological fate of mercury compounds in the body.[24] The complex of methylmercury and cysteine may act as a "molecular mimic" for the amino acid methionine and gain entry into the central nervous system via the same mechanism methionine uses to cross the blood-brain barrier.[25] Endogenous thiols transport mercury compounds and act to protect them from binding to other proteins, preventing functional damage in that tissue. In general, the higher the cysteine or thiol concentration in a cell medium, the lower the concentration of intracellular divalent mercury. In other words, higher concentrations of thiols appear to protect against accumulation of mercury, both *in vivo* and *in vitro*.[22]

Glutathione in Heavy Metal Binding

Glutathione is the most common low-molecular weight sulfhydryl-containing compound in mammalian cells, present in millimolar amounts in most cells.[26] As a result of the binding of mercury to glutathione and the subsequent elimination of intracellular glutathione, levels of reduced glutathione are lowered in several specific types of cells on exposure to all forms of mercury. Glial cells,[27] human erythrocytes,[28] and mammalian renal tissue[24] have all been found to have significantly lowered levels

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of reduced glutathione, a major source of oxidant protection. Mercury, as well as cadmium, generates highly toxic hydroxyl radicals from the breakdown of hydrogen peroxide, which further deplete glutathione stores.[27] There is evidence that glutathione depletion can lead to neurological damage; low levels of glutathione have been found in Parkinson's disease and cerebral ischemia-reperfusion injury.[29]

Glutathione, as both a carrier of mercury and an antioxidant, has three specific roles in protecting the body from mercury toxicity. First, glutathione, specifically binding with methylmercury, forms a complex that prevents mercury from binding to cellular proteins and causing damage to both enzymes and tissue.[30] Glutathione-mercury complexes also reduce intracellular damage by preventing mercury from entering tissue cells and becoming an intracellular toxin.

Second, glutathione-mercury complexes have been found in the liver, kidney, and brain, and appear to be the primary form in which mercury is transported and eliminated from the body.[24] The transport mechanism is unclear, but complexes of glutathione and mercury are the predominant form of mercury in both the bile and the urine.[31] Glutathione and cysteine, acting as carriers of mercury, actually appear to control the rate of mercury efflux into bile; the rate of mercury secretion in bile appears to be independent of actual bile flow. When bile flow rate is increased or decreased, the content of mercury in the bile changes inversely so net mercury efflux from the liver remains unchanged.[32] However, increasing bile levels of both glutathione and cysteine increases the biliary secretion of methylmercury in rats.[13] Other studies have confirmed this data in animal models.[33-35] Conversely, glutathione depletion inhibits biliary secretion of methylmercury in animal models and blocking glutathione production appears to shut down biliary release of mercury.[35]

Cells of the blood-brain barrier (brain capillary endothelial cells) release mercury in a glutathione complex. Inhibiting glutathione production in these cells inhibits their ability to release mercury.[23] Mercury accumulates in the central nervous system primarily in astrocytes, the cells that provide the first line of defense for the central nervous system against toxic compounds.[36] Astrocytes are the first cells in brain tissue to encounter metals crossing the blood-brain barrier. They also contain high levels of metallothionein and glutathione, both carriers for heavy metals. It is hypothesized that astrocytes are the main depot of mercury in the brain.[37] In studies with astrocytes, the addition of glutathione, glutathione stimulators, or glutathione precursors significantly enhances the release of mercury from these cells in a complex with glutathione. Fujiyama et al[38] also suggest that conjugation with glutathione is the major pathway for mercury efflux from astrocytes. Glutathione also increases mercury elimination from renal tissue. Studies in mammalian renal cells reveal glutathione is 50 percent as effective as the chelating agent DMSA (2,3-dimercaptosuccinic acid) in preventing inorganic mercury accumulation in renal cells.[39]

Third, glutathione increases the antioxidant capacity of the cell, providing a defense against hydrogen peroxide, singlet oxygen, hydroxyl radicals, and lipid peroxides produced by mercury.[30] The addition of glutathione to cell cultures exposed to methylmercury also prevented the reduction of cellular levels of glutathione peroxidase, a crucial antioxidant enzyme necessary for protection against the damaging effects of lipid peroxidation.[30]

As an antioxidant, glutathione appears to protect against renal damage resulting from inorganic mercury toxicity. The co-incubation of rat renal cells with glutathione and inorganic mercury was significantly more protective of renal cell injury when compared to inorganic mercury exposure alone.[40] Antioxidant levels – specifically glutathione, vitamin E, and ascorbic acid – are depleted in renal tissue exposed to mercuric chloride (inorganic mercury), and the addition of glutathione increased levels of both vitamin E and ascorbic acid in renal cells exposed to mercuric chloride.[24]

Mammalian cell lines resistant to mercury toxicity have been cloned.[41] They do not readily accumulate mercury and are resistant to the toxic

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effects of methylmercury or inorganic mercury. An outstanding characteristic of this cell line is that glutathione levels are five times greater in these cells than the parent cells from which they originated. The authors of this study conclude that the mechanisms of resistance were primarily due to glutathione's ability to facilitate mercury efflux from cells and the protective binding of mercury by glutathione to prevent cellular damage.

The Role of alpha-Lipoic Acid

In 1966, German physicians began using alpha-lipoic acid (ALA) therapeutically in patients with diabetic polyneuropathy and liver cirrhosis because of their observation that these patients had lower levels of circulating lipoic acid.[42] The application was subsequently extended to heavy metal intoxication and toxic mushroom poisoning.

According to Jones and Cherian,[43] an ideal heavy metal chelator should be able to enter the cell easily, chelate the heavy metal from its complex with metallothionein or other proteins, and increase the excretion of the metal without its redistribution to other organs or tissues. Although no human clinical trial has investigated the use of ALA as a chelating agent in mercury toxicity, there is evidence ALA satisfies at least two of the above criteria; i.e., absorption into the intracellular environment and complexing metals previously bound to other sulfhydryl proteins.

ALA produced endogenously is bound to proteins, but can also be found unbound in the circulation, after exogenous lipoic acid supplementation.[41] In this form it is chemically able to trap circulating heavy metals, thus preventing cellular damage caused by metal toxicity.[41] Lipoic acid is lipophilic and is able to penetrate cell membranes and reach high intracellular concentrations within 30 seconds of its administration.[44]

The fact that free ALA crosses the blood-brain barrier is significant because the brain readily accumulates lead and mercury, where these metals are stored intracellularly in glial tissue.[36,45] Oral doses of 10 mg/kg ALA in rats have reached peak levels in the cerebral cortex, spinal cord, and peripheral nerves within 30 minutes of administration, and studies of chronic daily dosing conclude ALA reaches all areas of the CNS and peripheral nervous system.[46] ALA has been shown to decrease lipid peroxidation in brain and sciatic nerve tissue[47] and when given orally to rats, decreased lipid peroxidation in brain tissue by 50 percent.[46] In diabetic neuropathy, free lipoic acid may prevent glucose-related oxidative damage by entering nerve tissue where it acts as both an antioxidant and heavy metal-binding agent.[28]

ALA has been administered to humans in doses up to 1,200 mg intravenously without toxicity, and in oral daily doses of as much as 600 mg three times daily. The only side effects reported are infrequent nausea and vomiting. No side effects have been reported in oral administration of up to 1,800 mg daily.[41,48] Doses of 500-1,000 mg have been well tolerated in placebo-controlled studies.[49] Extrapolation of pharmacokinetic and toxicity data demonstrate safe human dosages would not be exceeded with oral doses of several grams per day.[41]

ALA has been shown to increase both intra- and extracellular levels of glutathione in T-cell cultures, human erythrocytes, glial cells, and peripheral blood lymphocytes.[50] In rats, oral dosing of 150 mg/kg/day for eight weeks significantly increased glutathione levels in the blood and liver.[51] ALA has been shown to increase intracellular glutathione by 30-70 percent in murine neuroblastoma and melanoma cell lines, and in the lung, liver, and kidney cells of mice that had received intraperitoneal injections of 4, 8, or 16 mg/kg ALA for 11 days.[52,53] Levels of intracellular glutathione have been shown to increase by 16 percent in T-cell cultures at concentrations of 10-100 μM (concentrations achievable with oral and intravenous supplementation of ALA).[50] A single oral dose of 600 mg ALA was able to produce a serum concentration of 13.8 ± 7.2 μM and levels of 100-200 μM have been reported after 600 mg intravenous administration.[54]

Increases in glutathione levels seen with ALA administration are not only from the reduction of oxidized glutathione (one of the functions of ALA) but also from the synthesis of glutathione.[46] ALA is reduced to dihydrolipoic acid

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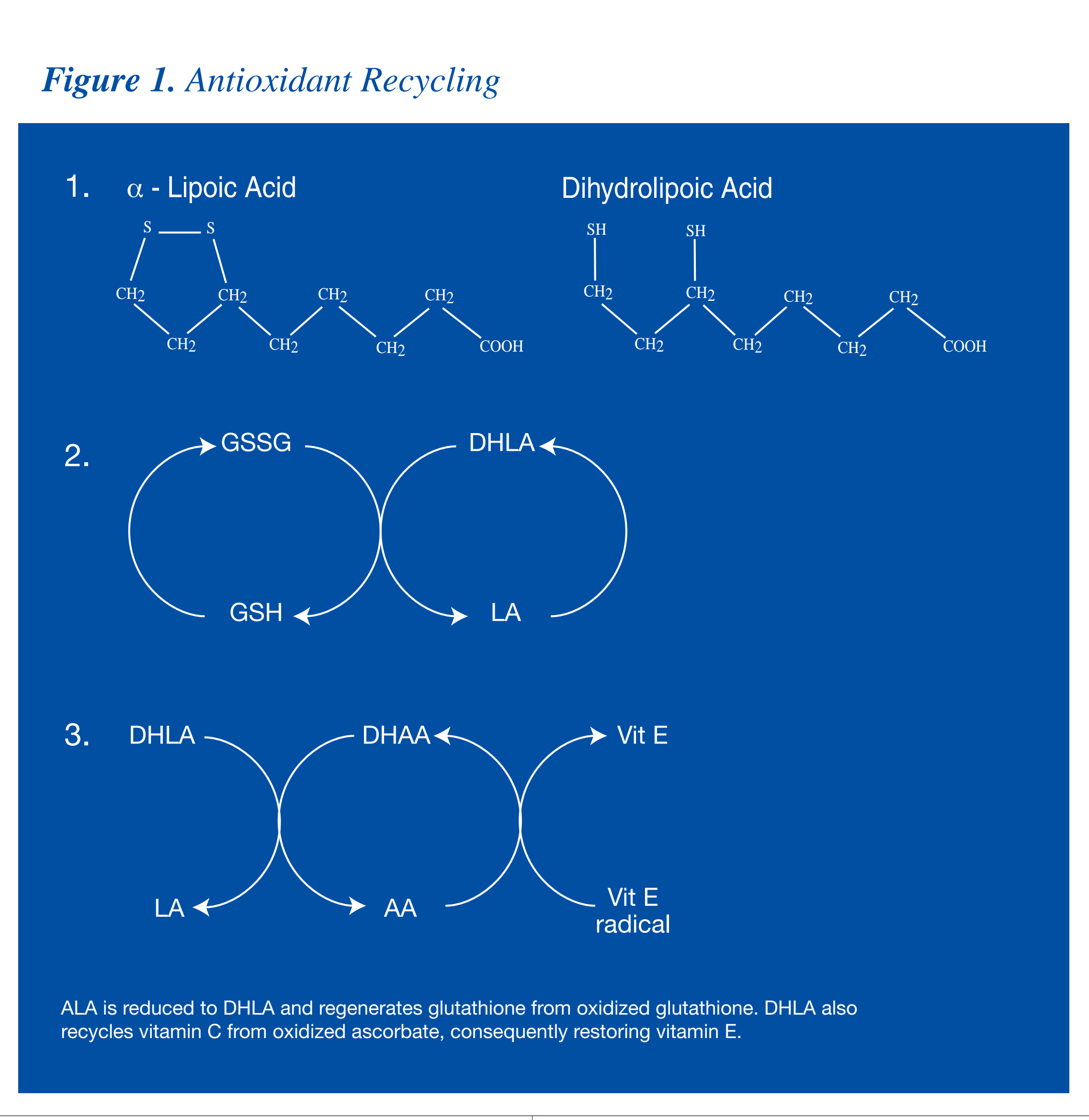
Figure 1. Antioxidant Recycling



ALA is reduced to DHLA and regenerates glutathione from oxidized glutathione. DHLA also recycles vitamin C from oxidized ascorbate, consequently restoring vitamin E.

(DHLA), itself a potent antioxidant. DHLA is able to regenerate oxidized ascorbate, glutathione, coenzyme Q, and vitamin E,[28] and is responsible for the ability of ALA to increase intracellular glutathione levels (Figure 1).[55]

ALA, through its reduction to DHLA and oxidation back to ALA, has the ability to continuously provide cysteine, the rate-limiting amino acid for glutathione production. ALA is rapidly reduced to DHLA and released in the extracellular environment where it reduces extracellular cystine to cysteine and increases the uptake of cysteine into the cell,[50] increasing glutathione production. ALA does this through enzyme-catalyzed reactions using NADH or NADPH, the metabolic power resulting from glucose metabolism (Figure 2).[51]

ALA and Binding of Copper, Iron, Platinum, and Lead

ALA and DHLA have been shown to form complexes with manganese ($Mn2^+$), zinc ($Zn2^+$), cadmium ($Cd2^+$), lead ($Pb2^+$), cobalt ($Co2^+$), nickel ($Ni2^+$), and iron ($Fe2^+$) ions.[55] In many cases, ALA-mediated heavy-metal binding prevents free-radical caused tissue damage or enzyme inactivation.[56]

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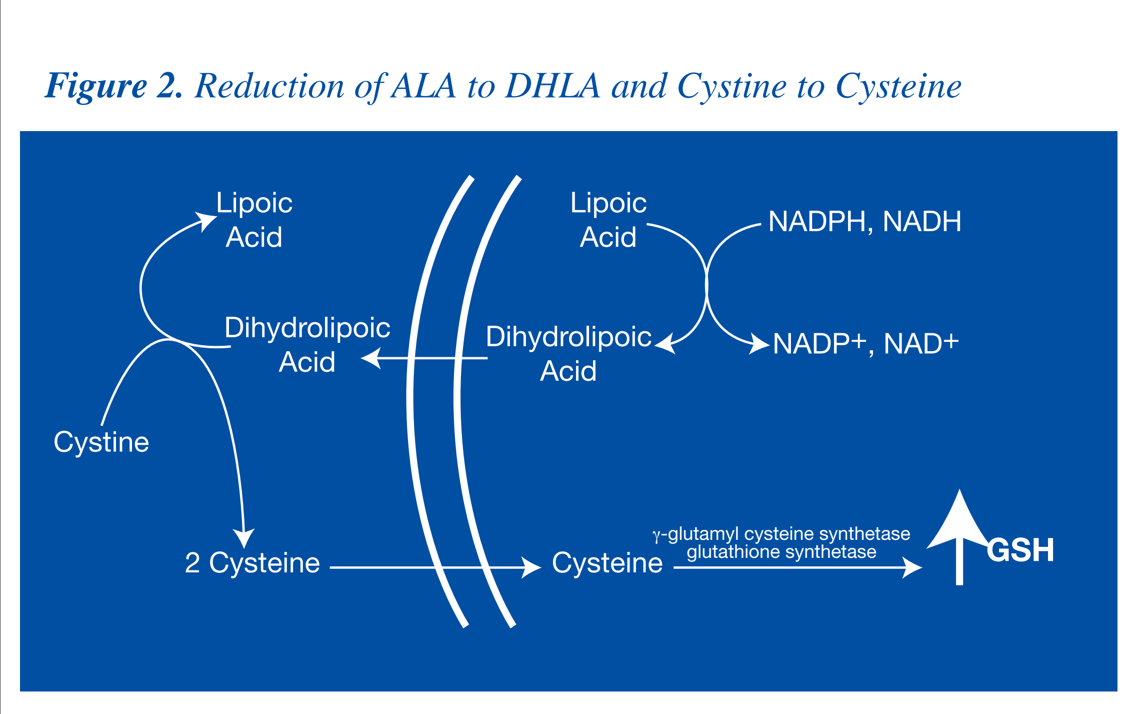
Figure 2. Reduction of ALA to DHLA and Cystine to Cysteine



In the case of iron and copper, complexing with ALA can protect cells from damage caused by iron- or copper-induced lipid peroxidation.[41] ALA has been shown to bind copper in human lipoproteins[57] and, as a result, to inhibit copper-induced peroxidation of low density lipoproteins. ALA has been used to treat Wilson's disease, effectively increasing renal copper excretion and normalizing liver function.[58]

ALA is also able to form complexes with ferritin-bound iron both *in vitro* and *in vivo*.[59] ALA has the ability to displace protein or vitamin C bound to iron and bind to $Fe2^{+}$. DHLA can facilitate the release of iron from the ferritin molecule and bind iron.[41]

The brain, particularly the substantia nigra and the globus pallidus, contains high levels of iron.[46] The high iron content and an increased level of unsaturated fatty acids lead to increased levels of tissue peroxidation.[46] ALA has been found to suppress the free radicals initiated by reactions with iron in the substantia nigra and other parts of the CNS.[46]

ALA has also been shown to protect against cisplatin-induced renal damage in rats by binding to platinum that is responsible for renal toxicity.[60] At dosages of 25-100 mg/kg (equivalent to 7 grams per 70 kg human adult), ALA restored normal levels of antioxidant enzyme activity, increased reduced glutathione levels, and significantly decreased renal tissue platinum content. The dose of cisplatin used in the study (16 mg/kg) is similar to clinical use in cancer treatment. Although the potential toxicity of this high dose of ALA is unknown, it is much higher than the 300-1800 mg typically used clinically.[46,48,49]

An intraperitoneal injection of 25 mg/kg ALA given to rats for seven days was able to significantly alter the oxidative stress induced by lead toxicity.[61] ALA administration increased glutathione levels 207 percent in the lead-exposed rats and decreased malondialdehyde levels in the brain, kidneys, and red blood cells, three of the four main targets of lead toxicity.[61] Further studies in cell lines of the fourth target, the reproductive system, found ALA had a protective effect in hamster ovarian cells, decreasing oxidative stress that causes cellular damage and death as a result of lipid peroxidation.[61] Because lead exposure was high (2,000 ppm injected daily into rats for five weeks) and the length of time ALA was administered was short (seven days), there may not have been

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enough time to see decreases in levels of lead in the brain or kidneys, if that effect were to take place. There were significant improvements in cell viability in ovarian cells exposed to lead that did not result from direct ALA-iron binding, suggesting ALA has a protective effect in lead toxicity aside from its ability to bind and excrete lead.[61]

ALA and Cadmium, Arsenic, and Mercury

Cadmium, arsenic, and mercury toxicity all involve similar pathways of cellular damage; i.e., mitochondrial damage, inhibition of mitochondrial enzymes, suppression of protein synthesis, and production of free radicals.[62] All three have a strong affinity for sulfhydryl-containing ligands (glutathione, alpha-lipoic acid, etc.), and each result in depressed levels of reduced glutathione.[63] The efficacy of ALA as an antioxidant and heavy metal-complexing agent in cadmium, arsenic, and mercury toxicity has been studied in animals – with results that may be applicable to heavy metal toxicity in humans.

ALA, at concentrations of 5 mM, was able to protect rat hepatocytes from cadmium toxicity (200 μM) by preventing decreases in total glutathione and increases in lipid peroxidation.[63] Another cadmium study investigated 1.5-6.0 mM concentrations of ALA or 17-89 μM DHLA in rat hepatocytes exposed to cadmium.[64] Both protocols decreased cadmium uptake by hepatocytes and normalized hepatocyte glutathione levels, leading to increased cell viability and survival despite the cadmium toxicity. ALA has also been shown (at a 30 mg/kg injected dose) to completely prevent damage that occurs from cadmium-induced lipid peroxidation in rat brain, heart, and testes.[65] In addition, ALA completely restored glutathione levels in the rat brain that had declined 63 percent with cadmium exposure.

A frequently quoted article referring to ALA as a heavy metal-complexing agent is the study by Grunert.[66] Published in 1960, the investigation used a dog and rat model in which simultaneous injection of sodium arsenate and ALA in both animals protected them from fatal arsenic toxicity. It has been shown that in acute arsenic intoxication, lipoic acid can form a complex with arsenic that renders the arsenic nontoxic.[41] Studies dosing mice with arsenic have shown ALA prevents intestinal uptake of arsenic and reduces the toxic effect of arsenic on enzyme inhibition.[49]

ALA has been shown to affect the release of glutathione into bile secretions. In animal studies, increasing amounts of glutathione in bile has been shown to dramatically increase the release of inorganic mercury. ALA given intravenously to rats at doses of 37.5-300 μM/kg was shown to increase inorganic mercury release in bile by 1,200-4,000 percent immediately after mercury exposure.[67] Levels of released inorganic mercury remained at a 300-700 percent elevation, even three hours after dosing with ALA. If mercury was injected 24 hours prior to the administration of ALA, the increase in release of inorganic mercury was substantially less, but was still elevated 140-330 percent. A lower dose of ALA (37.5 μM/kg) was more effective than higher doses at increasing the biliary elimination of methylmercury.

There was disconcerting evidence from this study, however, that ALA may also alter the tissue distribution of mercury and other heavy metals.

Although levels of inorganic mercury and methylmercury in the kidney dropped significantly, levels of inorganic mercury also increased significantly in the brain, lung, heart, and liver tissue. Methylmercury levels had also increased in the brain, intestine and muscle of the rats given ALA. The same phenomenon occurred in rats exposed to cadmium and given the same doses of ALA. Levels of cadmium in the liver dropped (where cadmium is most frequently stored) but increased in the kidney and muscle. The same was true in rats given copper and ALA; all tissues examined had increased levels of copper, except for the liver (where copper usually accumulates) where levels had dropped.[67] In all cases the pattern was the same; the tissues that concentrated the metal (blood, spleen, and kidneys in the case of methylmercury) had reduced concentrations, while other tissues appeared to have a greater concentration.

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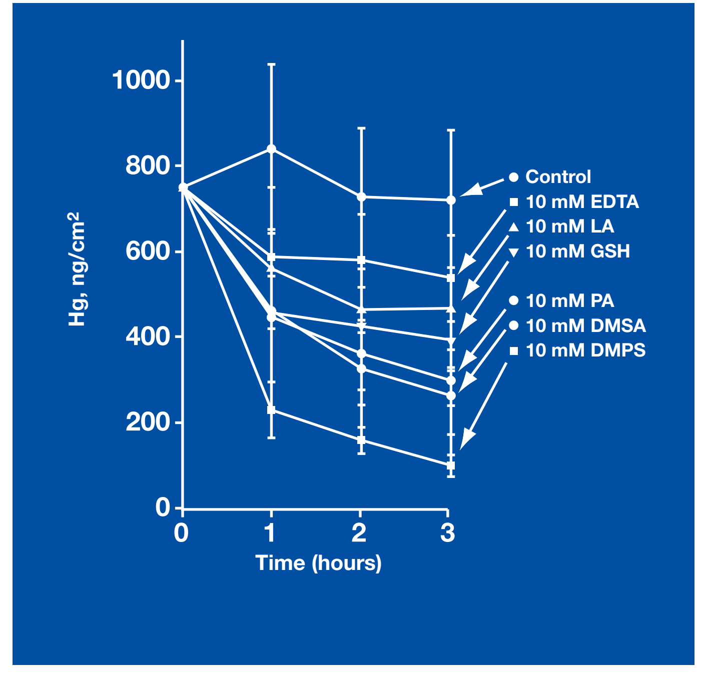

Figure 3. *Ability of Chelating Agents to Lower Mercury Content of Renal Tissue* in vitro *from Rabbits Injected with Mercuric Chloride*

Grunert subjected mice to lethal doses of mercuric chloride accompanied with ALA.[66] He found the ALA-to-mercury ratio was crucial in determining the outcome. A ratio of 6-8 moles ALA per mole mercuric chloride was necessary to allow the mice to survive mercury poisoning. A lower level of ALA actually increased the mercury toxicity (a molar ratio of 2 moles ALA to 1 mole mercuric chloride or lower) above control levels. The level of mercuric chloride used in this experiment, 20 mg/kg, is high and would only be seen in acute mercury poisoning.

In another study of mercury intoxication, an injection of 10 mg/kg/day ALA in rats given an injection of 1 mg/kg/day mercuric chloride prevented damage to nerve tissue caused by lipid peroxidation.[68] ALA significantly reduced lipid peroxidation in the mercury-exposed rats while elevating levels of the antioxidants glutathione, ascorbate, and tocopherol. The mechanism of protection was hypothesized to be the scavenging of peroxyl radicals formed in the brain and nervous system, although the authors believed direct complexing of inorganic mercury by ALA was also a possibility.

ALA versus Dithiol-based Chelating Agents (DMPS, DMSA)

The ability of ALA to bind inorganic mercury from rabbit renal tissue was compared to glutathione and the chelators 2,3-dimercaptopropane-1-sulfonate (DMPS), meso-2,3-dimercapto- succinic acid (DMSA), penicillamine, and ethylenediaminetetra acetic acid (EDTA) (Figure 3).[69]

DMPS was the most efficient chelator, removing 86 percent of the mercury in three hours, with DMSA being the next-most efficient, removing 65 percent of the mercury. In the same time period, penicillamine removed 60 percent, glutathione removed 50 percent, ALA removed 35 percent, and EDTA removed 20 percent. Only the levels reached by DMSA and DMPS, however, were statistically significantly different from baseline ($p<0.05$). Therefore, the effect of ALA and glutathione may show only a trend or an apparent effect and are not comparable to DMPS and DMSA. Although the actual effect of a chelator or heavy metal-complexing agent cannot be determined in a three-hour time period, and acute doses of 10 mg/kg of inorganic mercury would be considered highly toxic in an adult human, there is evidence from this study that ALA is a less efficient binder of inorganic mercury than the recognized chelating agents, DMSA and DMPS. All of the substances were used at a concentration of 10 mM, a level difficult to reach with ALA oral supplementation.

In another comparison study, ALA (25 mg/kg/day) resulted in an insignificant decrease in blood and tissue lead in rats with lead toxicity when compared to the dithiol-based chelating agent, DMSA (dosed at 90 mg/kg/day) (Table 2).[61]

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Table 2. Blood Lead Levels from Fischer 344 Rats

	Control	Pb only	Pb + LA	Pb + DMSA
Blood lead levels (mcg/dL)	0.2 ± 0.5	36.4 ± 4.4*	28.7 ± 4.1	2.0 ± 1.0**

All values represent mean ± SD for 5-10 samples
*p < 0.001, compared to the corresponding value of control group
** p < 0.005, compared to the corresponding value of lead group

Both DMSA and DMPS have been shown to be clinically effective heavy metal chelators in human studies of mercury toxicity,[70-75] particularly since they both chelate inorganic and organic mercury.[71] DMSA acts only as an extracellular chelator, whereas DMPS enters hepatocytes[73] and renal cells,[76] although it is still considered primarily an extracellular chelator.[73] DMSA is less toxic because of its inability to enter cells or bile,[73] with an LD_{50} of 13.73 mM/kg, approximately twice the LD_{50} of DMPS, which is 6.53 mM/kg.[73] While DMSA has been found to be more effective than DMPS at removing mercury from the brain,[77] DMPS appears to be more effective at removing mercury from the kidney.[78]

Conclusion

Many unanswered questions remain regarding ALA and heavy metal detoxification, especially pertaining to mercury. The amount of ALA supplemented versus the amount of toxic metal stored in the tissues is important, and has been clearly detailed in animal trials. A molar ratio of 6-8:1 (ALA:mercury) is necessary for protection and viability in mercury studies; a ratio of 2:3 has been seen in arsenic studies.[66] The ability of ALA to assist or prevent movement of heavy metals from the liver appears to be element-specific. In a previously mentioned study, the biliary release of methylmercury, cadmium, zinc, and copper was inhibited by ALA.[69]

The evidence that ALA may mobilize heavy metals to other tissues from tissues where the metals are most concentrated, specifically the brain, is troublesome. An explanation for this finding may lie in the complexing of heavy metals with glutathione and lipoic acid. Inorganic mercury forms stable complexes with ALA or DHLA and could be excreted with DHLA independent of available glutathione.[67] As Gregus et al[67] hypothesize, injected lipoic acid could complex with glutathione as it passes through the liver, preventing glutathione from carrying other heavy metals such as cadmium, or transition metals such as zinc and copper, into bile. Speculation aside, there is clear evidence ALA and its reduced form DHLA have the ability to act as both intra- and extracellular heavy metal-complexing agents, with little known toxicity and patterns of heavy metal mobilization and transport not yet understood in humans. In the absence of data from human trials, however, it can only be suggested that ALA be used as an adjunct to chelation with the standard dithiols, DMPS and DMSA.

Mercury toxicity is a significant clinical entity, as it is ubiquitous in the environment and poses serious risk to human health. The pathology of mercury toxicity in humans is diverse and encompasses direct damage to tissues and enzyme function as well as indirect damage as a result of oxidant stress.

Glutathione has been shown to be a significant factor in heavy metal mobilization and excretion, specifically with application to mercury, cadmium, and arsenic. Glutathione depletion and glutathione supplementation have specific effects on mercury toxicity, both by altering antioxidant status in the body and by directly affecting excretion of mercury and other heavy metals in the bile.

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Lipoic acid has been shown, by its increasing of cellular glutathione levels, to support the mobilization and excretion of mercury, and to decrease cellular damage and neurotoxicity. The reduced form of ALA, DHLA, appears to have direct heavy metal-binding effects. When compared to pharmaceutical dithiol-chelating agents, ALA appears to be able to bind and mobilize heavy metals from tissue, although with much weaker an effect.

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J Psychiatr Res. Author manuscript; available in PMC 2013 October 01.

Published in final edited form as:
J Psychiatr Res. 2012 October ; 46(10): 1326–1332. doi:10.1016/j.jpsychires.2012.06.008.

Oxidative Stress and Glutathione Response in Tissue Cultures from Persons with Major Depression

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Abstract

There is evidence that major depressive disorder (MDD) is associated with increased peripheral markers of oxidative stress. To explore oxidation and antioxidant response in MDD, we assayed human dermal fibroblast cultures derived from skin biopsies of age-, race-, and sex-matched individuals in depressed and normal control groups (n=16 each group), cultured in glucose and galactose conditions, for relative protein carbonylation (a measure of oxidative stress), glutathione reductase (GR) expression, and total glutathione concentration. In control-group fibroblasts, galactose induced a significant increase from the glucose condition in both protein carbonylation and GR. The cells from the MDD group showed total protein carbonylation and GR expression in the glucose condition that was significantly higher than control cells in glucose and equivalent to controls in galactose. There was a small decrease in protein carbonylation in MDD cells from glucose to galactose and no significant change in GR. There was no difference in total glutathione among any of the groups. Increased protein carbonylation and GR expression, cellular responses to oxidative stress induced by galactose in control fibroblasts, are present in fibroblasts derived from MDD patients and are not explainable by reduced GR or total glutathione in the depressed patients. These studies support the role of oxidative stress in the pathophysiology of MDD. Further confirmation of these findings could lead to the development of novel antioxidant approaches for the treatment of depression.

Keywords

human dermal fibroblasts; oxidative stress; glutathione; glutathione reductase; protein carbonylation; major depression

Introduction

Major depressive disorder (MDD) is a chronic, highly disabling condition, and both short and longer-term treatment outcome is not optimal (Kessler et al., 2005; Schmidt et al., 2011;

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Shelton & Tomarken, 2001). Research has linked the pathophysiology of MDD to a wide range of molecular biomarkers in blood and other peripheral tissues, demonstrating the complexity of the disorder (Schmidt et al., 2011). One important finding is a reduction in certain signal transduction proteins in MDD, particularly protein kinases A (PKA) and C (PKC; Dwivedi et al. 2004; Perera et al., 2001; Perez et al., 2002; Shelton et al., 2009 A; Pandey et al., 1998; Pandey et al., 1997). Although the mechanism for alteration of these signal transduction proteins remains obscure, there is evidence for redox regulation (Humphries et al., 2005; Humphries et al., 2007; Giorgi et al., 2010), and a growing number of studies link MDD with an increased state of oxidative stress (Ng et al., 2008).

Reactive oxygen species (ROS) are formed in the human body through a variety of processes, including normal cell metabolism, especially the mitochondrial electron transport chain (Halliwell, 1991), as well as ultraviolet light (Heck et al., 2003) and heat stress (Cho et al., 2009). ROS are buffered through antioxidant defenses, mostly thiol compounds that act as substrates for oxidation, and oxidative damage to cells occurs when the balance between ROS produced and concentration of protective antioxidants favors ROS (Halliwell, 1991). Therefore, increasing levels of oxidation could be explained by disrupted function of a major antioxidant mechanism, such as the production and redox efficiency of glutathione.

Glutathione is the most abundant thiol compound in cells of all organs and plays an important protective role against oxidative stress in the brain (Dringen et al., 2000; Dringen, 2000; Gawryluk et al., 2011). Glutathione reductase (GR) is the enzyme responsible for recycling oxidized glutathione to the reduced, antioxidant form and has been shown to be up-regulated in response to oxidative stress (Gawryluk et al., 2011; Schuliga et al., 2002). For example, GR was seen to increase in activity with increasing oxidation in human keratinocytes and fibroblasts exposed to increasing concentrations of arsenic (Schuliga et al., 2002). The role of oxidative stress, glutathione, and GR has been implicated in the pathogenesis of both psychiatric and non-psychiatric diseases including Leber's Hereditary Optic Neuropathy (LHON; Floreani et al., 2005), Type 2 Diabetes mellitus (Calabrese et al., 2011), Huntington's Disease (del Hoyo et al., 2006), schizophrenia (Gysin et al., 2009; Mahadik & Mukherjee, 1996), bipolar disorder (BD; Andreazza et al., 2008; Yumru et al., 2009), autism and ADHD (Ng et al., 2008).

Although work has been done regarding oxidation and glutathione response in MDD using serum (Kodydková et al., 2009) and post-mortem brain tissue (Gawryluk et al., 2011), little has been tested using human cell culture. Dermal fibroblasts are not only easy to establish, control, reproduce, and eliminate outside factors, but they have proven to be a relevant study system for signal transduction pathways in MDD (Akin et al., 2004; Manier et al., 2000; Shelton et al., 1996). In addition, fibroblasts have been used to study glutathione, GR, and oxidative stress with success in other diseases such as Leber's hereditary optic neuropathy (LHON) (Floreani et al., 2005), Type 2 Diabetes (Calabrese et al., 2011), and schizophrenia (Gysin et al., 2009; Gysin et al., 2007).

Our study explores levels of protein oxidation, glutathione concentration, and GR expression in dermal fibroblasts extracted from skin biopsies of a population of patients diagnosed with MDD [n=16] compared to sex-, age-, and race-matched healthy controls [n=16] and grown in glucose and galactose conditions in order to identify differences in oxidative damage and antioxidant capacity associated with MDD in peripheral tissue.

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Materials and Methods

Population

The Vanderbilt University Institutional review board (IRB) approved the study and written informed consent was obtained from all study participants before any procedures were conducted. The study population was composed of 16 human patients diagnosed with MDD and 16 age, race, and sex-matched healthy control participants (Table 1). Recruitment and diagnosis procedures for patients and controls have been described in previous publications (Shelton et al., 1996; Manier et al., 1996). In brief, all participants were assessed using the Structured Clinical Interview for DSM-IV (SCID; First et al., 2009). Exclusion criteria included other primary DSM-IV diagnosis (American Psychiatric Association, 1994), a history of bipolar disorder or non-mood psychotic disorder, or any medical condition that would preclude the biopsy (e.g., hemophilia). The groups included 8 males and 24 females, ages ranging from 20 to 53 years (Table 1).

Collection and processing of skin biopsies and establishment of fibroblast cultures

Specimens for fibroblast cultures were obtained by skin punch biopsies (1×2 mm) taken from the upper arm on participants using the method previously described in detail (Shelton et al., 1996; Manier et al., 1996). Skin biopsies were placed in plain DMEM medium and processed the same day as previously described (Shelton et al., 1996; Manier et al., 1996). The medium was changed three times a week. In about 2-3 weeks, the fibroblasts divided and became confluent. All fibroblast samples obtained for this study were able to propagate. The fibroblasts were subcultured using 0.5% Trypsin-EDTA (Invitrogen) as previously published (Akin et al., 2004) and expanded for freezing in a liquid nitrogen cell repository.

All fibroblasts used for experiments were cultured between passages 5 and 10 in order to minimize any effect from exposure *in vivo* to factors such as hormones, neurotransmitters, cytokines, or drugs (Akin et al., 2004). All fibroblasts were taken from liquid nitrogen storage and maintained in Dulbecco's modified Eagle's medium™ (DMEM; Mediatech, Manassas, VA, USA) containing 25 mM glucose and 1 mM sodium pyruvate supplemented with 2mM L-glutamine (Mediatech), 10% fetal bovine serum (FBS; Thermo Scientific HyClone, Logan, UT, USA), and antibiotic/antimycotic solution (A/A; Invitrogen) at 37°C and 5% CO_2. For exposure to galactose, cells were cultured in DMEM deprived of glucose (Mediatech) supplemented with 10mM galactose, 2mM L-glutamine, 10% FBS, 1mM, A/A, and sodium pyruvate (Sigma Aldrich). The cells from matching pairs of control and MDD subjects were cultured simultaneously under same conditions until reaching confluence, trypsinized, and counted using a Bright-Line Hemacytometer (Hausser Scientific, Horsham, PA). Cells, 0.5×10^6/plate were grown in two 60mm dishes (Sarstedt, Newton, NC, USA) in glucose-containing medium. After 24 hours, once fibroblasts adhered, the medium in one plate was changed into fresh glucose medium and the other to galactose medium. Cell growth and proliferation were checked and were not affected by the galactose condition in either group (data not shown). After another 24 hours, the cells were washed in 1X PBS twice on ice and collected with a cell scraper in cooled 1X PBS (Mediatech). The cell suspension was pelleted by centrifugation at 280g for 10 min at +4°C. Excess PBS was removed, and cells were resuspended in NP-40 lysis buffer (150 mM NaCl, 50mM Tris-Cl [pH 7.4], 5mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate) supplemented with PMSF, protease inhibitor cocktail (Sigma Aldrich) and phosphatase inhibitor cocktail (Sigma Aldrich). After 30 min of lysis on ice, cell lysate was cleared by centrifugation at 14,000Xg for 5 min at +4°C. Protein concentration w as determined using the D_c protein assay (Bio-Rad, Hercules, CA). Twenty μg of protein lysate was used immediately for determining protein carbonylation.

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Protein carbonylation determined by OxyBlot™ Protein Detection kit

Protein carbonyl levels were measured using protocol found in the commercially available OxyBlot™ Protein Detection kit (Millipore, Billerica, MA, USA). For each sample, two reactions were prepared: derivatized and negative control. Carbonyl groups in the proteins were derivatized to 2,4-dinitrophenylhydrazine (DNPH). Equal loading was assessed by restaining PDVF membranes with an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling, Danvers, MA, USA). All blots were analyzed and normalized by measuring the pixel density of bands appearing in the final blots using UN-SCAN-IT (Silk Scientific Corporation, Orem, UT, USA) as previously described (Shelton et al., 2009 B). Refer to Figure 1A for a representative blot.

Measurements of Glutathione and Glutathione Reductase

Total glutathione levels were assayed using the published enzymatic method (Rahman, 2006) and normalized to total protein amount as determined by D_c protein assay (Bio-Rad Laboratories, Hercules, CA).

Glutathione Reductase (GR) protein expression was assayed by western blot using 20 μg total protein from the same total lysate used for DNPH derivitization, normalized to GAPDH as above, and again analyzed using UN-SCAN-IT. Membranes were stained according to manufacturer's recommendations with anti-GR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and with anti-mouse IgG secondary antibody (Sigma). Refer to Figure 1B for example blot.

Statistical Analyses

In the 16 controls and 16 MDD, both protein carbonylation and GR were analyzed based on intensity of individual protein bands in the final western blot in fibroblasts from all participants relative to the corresponding pair. Data was then grouped into control and MDD plus the condition, glucose or galactose, for final comparison and analysis. Differences in treatments and controls were analyzed using means, standard error, and between-group comparisons conducted with Student's *t*-tests. Statistical significance was defined as p 0.05.

Results

Increased protein carbonylation in cultured MDD human fibroblasts

Protein carbonylation was assessed in cultured fibroblasts established from control and MDD participants. The majority of DNPH detectable protein products, as illustrated in Figure 1A, span molecular weight species from ~ 20 to ~ 250kDa. Control cells grown in glucose showed a very low number of DNPH protein products – markedly different from MDD cells under the same condition. Quantification of western blots showed that the sum of DNPH products was 1.85 times higher in MDD than in controls in the glucose condition (p 0.01) (Figure 2).

Cultured fibroblasts were exposed to galactose in the place of glucose in the culture medium in order to induce oxidative stress (Marroquin et al., 2007). Under these conditions, we observed significant increase in protein carbonylation in control fibroblasts from the glucose condition; quantification of western blots showed that the sum of DNPH products was 1.73 times higher in the galactose than in glucose-containing medium (p 0.01) (Figure 2). We saw an opposite response in MDD cells when exposed to galactose. There was a small but significant decrease in protein carbonylation in the MDD cells when grown in the galactose condition compared to the glucose; the sum DNPH products in the galactose condition was 0.81 times the measured in the glucose condition (p 0.01). These results indicate that the

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cells from participants with MDD have increased protein carbonylation relative to controls in the glucose condition, and under conditions of increased oxidative stress, the MDD fibroblasts do not respond with an increase in protein oxidation as do control fibroblasts.

Increased Glutathione Reductase protein expression in MDD fibroblasts without changes in total glutathione

To determine if increased protein carbonylation in MDD fibroblasts could be the result of diminished antioxidant capacity, we measured total glutathione concentration in the cells. We found no significant difference in the level of total glutathione among any of the groups (Figure 3). This result indicates that total the level of glutathione in fibroblasts is not significantly different between control and MDD, and levels are not significantly altered by the presence of galactose in either group.

Levels of reduced glutathione in the cytosol are maintained by GR[19]. Therefore we compared the protein levels of this enzyme in control and MDD fibroblasts under glucose and galactose condtions via western blot (Figure 1B). In control cells, we found GR levels to increase in the galactose condition; controls in the galactose condition showed 1.78 times more GR than controls in glucose condition (p 0.01) (Figure 4). MDD cells in the glucose condition showed 1.99 times the level of GR of control cells in glucose (p 0.05) (Figure 4). There was no significant difference in GR between MDD cells in the glucose and galactose conditions; our data shows GR in MDD cells in the galactose condition to be 0.96 times the glucose condition (p=0.81). These results indicate that GR expression is up-regulated in MDD in the glucose condition as it is in control cells when stressed with galactose with no accompanying changes in glutathione.

Discussion

To our knowledge, this is the first clear demonstration of increased oxidative stress in actual living tissue culture derived from persons with MDD. Under normal glucose culture conditions the amount of total protein carbonylation, a measure of protein modification in response to oxidative stress (Stadtman, 1993), was significantly higher in MDD fibroblasts than in those derived from control subjects (Figure 2) with no significant difference in total glutathione concentration (Figure 3). In addition, our study shows GR expression to be significantly higher in MDD cells when compared to controls in the glucose condition (Figure 4), another cellular response to the presence of oxidation (Gawryluk et al., 2011; Schuliga et al., 2002). Other studies have confirmed the involvement of oxidative stress in MDD, but our findings regarding glutathione and glutathione reductase differ from some previously published work. In a 2011 study, Gawryluk et. al found reduced levels of total glutathione and no change in GR in MDD in post-mortem, pre-frontal cortex brain tissue (Gawryluk 2011). Furthermore, depleted levels of glutathione and reduced GR activity have been found in a rodent depression model rodents (Zafir 2009). In the current study, total glutathione levels did not differ between groups. As well, GR showed an increase in the glucose condition, consistent with compensation for increased oxidative stress. Therefore, our findings do not support a role for either glutathione or GR in increased oxidative stress in MDD.

Removing glucose and adding galactose to cell culture medium is a commonly used practice to induce oxidative stress. Galactose metabolism doubles O_2 consumption and increases cell susceptibility to mitochondrial toxicants by shifting metabolism toward oxidative phosphorylation (OXPHOS; Marroquin et al., 2007). In our study, the galactose condition increased protein carbonylation in control fibroblasts but did not have the same effect in MDD cells. In fact, there was a slight decrease in overall protein carbonylation in MDD in the galactose condition when compared to the glucose (Figure 2). Furthermore, while GR

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was increased in the galactose condition in control cells, it was not significantly changed in MDD (Figure 4). We speculate that GR is maximally expressed in the glucose condition in MDD, and when galactose is introduced, there is no further increase in oxidative stress. Further exploration of the OXPHOS pathway in MDD is necessary for a more clear understanding of these results and could lead to important implications of the role of oxidative stress in the disease.

The current study was done in peripheral tissue in culture, and the results may or may not be relevant for brain (Teyssier et al., 2011). However, human fibroblasts have been used as a study system for neurological disorders, and they share most signal transduction enzymes and receptors with neurons (Manier et al., 2000). Assuming the connection can be made, the results of this study have important implications for the role of the oxidant anti-oxidant balance in MDD. The human brain metabolizes 20% of the oxygen in the body while only accounting for 2% of the body weight (Dringen et al., 2000; Dringen, 2000). As a result, ROS are generated at high rates, and glutathione is the organ's main protective antioxidant (Dringen et al., 2000; Dringen, 2000).

A future research objective is to identify MDD-relevant proteins that may be consistent targets for modification in the presence of increased oxidative stress. Protein carbonylation most often occurs at specific amino acids proline, threonine, lysine, and arginine (Stadtman, 1993) and can significantly alter or destroy protein activity and function (Oliver, 1987). GR has been shown to be susceptible to oxidation in the presence of oxidative stress (Tbatabaie & Floyd, 1994), and it is important to note that although the results of our study indicate that that GR production is up-regulated in response to oxidative stress in MDD, they do not conclude that the enzymatic function of GR is unaltered. Further studies are needed to make definitive conclusions on the functionality of glutathione and GR in the presence of oxidative stress in MDD fibroblasts as well as the activity of other enzymes such as glutathione s-transferase and glutathione peroxidase.

Other proteins shown to be susceptible to oxidation include PKA and PKC (Humphries et al., 2005; Humphries et al., 2007; Giorgi et al., 2010), important components of signaling pathways that previous studies found to be reduced in MDD fibroblasts (Dwivedi et al. 2004; Perera et al., 2001; Perez et al., 2002; Shelton et al., 2009 A; Pandey et al., 1998; Pandey et al., 1997). The catalytic subunit of PKA is inactivated by thiol oxidation of cysteine 199 in the activation loop of the kinase, particularly in conditions of high oxidation stress (Humphries et al., 2007). PKA typically exists as an inactive heterotetramer, with two regulatory and two catalytic subunits (Beave et al., 1974 A; Beavo et al., 1974 B; Meinkoth et al., 1993). It is activated by the binding of two cyclic AMP molecules to each regulatory subunit, which releases the catalytic subunits leading to phosphorylation of protein substrates, including cyclic AMP response element binding protein (CREB; Meinkoth et al., 1993; Taylor et al., 1992). This PKA activation continues until cyclic AMP is hydrolyzed by phosphodiesterases (Jeon et al., 2005) or the catalytic subunit is inactivated by protein kinase inhibitor (Fantozzi et al., 1994; Wen et al., 1994). The catalytic PKA subunit is susceptible to oxidation primarily in the free, activated state in the cytosol (Humphries et al., 2002).

Looking at MDD through the perspective of oxidative damage and glutathione response could lead to a new approach to treatment. Previous studies have shown an increase in oxidative stress markers and decrease in antioxidants in serum to be correlated with the severity of depression (Yanik et al., 2004; Cumurcu et al., 2009; Sarandol et al., 2007). This correlation poses the possibility of reversing the oxidation by increasing natural antioxidant systems or administering antioxidant drugs to decrease the symptoms of depression. A number of stimuli can induce or inhibit glutathione (Soltaninassab et al., 2000), and some

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selective serotonin reuptake inhibitors (SSRIs) have been shown to behave as antioxidants (Khanzode et al., 2003; Zafir et al., 2009). Standard SSRIs fluoxetine and citalopram, for example, were shown to reverse effects of oxidative damage in serum of MDD subjects (Khanzode et al., 2003). In addition, administering fluoxetine as well as imipramine and venlafaxine, two other SSRIs, to rats with depressed phenotypes was shown to significantly reverse the biochemical effects of oxidation, including protein carbonylation, in brain tissue (Zafir et al., 2009).

Although the increased oxidation has been observed in a number of studies, using biological markers of oxidative stress primarily in blood plasma or serum samples (reviewed by Ng et al., 2008), demonstration of increased oxidation in our cell culture model opens a new area of research. In this cell culture model we will be able to assess the underlying mechanism and specific pathway involved and screen for potential remedies. Further exploration of carbonylated proteins in MDD may lead to the identification of diagnostic (pre-symptomatic) biomarker for oxidative damage and establishment of an effective antioxidant therapy, offering novel approaches to anti-depressant treatment.

Acknowledgments

This work was supported by grant from the Brain & Behavior Research Foundation (NARSAD) and by Vanderbilt Kennedy Center for Research on Human Development.

This research was supported by the Vanderbilt Psychiatric Genotype/Phenotype Project and the Vanderbilt Institute for Clinical and Translational Research (1-UL1-RR024975 NCRR/NIH). We would also like to thank the Vanderbilt Kennedy Center and Drs. Beth Ann McLaughlin and Karoly Mirnics for their assistance in this project.

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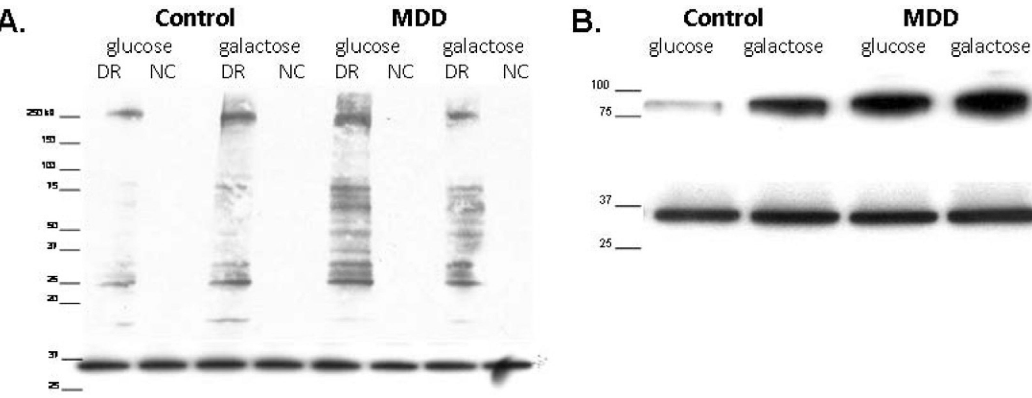

Figure 1.

A) Representative DNPH blot. Western blot probed with DNPH antibody (top) and re-probed with GAPDH antibody (bottom). Blot shows one control-MDD matched pair, derivatized reaction (DR) and negative control (NC) for each sample with molecular weight marker; from left to right lanes read: control in glucose medium, control in galactose medium for 24 hours, MDD in glucose medium, and MDD in galactose medium for 24 hours. Directly below the DNPH blot is the GAPDH restain of the same PVDF membrane displaying equal protein loading. **B) Representative GR blot.** Western blot probed with Glutathione Reductase antibody (top) and re-probed with GAPDH antibody (bottom). Blot shows one control-MDD matched pair with molecular weight marker; from left to right lanes read: control in glucose medium, control in galactose medium, MDD in glucose medium, and MDD in galactose medium. Directly below the GR blot is the GAPDH restain of the same PVDF membrane displaying equal protein loading.

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Figure 2. Protein Carbonylation

Normalized pixel density measurements from stained western blots for each experimental condition. Condition and control or subject are as labeled.

Bars represent standard error.

* = significantly different from the control in glucose condition, p-value 0.05

** = significantly different from the control in glucose condition, p-value 0.01

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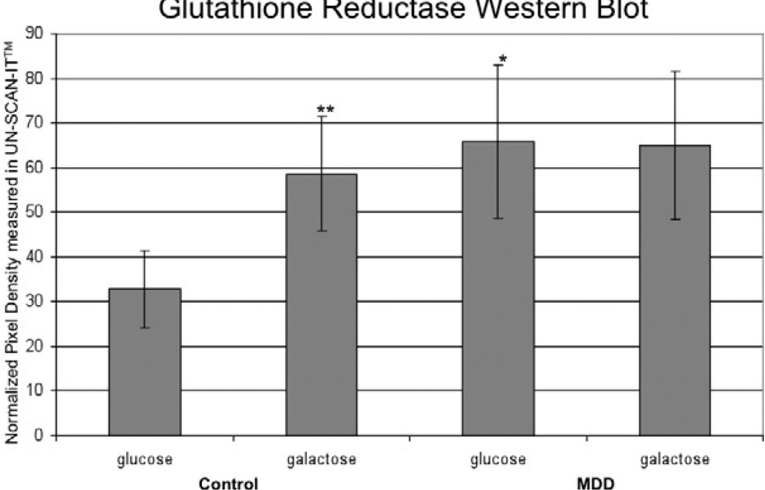

Figure 3. Total Glutathione

Total glutathione concentrations in total cell lysate measured using the enzymatic method and expressed in nM/μg protein. Graph labeling same as in Figure 2. No significant difference was found between any of the groups.

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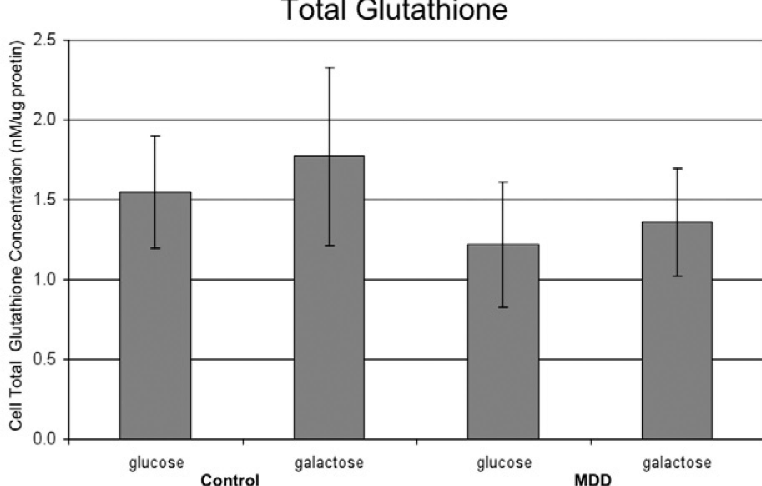

Figure 4. GR Expression

Normalized pixel density measurements from western blots stained with anti-GR antibody for each experimental condition. Graph labeling same as Figure 2.

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Table 1

Study Population

The age, race, sex, and current medical conditions and medications of study participants, both healthy controls and those diagnosed with MDDD. Controls from the left were matched for relative comparisons with members of the MDD group in the same row.

Control					MDD				
Age	Race	Sex	Medical Conditions	Current Medications	Age	Race	Sex	**Medical Conditions	Current Medications
36	W	Male	-	-	33	W	Mala	Diabetes mellitus type 1	*Escitalopram 10 mg.*, Exenatide 20 mcg
34	AA	Male	-	-	33	AA	Male	-	*Venlafaxine 75 mg.*
30	W	Male	Gastroesopahgeal reflux disease	Omeprazole 40 mg.	29	W	Male	-	
48	W	Male	-	-	46	W	Male	-	-
20	W	Female	-	-	22	W	Female	-	*Escitalopram 15 mg.*
27	W	Female	-	Estradiol 24 mg.	27	W	Female	-	*Amitriptyline 25 mg.*
22	W	Female	-	-	22	W	Female	-	*Fluoxetine 40 mg.*
51	AA	Female	-	-	51	AA	Female	Gastroesophageal reflux disease	*Escitalopram 30 mg.*
23	AA	Female	-	-	23	AA	Female	-	-
52	W	Female	-	*Alprazolam 1 mg.*	53	W	Female	C4-C5 hemiated disc, hypertension	*Escitalopram 20 mg. Ziprasidone 160 mg.*, Lisinopril 40 mg.
44	W	Female	Intersticial cystitis	Topiramate 100 mg.	43	W	Female	hypertension	*Escitalopram 10 mg.*, Lisinopril 20 mg. Verapamil 240 mg.
27	AA	Female	Cervical dysplasia		26	AA	Female		
22	W	Female	-	drospirenone/ethinyl estradiol	23	W	Female	-	*Fluoxetine 10mg*
49	W	Female	-	-	52	W	Female	-	*Escitalopram 20 mg. Ziprasidone 20 mg.*
35	W	Female	C5-C6 herniated disc, hypertension	Triamterine/Hydrochlorthiazide	34	W	Female		
40	W	Female	-	-	37	W	Female	-	*Escitalopram 10 mg.* Topiramate 100 mg..

*Abbreviations: W, white; AA, African American

Italicized medications are psychiatric medications

** Non-psychiatric

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Prog. Neuro-Psychopharmacol. & Biol. Psychiat. 1996, Vol. 20, pp. 1159–1170
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PII S0278-5846(96)00103-0

REDUCED INTRAVENOUS GLUTATHIONE IN THE TREATMENT OF EARLY PARKINSON'S DISEASE

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(Final form, July 1996)

Abstract

Sechi GianPietro, Maria G. Deledda, Guido Bua, Wanda M. Satta, Giovanni A. Deiana, Giovanni M. Pes, and Giulio Rosati: Reduced intravenous glutathione in the treatment of early Parkinson's Disease. Prog. Neuro-Psychopharmacol & Biol. Psychiat. 1996. 20, pp. 1159–1170

1. Several studies have demonstrated a deficiency in reduced glutathione (GSH) in the nigra of patients with Parkinson's Disease (PD). In particular, the magnitude of reduction in GSH seems to parallel the severity of the disease. This finding may indicate a means by which the nigra cells could be therapeutically supported.
2. The authors studied the effects of GSH in nine patients with early, untreated PD. GSH was administered intravenous, 600 mg twice daily, for 30 days, in an open label fashion. Then, the drug was discontinued and a follow-up examination carried-out at 1-month interval for 2-4 months. Thereafter, the patients were treated with carbidopa-levodopa.
3. The clinical disability was assessed by using two different rating scale and the Webster Step-Second Test at baseline and at 1-month interval for 4-6 months. All patients improved significantly after GSH therapy, with a 42% decline in disability. Once GSH was stopped the therapeutic effect lasted for 2-4 months.
4. Our data indicate that in untreated PD patients GSH has symptomatic efficacy and possibly retards the progression of the disease.

Keywords: Parkinson's Disease; reduced glutathione.

Abbreviations: Columbia University Rating Scale (CURS); Parkinson's Disease (PD); Patients Global Impressions (PGI); reduced glutathione (GSH); resting tremor (RT); Webster Step-Second Test (W.S.S.T.).

Introduction

The mechanisms underlying dopamine cells death in the zona compacta of substantia nigra in Parkinson's disease (PD) remain unclear. However, current concepts of this process indicate that free radicals generated by oxidation reactions may play a key role (Jenner et al., 1992). Indeed, in postmortem tissues from patients with PD there is evidence for inhibition of complex 1 of the mitochondrial respiratory chain, altered iron metabolism and decreased levels of reduced glutathione (GSH) (Riederer et al., 1989, Jenner, 1993). Of these defence mechanisms implicated in the prevention of free-radical-induced tissue damage, only the reduction in the levels of GSH in substantia nigra appears to be specific to PD (Jenner, 1993, Sian et al., 1994) and, noteworthy, this reduction has been also found in cases of incidental Lewy body disease (presymptomatic PD) (Perry et al., 1982, Sian et al., 1992). In particular, the magnitude of reduction in GSH seems to parallel the severity of PD and, in advanced stages, in the nigra, GSH is virtually undetectable (Riederer et al., 1989). In addition, data from animal studies have shown that an induced GSH depletion in mice produces morphological changes in nigral dopamine neurons resembling those seen in normal aging and in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) neurotoxicity (McNeil et al., 1986). These observations have led us to determine the effect of intravenous (i.v.) GSH in patients with early, untreated PD.

Methods

Patients

After giving informed consent, 9 consecutive patients with idiopathic PD were enrolled in the study. There were 6 men and 3 women, age 66 ± 9 years (mean ± SD) (range, 49 to 77); Hoehn and Yahr stage of parkinsonism 2 ± 0.9 (mean ± SD) (range 1 to 4), with a disability duration of 13 ± 4.5 months (mean ± SD) (range, 8 to 24). The patients were considered eligible for the study, provided that they had not been treated previously with any antiparkinsonian drug or other agents active on the central nervous system including deprenyl or vitamin E. The patients with dementia (Mini-Mental State Examination), or depression (Hamilton Rating Scale) were excluded.

Procedures

GSH was administered i.v. 600 mg in 250 ml saline, as 1-hour infusion, twice daily, at 8.00 A.M. and 4.00 P.M, for 30 days, in an open label fashion. The patients were assessed at baseline and 30 days after the treatment. Then, GSH

was discontinued and, if the patient status improved, a follow-up examination was carried-out at 1-month interval, until the patient's clinical status returned to baseline, or when the patient felt he or she was worsened. Thereafter, the patients were treated with carbidopa-levodopa (25-250 mg), half-tablet three times daily, and a new examination carried-out after 30 days, about two hours after the intake of the drug.

Assessments

On each visit, the clinical disability was assessed according to a modified Columbia University Rating Scale (CURS) (Yahr et al., 1969), and to the Webster Step-Second Test (W.S.S.T.) (scoring method: time in seconds to stand and walk a prescribed course and sit again) (Webster, 1968).

The subscores evaluated at the modified CURS were: speech, hypomimia, tremor at rest, action or postural tremor of hands, rigidity, finger taps, hand movements, pronation and supination of hands, foot taping, arising from chair, posture, gait, balance and hypokinesia.

For the W.S.S.T., three sequential trials were performed for each patient, at baseline and at each control, with a fixed intertrial interval of 15 s. In the tabulation of the results the authors used the mean (± SD) of the three W.S.S.T. values obtained, for each patient, at the beginning of the experiment and after each of the trial periods.

Clinical response was also self assessed by patients according to Patients Global Impressions (PGI) (Guy, 1976). This scale (ranging from 1 = very much better, to 7 = very much worse) was used to assess the change in severity of the disease from the beginning of the study and from the previous visit.

All patients were evaluated by the same examiner throughout the study. On each examination, they were observed over two consecutive days. In addition, in patients with tremor, at baseline and at each examination, at approximately the same time of day, tremograms were recorded using an accelerometer transducer attached to the index finger of the hand and recorded on an EEG polygraph. Tremor frequency (in Hertz) and visual mean amplitude (mean value of tremor estimated visually in microvolts) were measured from the tracings.

Laboratory Assessments

The following laboratory tests were performed at entry and after 30 days of GSH therapy: routine blood chemistries, liver function tests, blood counts, urinalysis and ECG. A chest X-ray and a brain CT, performed without contrast, were conducted in all patients before study entry.

Data Analysis

A statistical analysis of W.S.S.T. values was made, for each patient, by Student's *t* test for paired samples. A nonparametric statistical method was used to compare clinical parkinsonian scores (Wilcoxon Matched-pairs Signed-ranks Test) and the PGI (McNemar's Test). In addition, the sums of clinical parkinsonian scores for each patient, at baseline, were correlated either with the percent improvement calculated through the same scale, or with the percent improvement at W.S.S.T. after GSH therapy. The percentage of change was calculated by the following formula:
prestudy value - treatment value / prestudy value x 100 = % change. The level of significance was $p < 0.05$.

Results

All 9 patients enrolled completed the study. There were no serious complications from i.v infusion of GSH. Two patients during the third week of i.v GSH treatment had fever (axillary, peak temperature, 38.2°C), erithemia of the skin, irritation and hardness at injection site, likely due to infusion thrombophlebitis. The irritation and fever cleared up after 5 days of antibiotic and antiphlogistic therapy. Patient 6 after completion of the wash-out period suffered a thigh-bone fracture. GSH did not induce clinically significant changes in any laboratory test compared with basal conditions. The brain CTs showed a mild cortical atrophy in two patients and no definite abnormalities in seven of them. The frequency of resting tremor (RT) was 5 to 6 Hz. In patient 1, the mean amplitude of RT, compared with the baseline period, was reduced, approximately, by 50% after GSH therapy, and by 25% after carbidopa-levodopa (Fig 1). No definite variations in the mean amplitude of RT were noted in the other patients, in the various sequences of treatment, with respect to baseline, or to the wash-out period. At W.S.S.T. all patients improved significantly after GSH, with respect to baseline (from $p < 0.05$, to $p < 0.01$); instead, after levodopa-carbidopa, only five patients improved significantly, with respect to the wash-out period (from $p < 0.02$, to $p < 0.01$). (Table 1). In our opinion, for the dosages of levodopa-carbidopa and GSH used, the transient improvement induced by these drugs was roughly comparable. The total scores for parkinsonian disability (modified CURS) were significantly lower either after GSH therapy, with respect to baseline ($p < 0.007$), or after levodopa-carbidopa with respect to the wash-out period ($p < 0.01$). (Table 2). A significant improvement after GSH therapy, with respect to baseline, of modified CURS subscores, was evidenced for speech, hypomimia, rigidity,

pronation and supination of hands, foot taping, posture, gait, balance and hypokinesia (from $p < 0.02$, to $p < 0.007$).

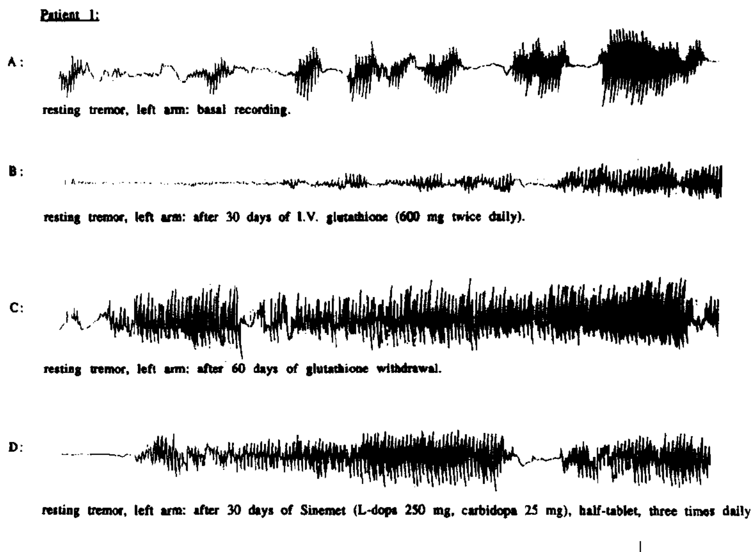

Fig 1. Representative resting tremor recordings in patient 1, at baseline (A); 30 days after therapy with reduced glutathione (B); 60 days after withdrawal of reduced glutathione (C); and 30 days after carbidopa-levodopa (D): Vertical calibration is 200 microvolts; horizontal scale is 1 second.

A similar improvement was noted after carbidopa-levodopa, with respect to the wash-out period (from $p < 0.04$, to $p < 0.01$). As seen from Tables, the values of the modified CURS scores and the values of W.S.S.T., after withdrawal of GSH therapy, reached the baseline values after 2.6 ± 0.7 months (range, 2 to 4 months). The correlation coefficient between total CURS scores at baseline, and the percent improvement calculated through the same scale, after GSH therapy, is shown in Fig 2. In Fig 3, is shown the correlation coefficient between total CURS scores at baseline and the percent improvement at the W.S.S.T. after GSH therapy. The slope of this late correlation is significantly different than zero ($r = 0.6813$; $p = 0.0433$), while the correlation shown in Fig 2 is non-significant.

Table 1

Webster Step-Second Test (W.S.S.T): Scoring Method: Time in Seconds to Stand and Walk a Prescribed Course and Sit Again (mean ± SD, of 3 sequential trials).

Patient	W.S.S.T. At Baseline	W.S.S.T. After GSH	% Improv.$_1$	Wash-out (months)	W.S.S.T. After Wash-out	W.S.S.T. After Lev.+DCI	% Improv.$_2$
1	46.8 ± 1.6	41.2 ± 0.3**	12	3	45.5 ± 0.86	43.0 ± 0.5**	5.5
2	58.7 ± 0.6	57.2 ± 0.6*	3	4	65.7 ± 1.1	63.7 ± 1.1	3
3	34.0 ± 0.1	33.0 ± 0.5*	3	3	37.7 ± 0.58	34.7 ± 0.57**	8
4	34.3 ± 0.5	33.3 ± 0.3*	3	2	42.7 ± 0.58	42.0 ± 0.5	2
5	59.0 ± 1.8	52.0 ± 1.8**	12	2	51.2 ± 0.3	50.0 ± 0.1**	2.5
6	32.9 ± 4.3	25.6 ± 1.5*	22	2	32.3 ± 2.6	-	-
7	44.3 ± 1.15	39.7 ± 0.58**	10	2	45.0 ± 1.2	39.6 ± 1.9***	12
8	51.7 ± 0.8	49.4 ± 1.0*	4.5	2	52.0 ± 1.2	48.2 ± 1.3***	7
9	25.0 ± 0.1	22.7 ± 1.1*	9	3	24.3 ± 0.6	24.0 ± 0.1	1

Lev.+DCI=Levodopa+Decarboxylase Inhibitor; W.S.S.T. values after GSH were compared with baseline; W.S.S.T. values after Lev.+DCI were compared with the wash-out period; *$p<0.05$; **$p<0.01$; ***$p<0.02$ (Student's t Test for paired samples).

Table 2

Modified Columbia University Rating Scale: Total Scores in 9 Patients with Parkinson's Disease Treated with GSH (600 mg/day, I.V.) or Levodopa (375 mg/day + DCI, per os).

Patient	Baseline T. Scores	T. Scores After GSH	% Improv.$_1$	Wash-out (months)	T. Scores After Wash-out	T. Scores After Lev.+DCI	% Improv.$_2$
1	25	15	40	3	24	14	42
2	12	3	75	4	16	3	81
3	29	20	31	3	27	20	26
4	15	7	53	2	18	7	61
5	35	17	51	2	30	14	53
6	42	26	38	2	42	-	-
7	20	15	25	2	21	14	33
8	31	19	39	2	32	21	34
9	34	25	27	3	35	26	26
Mean ± SD	27 ±10	16 ± 8*	42 ± 16	2.6 ± 0.7	27 ± 8	15 ± 7**	44.5 ± 19

Lev.+DCI=Levodopa+Decarboxylase Inhibitor; T. Scores=Total Scores; T. Scores values after GSH were compared with baseline; T. Scores values after Lev.+DCI were compared with the wash-out period; *$p<0.007$; **$p<0.01$; (Wilcoxon Matched-pair Signed-ranks Test).

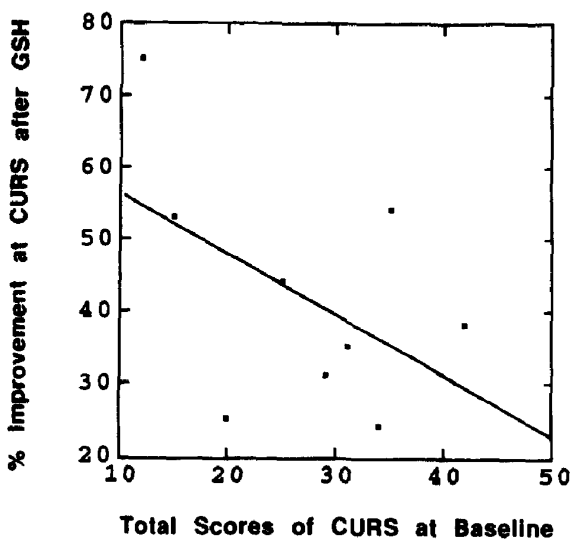

Fig 2. Correlation coefficient between total CURS scores at baseline and the percent improvement calculated through the same scale after therapy with reduced glutathione (r= - 0.5027; p=n.s.).

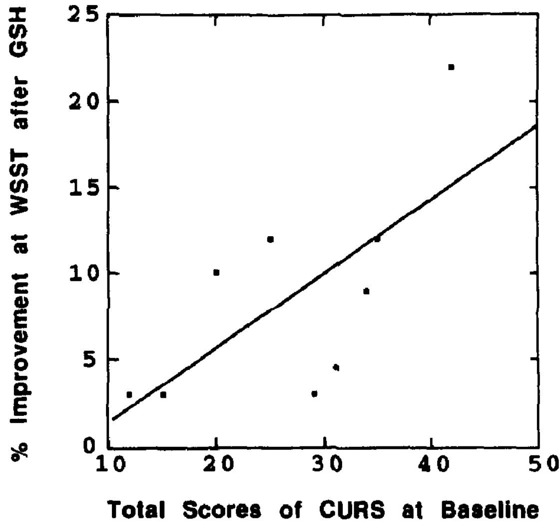

Fig 3. Correlation coefficient between total CURS scores at baseline and the percent improvement at the W.S.S.T. after therapy with reduced glutathione (r=0.6813; p=0.0433).

Total scores of PGI were significantly lower either after GSH therapy, with respect to wash-out period (or, baseline) ($p < 0.0039$) or after levodopa-carbidopa, with respect to the wash-out period ($p < 0.0039$), where, no significant difference was found at PGI between GSH therapy and levodopa-carbidopa. One patient (n.9) with a marked sialorrhea, reported the disappearance of this symptom after GSH therapy. After withdrawal of GSH the benefit lasted for about 3 months. Levodopa-carbidopa therapy was ineffective on sialorrhea in the same patient.

Discussion

The crucial observation, that in idiopathic PD the magnitude of reduction in GSH in substantia nigra seems to parellel the severity of the disease, may indicate a means by which the nigra cells could be therapeutically supported (Riederer et al., 1989).

GSH and the Blood-Brain Barrier

GSH is a tripeptide (gamma-glutamyl-cysteinyl-glycine) which in physiological conditions is believed to be extracted in minimal amount at the blood-brain barrier (Cornford et al., 1978). In addition, as it is a naturally occurring peptide, the possibility exists that there may be a breakdown of glutathione in plasma by peptidases, as in other tissues and at blood-brain barrier itself (Meister and Tate, 1976). Therefore, its clinical value as a therapeutic agent, if administered by a peripheral route, should be minimal. However, since recent investigations support the concept of a selective transcytosis for many peptides across an intact blood-brain barrier (Pardridge, 1986), and since the finding that in idiopathic PD the locus coeruleus, which helps to preserve the integrity of blood-brain barrier functions, is damaged (Tomonaga, 1983, Harik and McGunigal, 1984), the authors administered GSH as 1-hour infusion two times daily for 30 days in PD patients, to investigate a possible therapeutic effect of this peptide, after peripheral administration. Actually, recent experimental evidences have shown blood-brain extraction of circulating GSH in a brain perfusion model, and the transcytosis of intact GSH into the brain parenchyma without breakdown (Zlokovic et al., 1994).

Effects of Intravenous GSH in Parkinson's Disease

The results of our open study indicate that in PD this peptide given i.v. may reach its specific target in the brain (i.e, the nigra cells) and may have a significant beneficial effect on several parkinsonian signs. In particular, as

shown in Fig 3, the therapeutic effect of GSH on hypokinesia appears to be correlated to the severity of the symptom. This peptide was also effective in reducing the RT in one patient, but failed in other four. In this patient, GSH apparently improved the RT more than levodopa-carbidopa. In our opinion, since the dosages of levodopa-carbidopa and GSH used are not comparable, to draw this conclusion is incorrect. Once GSH was stopped the therapeutic effect lasted for 2-4 months. This finding, in our opinion, is a strong evidence against a placebo effect and this may indicate a protective effect of the drug on the rate of progression of PD. However, this does not necessarily exclude a symptomatic effect of GSH. These concepts are supported by the results of two double-blind studies on the use of selegine, or bromocriptine versus placebo in PD (Teychenne et al., 1982, Myllylä et al., 1992). Indeed, in these studies, the mean CURS scores in the placebo group returned to baseline after about 1-month, and the symptomatic effect of bromocriptine, once stopped, did not last for more than four weeks (Teychenne et al., 1982, Myllylä et al., 1992).

Hypothetical Mechanisms Underlying the Therapeutic Effect of GSH

The mechanism underlying the therapeutic effect of GSH in PD is unknown. According to the most basic neurochemical abnormality in the brain of PD patients (i.e., the marked loss of dopamine in the nigrostriatal neuron system) (Ehringer and Hornykiewiez, 1960), an action of GSH at dopaminergic synapses (presynaptically or postsynaptically) can be hypothesized. In particular, based on the theoretical notion that decreasing the oxidative load in substantia nigra may slow disease progression, it would seem that GSH, because of its antioxidant properties (e.g., reduced formation of hydrogen peroxide), may be able to protect the striatonigral cells and foster dopaminergic activity (Jenner, 1993). Recent evidences of glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes fit this hypothesis (Volterra et al., 1994).
Another important biological function that has been ascribed to glutathione is the role in translocation of amino acids, and possibly also peptide, across cell membranes (Meister and Tate, 1976). This function may be important for the transport of substrate and specific proteic neurotrophic factors into the dopaminergic neurons of the substantia nigra (Tooyama et al., 1993). Given the reduction in the levels of GSH in cells of substantia nigra, in PD this function is likely impaired. A replacement therapy with exogenous GSH may contribute to its reinstatement. A controlled study of this peptide for the treatment of PD seems warranted.

Conclusion

The findings indicate that in PD GSH given i.v. may reach its specific target in the brain (i.e., the nigra cells), it has a significant beneficial effect on several parkinsonian signs and possibly retards the progression of the disease.

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LT Hallman:

Thank you for contacting PCCA and NCPA as the nominators of glutathione for inclusion on the 503A Bulk Drug Substances list. Given the relatively short timelines in FDA's request, the information provided here is not to be considered all-inclusive. Please note that glutathione does have a monograph in the current USP/NF, and thus meets the statutory requirement for use as an Active Pharmaceutical Ingredient under section 503A of the DQSA. Below is our response to FDA's questions 1-3 regarding glutathione:

1. PCCA does want to pursue review by the FDA and consideration by the PCAC of glutathione for inclusion on the 503A Bulks list.

2. Compounded glutathione is being nominated for:

Proposed Use	Dosage Form & Strength/ Concentration	Medical Literature
Chemotherapy Induced Neuropathy excluding paclitaxel/ carboplatin regimens	Intravenous Administration Dosed at 1500 mg/m^2	Cascinu S, Cordella L, Del Ferro E, Fronzoni M, Catalano G. Neuroprotective effect of reduced glutathione on cisplatin-based chemotherapy in advanced gastric cancer: a randomized double-blind placebo-controlled trial. J Clin Oncol. 1995;13:26–32 [PMID: 7799029] Cascinu S, Catalano V, Cordella L, *et al.* Neuroprotective effect of reduced glutathione on oxaliplatin-based chemotherapy in advanced colorectal cancer: a randomized, double-blind, placebo-controlled trial. J Clin Oncol. 2002;20:3478–3483. [PMID: 12177109]

		Milla P, Airoldi M, Weber G, Drescher A, Jaehde U, Cattel L. Administration of reduced glutathione in FOLFOX4 adjuvant treatment for colorectal cancer: effect on oxaliplatin pharmacokinetics, Pt-DNA adduct formation, and neurotoxicity. Anticancer Drugs. 2009;20:396–402. [PMID: 19287306] Leal AD, Qin R, Atherton PJ, Haluska P, Behrens RJ, Tiber CH, Watanaboonyakhet P, Weiss M, Adams PT, Dockter TJ, Loprinzi CL; Alliance for Clinical Trials in Oncology. Cancer. 2014 Jun 15;120(12):1890-7. [PMID:24619793]

Proposed Use	**Dosage Form & Strength/ Concentration**	**Medical Literature**
Cystic Fibrosis	Inhalation Dosing was reported at 66 mg/kg. The most common dose in adults was 600 mg.	Bishop C, Hudson VM, Hilton SC, Wilde C. A pilot study of the effect of inhaled buffered reduced glutathione on the clinical status of patients with cystic fibrosis. Chest. 2005;127(1):308-317. [PMID: 15653998] Roum JH1, Borok Z, McElvaney NG, Grimes GJ, Bokser AD, Buhl R, Crystal RG. Glutathione aerosol suppresses lung epithelial surface inflammatory cell-derived oxidants in cystic fibrosis. J Appl Physiol (1985). 1999 Jul;87(1):438-43. [PMID: 10409605] D.Hartl, et al. Inhaled glutathione decreases PGE_2 and increases lymphocytes in cystic fibrosis lungs. Free Radic Biol Med. 2005 Aug 15;39(4):463-72. [PMID:16043018]

3. PCCA has established the stability of glutathione in solution with and without preservative through 90 days. Stability has been confirmed via stability-indicating assay that has undergone method validation in accordance with ICH guidelines. We are also aware of pharmacies performing their own studies using stability-indicating assays as well.

We look forward to providing you further information as requested in the coming weeks.

Sincerely,

Jim Smith
PCCA President

Ronna Hauser, PharmD
NCPA Vice President of Pharmacy Affairs

March 16, 2018

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LT Hallman:

Thank you for contacting PCCA and NCPA as nominators of glutathione for inclusion on the 503A Bulk Drug Substances list.

The information provided here is not to be considered all-inclusive. Some clinicians may have further information that we were not able to collect by the due date requested. Also, please note that glutathione does have a USP monograph, and thus meets the statutory requirements for use as an Active Pharmaceutical Ingredient under section 503A of the DQSA.

Below are our responses to FDA's questions #4 and #5:

4. To the best of our abilities, approximately 30,000 prescriptions of compounded glutathione are estimated to be dispensed per year in the U.S. in the treatment of cystic fibrosis and chemotherapy induced neuropathy.

5. **Chemotherapy Induced Neuropathy**

   There are three professional societies whose position statements are not in favor of glutathione for chemotherapy induced neuropathy. A closer read of these position statements reveals some specific vital limitations to their lack of endorsement.

   The American Cancer Society (ACS) website discusses various ideas for preventing chemotherapy induced neuropathy, including glutathione. ACS states that study results have been mixed and more research is needed.

   The American Society of Clinical Oncology (ASCO) has a statement addressing "Glutathione for patients receiving paclitaxel/carboplatin chemotherapy": "Due to lack of high-quality, consistent evidence, the ASCO guidelines do not recommend any agents for the use in prevention of CIPN."

This same ASCO guidance document points to six randomized clinical trials that evaluated the protective effects of glutathione against platinum-based neurotoxicity with five of these trials reporting a "statistically significant reduction in neurotoxicity, in one form or another, with administration of GSH compared with placebo." The paclitaxel/carboplatin trial was the sixth and most recent, and the ASCO document states "the results of this study suggest that GSH is not an effective agent in the prevention of ***taxane-induced CIPN***." [emphasis added] This seems to form the basis of the ACS statement on mixed study results.

According to the authors of the paclitaxel/carboplatin study (Leal 2014), their results should not be used to dismiss the efficacy of glutathione for CIPN:

> "Although the current results support the finding that glutathione is not an effective agent in the prevention of taxane-induced CIPN when given in combination with CBDCA, these results may not be applicable for cisplatin-induced or oxaliplatin-induced neurotoxicity.
>
> A recently published study by Smith et al [26] supports the view that therapies for chemotherapy-induced neuropathy may be different for different chemotherapy agents. Their report included data from a randomized, double-blind, placebo-controlled, crossover trial that investigated the efficacy of duloxetine for the treatment of established CIPN in a cohort of patients with either taxane-induced or oxaliplatin-induced CIPN. Those authors observed a significant decrease in patient-reported average pain among those who received duloxetine compared with those who received placebo. However, in a subgroup analysis, it appeared that duloxetine was efficacious in patients with oxaliplatin-induced CIPN but not in those with taxane-induced CIPN. This may explain the differences between our current findings and the findings reported previously in other pilot trials that examined oxaliplatin-based or cisplatin-based therapies."

All of the studies cited by ASCO show a lack of glutathione-associated toxicity and lack of interference with antitumor activity.

<u>Cystic Fibrosis</u>

In 2013, the American Journal of Respiratory and Critical Care Medicine published the Cystic Fibrosis Pulmonary Guidelines: Chronic Medications for Maintenance of Lung Health and determined that the evidence is insufficient to recommend for or against chronic use of glutathione for patients 6 years old and up. However, more recent data suggests that chronic use of glutathione does show significant benefits.

> An article by Calabrese, et al. in 2015 reports: "FEV_1 still represents the most important single predictive factor of survival in CF [[24], [25]]. In patients with CF and bacterial chronic infection an annual decline of lung function has been reported [26]. In a recent study, an age-dependent annual decline in percentage predicted FEV_1 was assumed to be between 1% and 3% [27]. We confirmed these data, showing a significant decrease in FEV_1 in the adult placebo group during the study period that did not occur in the GSH group."
>
> A thorough analysis of the literature will support the value of glutathione for patients with cystic fibrosis.

We hope this information aids the Agency in your review of compounded glutathione. If you require further information, please contact us at your convenience.

Sincerely,

Jim Smith
PCCA President

Ronna Hauser, PharmD
NCPA Vice President of Pharmacy Affairs

Tab 2c

FDA Evaluation of Glutathione

DATE: May 2, 2022

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TO: Pharmacy Compounding Advisory Committee

SUBJECT: Evaluation of Glutathione for Inclusion on the 503A Bulk Drug Substances List

I. INTRODUCTION

Glutathione[1] (GSH; L-Glutathione; glutathione-SH; L-glutathione, reduced) has been nominated for inclusion on the list of bulk drug substances for use in compounding under section 503A of the Federal Food, Drug, and Cosmetic Act (FD&C Act). This evaluation considers glutathione for the following[2]:

- Skin lightening
- Cystic fibrosis
- Asthma
- Chronic obstructive pulmonary disease
- Chronic lung disease
- Oxidative stress
- Reduction of the side effects of chemotherapy
- Inhibition of chemical induced carcinogenesis
- Prevention of radiation injury
- Treatment of heavy metal poisoning (cadmium and mercury)
- Acetaminophen toxicity
- Autism spectrum disorder
- Alzheimer's disease
- Parkinson's disease
- Major depressive disorder
- Schizophrenia
- Helicobacter pylori infection
- Human immunodeficiency virus infection
- Tuberculosis
- Otitis media
- Peripheral obstructive arterial disease
- Anemia
- Diabetes
- Septic shock

Glutathione has been proposed to be administered as oral (capsule/troche), sublingual, topical (cream/gel), ophthalmic, nasal spray, rectal, injection (intravenous [IV], intramuscular [IM]), and inhalation preparations.

Oral glutathione is marketed in the United States as a dietary supplement formulated as a capsule, tablet, or liquid. There is not an applicable United States Pharmacopeia (USP) or

[1] Substances/compounds that contain the word "glutathione" but are a different substance/compound than glutathione are not intended to be addressed by this evaluation, e.g., glutathione disulfide (GSSG; oxidized glutathione; ex. BSS Plus NDA 018469), glutathione diethyl ester, glutathione amide, N-acetylglutathione, S-nitrosoglutathioine (GSNO), glutathione synthetase (GSS), glutathione peroxidase (GPx), S-acetylglutathione diethylester, glutathione monoethylester. Also, precursors of glutathione, e.g., N-acetylcysteine, are not intended to be addressed by this evaluation.

[2] Nominators proposed certain broad uses for glutathione: infectious disease, immune enhancement, and immune system protection. We looked at the supporting articles provided by the nominators to inform our understanding of these uses and, as discussed below, focused our evaluation on specific conditions or diseases identified in the supporting articles: helicobacter pylori infection, human immunodeficiency virus infection, and tuberculosis.

National Formulary (NF) drug monograph, glutathione is not a component of an FDA-approved drug.[3]

In evaluating substances considered for inclusion on the list of bulk drug substances that can be used to compound drug products in accordance with section 503A of the FD&C Act (503A Bulks List), FDA considers (1) the physical and chemical characterization of the substance; (2) any safety issues raised by the use of the substance in compounded drug products; (3) the available evidence of the effectiveness or lack of effectiveness of a drug product compounded with the substance, if any such evidence exists; and (4) historical use of the substance in compounded drug products, including information about the medical condition(s) the substance has been used to treat and any references in peer-reviewed medical literature (See 21 CFR 216.23(c)).

We have explained that it is necessary to evaluate a nominated bulk drug substance in the context of the uses proposed for compounded drug products that include the substance,[4] though we acknowledge that inclusion of a substance on the 503A Bulks List may not be limited to a specific use.

We have reviewed publicly available data on the physicochemical characteristics, safety, effectiveness, and historical use in compounding of this substance. For the reasons discussed below, we believe the evaluation criteria weigh against placing glutathione on the 503A Bulks List.

II. EVALUATION CRITERIA

A. Is the substance well-characterized, physically and chemically, such that it is appropriate for use in compounding?[5]

Databases searched for information on glutathione in this section included PubMed, SciFinder, Analytical Profiles of Drug Substances, the European Pharmacopoeia, British Pharmacopoeia, and Japanese Pharmacopoeia, and USP/NF.

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[3] FDA understands that the "FDA-approved irrigation product" containing glutathione disulfide referred to in the 503A renomination template submitted by McGuff Compounding Pharmacy Services, Inc. et al. is the approved BSS Plus ophthalmic irrigation solution (NDA 018469) that contains oxidized glutathione (i.e., each mL contains glutathione disulfide 0.184 mg and several other active ingredients). Another nominator claimed that the "salt form (glutathione disulfide)" is contained within two FDA approved products: Endosol Extra (Application Number [sic] N020079) and BSS Plus (Application Number [sic] N018469)." However, glutathione disulfide (GSSG) is not a salt of glutathione (it is a dimer of glutathione) and GSSG is not the same substance as glutathione. The UNII Code for glutathione is GAN16C9B8O, while the UNII Code for glutathione disulfide is ULW86O013H.

[4] See, for example, the final rule titled List of Bulk Drug Substances That Can Be Used To Compound Drug Products in Accordance With Section 503A of the Federal Food, Drug, and Cosmetic Act, 84 FR 4696.

[5] Among the conditions that must be met for a drug compounded using bulk drug substances to be eligible for the exemptions in section 503A of the FD&C Act is that the bulk drug substances are manufactured by an establishment that is registered under section 510 of the FD&C Act and that each bulk drug substance is accompanied by a valid certificate of analysis. Sections 503A(b)(1)(A)(ii) and (iii). A bulk drug substance is deemed to be adulterated if the methods used in, or the facilities or controls used for, its manufacture, processing, packing, or holding do not conform to or are not operated or administered in conformity with current good manufacturing practice. Section 501(a)(2)(B).

Figure 1: Chemical structure of glutathione

Glutathione is an endogenous tripeptide which plays many important roles in cellular functions. The compound can also be synthesized exogenously.

1. *Stability of the active pharmaceutical ingredient (API) and likely dosage forms*

As a solid, glutathione is stable at room temperature when carefully kept away from oxygen. According to the revised Generally Recognized as Safe (GRAS) notices regarding glutathione for use as a food ingredient,[6] glutathione is stable when kept in an airtight container at room temperature and normal relative humidity levels for up to 39 months. Only 65 - 80% of glutathione remains unchanged in aqueous solutions with various pH values after 7 days at room temperature. The instability of glutathione in solutions may be due to the rapid oxidation of the thiol group into a disulfide group (Harbin et al. 2004). However, with proper formulation techniques, sufficient stability of the aqueous formulations can also be achieved. For example, a reduced glutathione solution at an initial concentration of 189 mg/ml was stored under 5 °C at pH 6.4 (0.005M octylammonium orthophosphate buffer) for 112 days. No decrease in the concentration of the reduced glutathione was observed (Harbin et al. 2004). Therefore, glutathione is likely to be stable under room temperature in its solid formulations (capsules, oral and sublingual troche, etc.) when protected from oxygen. Similarly, with protection from oxygen and proper formulation techniques (e.g., proper buffer solutions, controlled pH and temperature), the substance can be stable when compounded as liquid formulations (such as injection and oral solutions) and semi-solid formulations (e.g., creams, gels, etc.).

2. *Probable routes of API synthesis*

Glutathione can be synthesized from yeast fermentation, followed by centrifugation, complexation, ultrafiltration, ion exchange, washing and recrystallization (Li et al. 2004).

3. *Likely impurities*[7]

[6] FDA GRAS Notices available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&sort=GRN_No&order=DESC&startrow=1&type=basic&search=glutathione. Accessed Apr 18, 2022.

[7] This evaluation contains a non-exhaustive list of potential impurities in the bulk drug substance and does not address fully the potential safety concerns associated with those impurities. The compounder should use the information about the impurities identified in the certificate of analysis accompanying the bulk drug substance to evaluate any potential safety and quality issues associated with impurities in a drug product compounded using that bulk drug substance taking into account the amount of the impurity, dose, route of administration, and chronicity of dosing.

Likely impurities include:

- Residual starting materials and reaction intermediates from fermentation
- Bioburden, such as residual yeast
- Glutathione disulfide from the oxidation of glutathione

4. *Toxicity of those likely impurities*

The impurities mentioned above are unlikely to be present at a highly toxic level.

5. *Physicochemical characteristics pertinent to product performance, such as particle size and polymorphism*

Glutathione is a white solid that is soluble in water. No further information on the influence of particle size and polymorphism on bioavailability were found in the literature.

6. *Any other information about the substance that may be relevant, such as whether the API is poorly characterized or difficult to characterize*

Glutathione is easily characterized with proton nuclear magnetic resonance spectroscopy, Carbon-13 nuclear magnetic resonance spectroscopy, Fourier transform infrared spectroscopy , and mass spectrometry.

Conclusions: Glutathione is an endogenous tripeptide that can also be synthesized in well-developed protocols and is easily characterized with various analytical techniques. Glutathione is likely to be stable when compounded as solid or liquid products with proper formulation and storage conditions. When compounded as aqueous solutions and semi-solid formulations, proper formulation and storage techniques, (controlled pH, protection from air and storage temperature) are needed to achieve sufficient stability. Ordinary storage conditions are acceptable to provide stability for solid products, if they are protected from air. Based on the available information, glutathione is well characterized. The solid form of glutathione is likely to be stable under room temperature storage when protected from oxygen. Additionally, with protection from oxygen and proper formulation techniques (e.g., proper buffer solutions, controlled pH and temperature) the substance can be stable when compounded as liquid formulations (such as injection and oral solutions) and semi-solid formulations (e.g., creams, gels, etc.).

B. Are there concerns about the safety of the substance for use in compounding?

1. *Nonclinical assessment*

The following databases were searched in the preparation of this section: PubMed, National Toxicology Program website, Embase, Web of Science, ToxNet, NIH dietary supplement label database, Google, GRAS notice inventory, and Drugs@FDA.

a. General pharmacology of the drug substance

Glutathione is a tripeptide (γ-L-Glutamyl-L-cysteinyl-glycine) that is endogenously synthesized in the human body. Although glutathione is synthesized from precursor amino acids in virtually all cells, the liver is the main source of plasma glutathione. It exists in two forms: the oxidized (glutathione disulfide, GSSG) and the reduced (glutathione, GSH) forms (Figure 2, Lomaestro and Malone 1995). Glutathione is also found in the brain, spinal cord, glial cells, and neuronal intracellular spaces. Most glutathione clearance from plasma occurs in the kidneys and liver (Cronkite et al. 1951).

Figure 2. Synthesis and Breakdown of Glutathione (Lomaestro and Malone 1995)

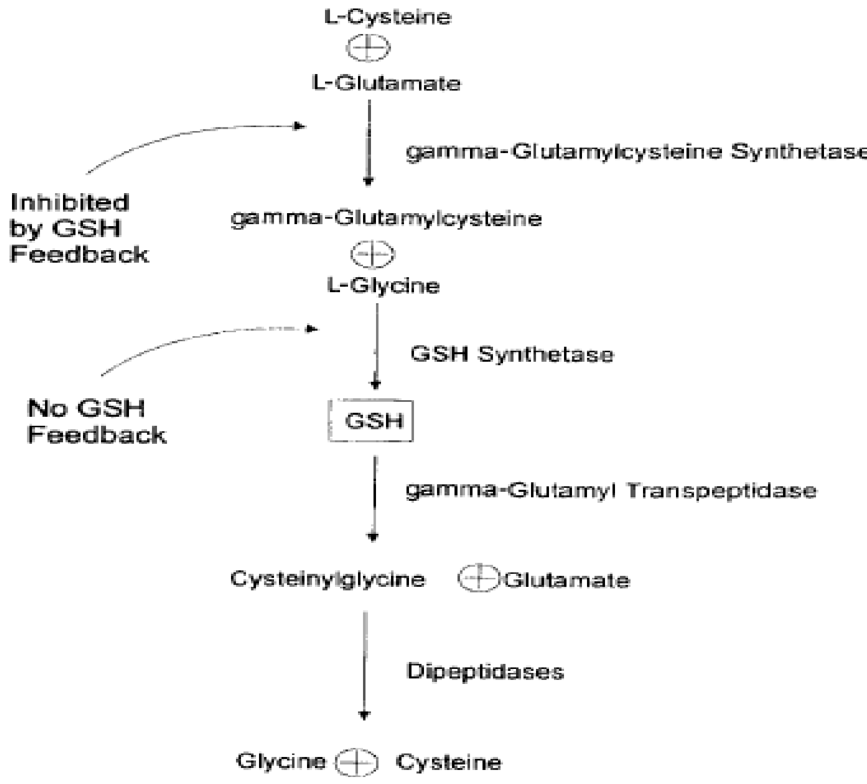


Figure 1. Synthesis and breakdown of glutathione (GSH).

When intracellular levels of glutathione decrease, exogenous uptake of glutathione takes place in various epithelial cells (e.g., enterocytes, alveolar cells, renal proximal globular cells, endothelial cells and retinal pigmented cells) to support the function of glutathione-dependent metabolic reactions. In contrast to epithelial cells, other cell types which do not have a direct transport capacity for intact glutathione, synthesize glutathione either by transporting the three amino acids (cysteine, glutamine and glycine) into the cells or by transpeptidase activity at the cell surface (Ballatori et al. 2009).

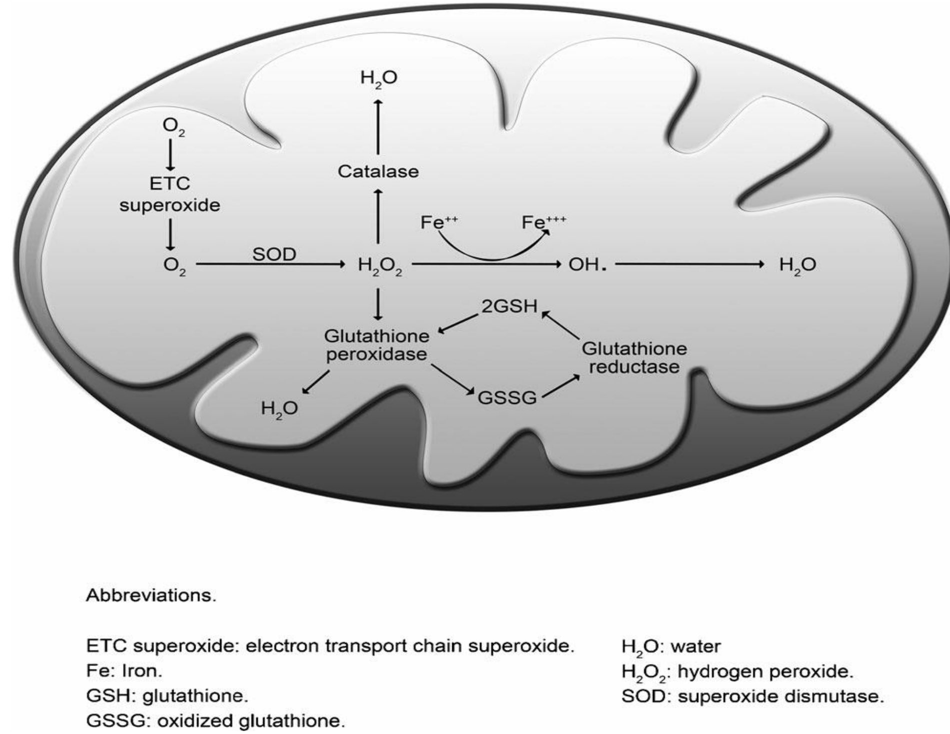
The main function of glutathione is as an antioxidant. Glutathione is an essential cofactor for numerous enzymes including the glutathione peroxidases and glutathione *S*-transferases in reactions that occur to inactivate various exogenous toxins (e.g., xenobiotics, environmental toxins, pharmaceuticals). See Figure 3. These toxins include reactive nitrogen species, reactive oxygen species, hypochlorous acid, hydroxy radicals and peroxides which are formed during cellular metabolism. As an example of glutathione detoxification of peroxides, glutathione interacts with the enzyme glutathione peroxidase in a reaction that converts glutathione to oxidized glutathione. GSSG can be reduced by the enzyme glutathione reductase in the presence of reduced nicotinamide adenine dinucleotide phosphate to regenerate glutathione. See Figure 4.

Figure 3. Glutathione Antioxidant Activity (Morris et al. 2014a)

Abbreviations.

B12: vitamin B12.
BHMT: betaine homocysteine methyltransferase.
DMA: dimethylarsinic acid.
DP: dipeptidase.
GGT: γ-glutamyltransferase.
GSH: glutathione.
GSSG: oxidized glutathione.
InAs: inorganic As.
MMA: monomethylarsonic acid.
MTR: methionine synthetase.
SAM: S-adenosylmethionine.
SAH: S-adenosylhomocysteine.
THF: Tetrahydrofolate.

Figure 4. Cellular Functions of Glutathione (Morris et al. 2014a)



Other cellular functions of glutathione include regulation of cellular differentiation, proliferation, and apoptosis (Meister 1992). Disturbances in glutathione homeostasis (e.g., reduced levels of glutathione or a disproportionate ratio of glutathione to GSSG) have been suggested to be associated with the development or progression of human diseases (Ballatori et al. 2009).

Glutathione in its oxidized and reduced forms exerts most of its effects in the central nervous system (CNS) by interacting directly or indirectly with glutamate receptors. Each of the three amino acids (glutamate, cysteine, and glycine) which form glutathione can interfere with glutamate-mediated neurotransmission through either direct or indirect receptor modulations; the glutamate group can bind to glutamate receptors, the cysteine-based thiol group affects the redox state of those receptors, while glycine is an N-methyl D-aspartate receptor agonist. (Morris et al. 2014a). These interactions play a role in the functioning of the CNS.

b. Pharmacokinetics (PK)/Toxicokinetics (TK)

Absorption:

When glutathione was orally administered in rats either as a liquid bolus (30 µmol) or in addition to food (2.5-50 mg/g of food), an increase in glutathione plasma concentration from 15 to 30 µM was seen with levels peaking at 90-120 min after its administration and remaining high for over 3hrs. Administration of the amino acid precursors of glutathione did not impact plasma glutathione levels. When glutathione synthesis was inhibited by L-buthionine- [S,R]-sulfoximine (an inhibitor of glutathione synthesis) and acivicin (an inhibitor of glutamyltransferase), the increase in plasma glutathione levels resulted mostly from absorption of intact glutathione rather than via its metabolism. Plasma protein bound glutathione also

increased after glutathione administration, with a time course similar to that observed for free plasma glutathione. Under the conditions of this study in rats, supplementation of dietary glutathione was absorbed and resulted in an increase in blood plasma glutathione (Hagen et al. 1990).

Distribution:

Cronkite et al. (1951) administered glutathione via injection in mice (subcutaneous, SC, 5 mg/kg) and rats (IV, 5 mg/kg). Under the conditions of this study, glutathione accumulated in the liver and spleen in mice (measured at 45 minutes after the injections) and in liver, spleen, and kidneys in rats (30 minutes after the injection). See Table 1.

Table 1. Distribution of Injected Glutathione (Cronkite et al. 1951)

TABLE I. Distribution of Injected Reduced Glutathione in the Tissue of Mice and Rats. Each value is the mean of 6 animals in mg/100 g of tissue.

Mice	Controls	45′ after 5 mg per g of mouse subcut.
Blood	39	44
Liver	344	435
Spleen	275	322
Rats		30′ after inj. 5 mg/g i.v.
Blood	41	48
Liver	338	476
Spleen	288	376
Testicles	293	292
Small intestine	278	295
Thymus	276	312
Muscle	56	63
Kidneys	205	310

The level of reduced glutathione was measured in dogs where glutathione was injected intravenously (either 1 g or 2 g) either alone or with cysteine (either 1 or 2 g). In the absence of cysteine, the mean glutathione plasma concentration was 35.7±7.2 mg/100 mL of blood.
The pharmacodynamic effects in the treated dogs (accelerated pulse, rapid respiration) decreased within 5 min after injection.

Clearance:

Glutathione was cleared within about 30 min post injection under the conditions of the study described above. See Figure 5.

Figure 5. Glutathione Blood Levels in Dogs (Cronkite et al. 1951)

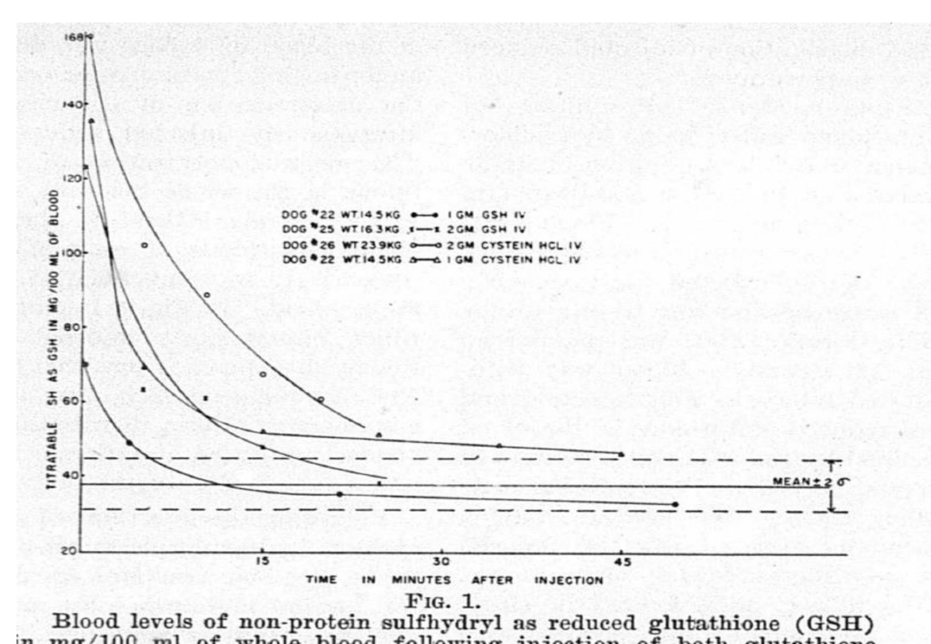


FIG. 1.
Blood levels of non-protein sulfhydryl as reduced glutathione (GSH) in mg/100 ml of whole blood following injection of both glutathione (pH 6.5) and cysteine (pH 6.5) intravenously in dogs.

c. Animal Models of Glutathione Effects

The information below summarizes the authors' conclusions of several published articles that were either submitted by nominators or located in the literature for the nonclinical evaluation of glutathione as it relates to its potential protective effects against various toxicities.

- Animal model of cisplatin-induced neurotoxicity: When glutathione (500 mg/kg) was intravenously administered to Wistar rats (twice weekly) five minutes before low dose cisplatin was injected (intraperitoneally at 1 mg/kg for 10 weeks), a decrease in neuropathy in the glutathione-cisplatin treatment group was observed as compared to control animals treated with cisplatin and placebo. Similar results were obtained when the same dose of glutathione was used in combination with a high dose of cisplatin (2 mg/kg for 5 weeks of treatment followed by 5 weeks of recovery) where glutathione was associated with a decrease in neuropathy. The authors concluded that glutathione prevented cisplatin-induced neuropathy under the conditions of this study and that it should investigated further in the clinic (Hamers et al. 1993).

- Animal model of detoxification: The effect of glutathione on detoxification of the insecticide, methyl parathion (MP) was investigated in a rat model (Jiang et al. 2010). Manifestations of MP acute poisoning are similar in humans and animals and include reduced cholinesterase levels in brain, erythrocytes, and plasma; clinical signs of neurological effects (e.g., tremors and convulsions); cardiac arrhythmia and liver toxicity (short term effects include disruption of liver plates, hepatocytes swelling, and deformity; long term effects include extensive cell fatty degeneration and chronic hepatic injury).

Because glutathione has a favorable effect on ischemic hepatic injury, this study was conducted to investigate whether exogenous glutathione can protect the liver from MP- induced injury using a single intragastric dose of 15mg/kg MP. Endpoints measured included plasma levels of acetylcholinesterase, glutamate pyruvate transaminase, glutamic oxalacetic transaminase in plasma, superoxide dismutase, and glutathione peroxidase (GPx) in the liver; as well as the histopathological assessment of the liver. Under the conditions of this study, the liver injuries were attenuated by glutathione treatment (600 mg/kg or 1200 mg/kg, intravenously) at 6 hours or 24 hours after MP poisoning.

- Treatment of acetaminophen poisoning: Transgenic mice overexpressing superoxide dismutase or plasma glutathione peroxidase, both of which are antioxidants which act as scavengers for free radicals, were resistant to acetaminophen toxicity. IV injection of glutathione peroxidase[8] provided normal mice with high level of protection against a lethal dose of acetaminophen. However, transgenic mice overexpressing intracellular GPx in the liver were significantly more sensitive to acetaminophen toxicity compared with non-transgenic littermates. This sensitivity appears to be due to the inability of these animals to efficiently recover glutathione which was depleted as a result of acetaminophen metabolism (Mirochnitchenko et al. 1999).

- Prevention of cadmium poisoning: In mice, pretreatment with glutathione provided no protection to an injection of 20 µmol/kg cadmium; however, pretreatment with glutathione monoisopropyl ester (10 mmol/kg) prevented mortality (No author. Nutrition Reviews 1988).

d. Acute toxicity[9]

A subcutaneous injection of glutathione (5g/kg) was lethal in mice. A single IV injection of glutathione in dogs (1g or 2g) caused accelerated pulse and rapid respiration, which were recovered 5 min after the injection (Cronkite et al. 1951).

e. Repeat dose toxicity[10]

A 26-week IV toxicity study was conducted in dogs using doses of 30, 100, and 300 mg/kg/day glutathione sodium. The text of the article was written in Japanese with the exception of the tables and figures which were written in English. According to the tables, glutathione sodium treatment is not associated with any effect on the body weight or food consumption of the treated dogs. No other data were captured from this study (Suzuki et al. 1972).

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[8] Glutathione peroxidase-1 (GPx-1) is an intracellular antioxidant enzyme that can scavenge free radicals by enzymatically reducing hydrogen peroxide to water to limit its toxicity.

[9] Acute toxicity refers to adverse effects observed following administration of a single dose of a substance, or multiple doses given within a short period (approximately 24 hours). Endpoints captured in acute toxicity studies usually include mortality and gross clinical observations. Acute toxicity data are usually superseded by data obtained from longer term toxicity studies.

[10] Repeated-dose toxicity studies consist of in vivo animal studies that seek to evaluate the toxicity of the test substance by observing the changes that emerge in clinical observations, clinical chemistry, gross pathology, and histology endpoints when the test substance is repetitively administered daily for a predetermined period of time.

No other repeat dose or chronic toxicity data were found for glutathione.

f. Genotoxicity[11]

Glutathione was negative in the Ames test in the absence of metabolic activation; however, in the presence of rat kidney S9 microsomal fractions (10-20mM), an increase in the number of revertants was seen in the *Salmonella typhimurium* TA100 strain (Glatt et al. 1983). Further studies have shown that a steep dose-response profile was obtained for glutathione where concentrations below 10^{-3}M were almost inactive whereas slightly higher concentrations were lethal to cells in culture (Thust and Bach, 1985). Further examination of the dose-response curve showed that the culture medium used in the experiment had an impact on the toxicity profile of glutathione. For instance, when glutathione was added to the Chinese hamster cell strain, V79-E cells (at concentrations 2.5 X 10^{-4} and 10^{-3}M) for 1 hr, it induced a dose-dependent cell cycle delay, sister chromatid exchanges and clastogenic damage. Peak damage was obtained at 24 hours post-treatment. Glutathione concentrations above 10^{-3}M were lethal. The highest tolerated dose corresponded to the intracellular glutathione level in this cell line. When a different culture medium was used (Hank's solution instead of minimum Essential Medium solution), none of the toxicities mentioned above were seen at similar concentrations of glutathione. See Table 2.

Table 2. Genotoxicity Timeline (Thust 1988)

TABLE 3
Time Course of Clastogenicity, Polyploidization and Mitotic Inhibition Induced by 10^{-3}M Glutathione in Complete Eagle's MEM

Post-treatment	Aberrant Metaphases	Aberrations									4 n Metaphases	MI (%)
		G	B′	B″	Exch.	Dic.	R.	Extr.	PCC	End.		
4 h	7%	6	1	—	—	—	—	—	—	—	2%	1.5
12 h	23%	2	8	1	21	2	3	1	—	—	4%	2.9
24 h	37%	1	4	10	20	3	3	12	3	1	5%	6.7
36 h	30%	4	2	6	2	—	3	16	9	—	4%	9.8
48 h	27%	2	1	3	2	3	1	12	11	—	7%	12.5
72 h	15%	3	—	—	4	4	—	2	3	—	11%	5.9
Control, 24 h	1%	1	—	—	—	—	—	—	—	—	3%	26.8

MI, mitotic index; for further abbreviations, see Table 1.

Further testing showed that the critical cofactor for glutathione genotoxicity was the presence of cystine in the culture media as no cytotoxicity was seen in culture media which lacked this amino acid (Thust 1988). The dependence of mutagenicity of glutathione on cysteine was confirmed using a different cell type, TA102 by other investigators. See Table 3.

[11] The genotoxicity assessment battery usually consists of a gene mutagenicity assay (for single dose trials) and a variety of clastogenicity/genotoxicity assays. To support multiple dose administration in humans, additional genotoxicity testing assessment is usually conducted to detect chromosomal damage in mammalian systems.

Table 3. Mutagenicity of Glutathione with Cysteine (Stark et al. 1989)

MUTAGENICITY OF THIOLS AT pH 7 IN THE PLATE TEST

µmoles/plate	Rev./plate	Net	µmoles/plate	Rev./plate	Net
GSH, $pK_{a(SH)} = 8.56$			L-Cysteine, $pK_{a(SH)} = 8.15$		
0	233 ± 22	–	0	233 ± 22	–
25	307 ± 24	74	10	312 ± 13	79
50	335 ± 20	102	20	391 ± 58	158
75	356 ± 26	123	30	789 ± 44	556
			40	1105 ± 123	872

Values are mean+/- S.D. of triplicate plates. Net revertants/mole for glutathione was 2.3

Glutathione was negative in the mouse lymphoma assay when tested at 50-10,000 µg/mL (Seifried et al. 2006).

g. Developmental and reproductive toxicity[12]

An embryofetal study was conducted where pregnant mice (gestational days [GD] 6-13) and rabbits (GD 8-16) were treated with glutathione sodium (mouse doses: 100, 300, and 1000 mg/kg/day; rabbit doses: 30 and 300 mg/kg/day) (Suzuki et al. 1972). The text of the article was written in Japanese except for the tables and figures which were written in English. According to the tables, there were no effects on body weight gain, fertility rates, male to female ratio of newborn pups, fetal survival, body weight of fetuses or malformations. The only exception was in the mouse study where 2 cases of cleft palate were seen; 1 case was seen in the 100 mg/kg/day group and another in the 1000 mg/kg/day group.

No other toxicity studies were found that evaluate the toxicity of exposure to glutathione during sensitive periods of reproduction and development in animal models.

In a mouse model where the effect of glutathione on embryos pretreated with thibendazole[13] (TBZ) on GD 9 of embryogenesis was tested, exposure to glutathione alone did not seem to be associated with an increase in embryonic adverse effects (see Table 4 below, column of glutathione 800 mg/kg and 0 TBZ). However, because no placebo control was included in this study, and because there were a few abnormalities recorded in the glutathione group alone (abnormalities consisted of 5 external findings: 2 cleft palate, 2 exencephalia, and 1 open eyelids and 2 skeletal malformations: 1 fused vertebral arches, 1 fused vertebral bodies and 1 fused rib), it is not possible to conclude whether glutathione is associated with an increase in malformations on GD9 mouse embryo. See Table 4.

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[12] Developmental and reproductive toxicity studies are usually designed to assess the potential adverse effects in humans of both sexes and include females from various age groups that will be exposed to the proposed substance. *Developmental toxicity* or *teratogenicity* refers to adverse effects (can include embryo-fetal mortality, structural abnormalities, functional impairment, or alterations to growth) and can occur in pups either as a result of the exposure of their parents to the substance, prior to the pups' birth, or by direct exposure of the pups to the substance after birth.

[13] Thiabendazole is a broad spectrum anthelmintic agent used predominantly in treatment of intestinal pinworm and strongyloides infection.

Table 4. Reproductive Toxicity (Ogata et al. 1989)

Table 2. Effects of pretreatment with glutathione on foetal malformations induced by treatment with thiabendazole on day 9 of gestation

Observation	GSH dose (mg/kg body weight) . . . / TBZ dose (mg/kg body weight) . . .		Incidence of malformations		
	GSH dose	800	0	400	800
	TBZ dose	0	1000	1000	1000
External malformations					
No. of litters with malformed foetuses/no. examined (%)		3/18 (16.7)	6/12 (50.0)	7/13 (53.8)	9/13 (69.2)
Malformations (%)†		2.2 ± 5.6	23.9 ± 31.8	25.5 ± 33.8	28.3 ± 34.6
No. of malformed foetuses/no. examined		5/230	11/73	20/108	25/99
No. of foetuses with:					
Reduction deformity of limbs		0	9	5	20
Short or non-tail		0	1	16	9
Anal atresia		0	0	1	4
Cleft palate		2	2	1	3
Exencephalia		2	0	0	0
Open eyelids		1	0	2	3
Skeletal malformations					
No. of litters with malformed foetuses/no. examined (%)		2/18 (11.1)	9/12 (75.0)	10/13 (76.9)	11/13 (84.6)
Malformations (%)†		0.8 ± 2.4	41.3 ± 36.4	42.4 ± 42.1	46.4 ± 34.6
No. of malformed foetuses/no. examined		2/230	21/73	37/108	44/99*
No. of foetuses with:					
Fusion of vertebral arches		1	14	35	37
Fusion of vertebral bodies		1	9	15	17
Fusion of ribs		1	3	9	10
Reduction deformity of skeleton antebrachii		0	8	4	7

GSH = glutathione TBZ = thiabendazole

†Calculated by averaging the percentage in each litter (i.e. no. of malformation/no. of foetuses), and shown as mean ± 1SD.
The value marked with an asterisk differs significantly from the corresponding value for treatment with TBZ alone (*$P < 0.05$).

h. Carcinogenicity[14]

A buccal hamster pouch model was used where glutathione (10 mg/kg dissolved in 0.5 mL mineral oil) was administered 3 times weekly to hamsters (n=10). The study results show that glutathione inhibited experimentally induced oral carcinogenesis in glutathione-treated hamsters compared to control hamsters (n=20) (Schwartz and Shklar 1996).

Administration of glutathione (100 mg, oral) and selenium (0.1 mg, oral) to Wistar rats inhibited/reduced the carcinogenic action of benzo[a]pyrene(BaP)-induced carcinogenesis compared to a control group. However, glutathione administration alone did not reduce the incidence rate of tumors in BaP-exposed rats. See Table 5.

Table 5. Carcinogenicity Potency (Charalapoulous et al. 2004)

Table 1. Tumor incidence, treatment, mean survival time, carcinogenic and anticarcinogenic potency, and p-value in the 3 groups studied

Group	*No. of animals*	*Tumor incidence (%)*	*Treatment*	*Mean survival time (days)*	*CP*	*AP*	*p-value (<0.001)*
G I	42	100	BaP	183.8±28	54.3		I *versus* II
G II	38	97.4	BaP+vit C	238.4±31	41.2	13.1	I *versus* III
G III	20	100	BaP+Se+GSH	344.9±48	28.9	25.4	II *versus* III

BaP: benzo[a]pyrene; vit C: ascorbic acid; Se: selenium; GSH: glutathione; CP: carcinogenic potency; AP: anticarcinogenic potency

[14] Studies that assess cancer risk in animals are used as predictive tools to evaluate the potential for drugs to result in tumors when used by humans on a chronic basis. Carcinogenicity studies are conducted if the clinical use is expected to be continuous for a minimum of 6 months of life, or if intermittent clinical use is expected to total 6 months or more of life.

Conclusions: Glutathione is a tripeptide that is endogenously synthesized in the human body. The pharmacology and metabolism of glutathione is well understood. However, insufficient nonclinical data exist to evaluate the toxicity profile of glutathione in repeat dose toxicity or developmental toxicity. Available acute toxicity studies in animals show that high levels of glutathione are tolerated. Available genotoxicity data from the Ames assay and the mouse lymphoma assay show that glutathione is not mutagenic in the absence of metabolic activation. Glutathione inhibited experimentally induced oral carcinogenesis in glutathione-treated hamsters.

Glutathione has been tested for its potential protective activity in animal models of diseases. Publicly available literature has reported on the potential use of glutathione in animal models. These include a link between glutathione dosing and a decrease in neuropathy in cisplatin treated rats when compared to control animals. In another animal model, IV injection of GPx was reported to provide mice with protection against a lethal dose of acetaminophen. The applicability of these findings to the clinical setting is not known at this time.

2. *Human safety*

The following databases were consulted in the preparation of this section: PubMed, EMBASE, FDA Adverse Event Reporting System (FAERS), The Center for Food Safety and Nutrition (CFSAN) Adverse Event Reporting System (CAERS), Cochrane Database of Systematic Reviews,[15] and ClinicalTrials.gov.[16]

a. Reported adverse reactions (FAERS, CAERS, and other regulatory Agencies)

FAERS

The FAERS database was searched from January 1, 2000 through December 31, 2021, and identified 15 United States (U.S.) cases associated with reduced glutathione. Two non-U.S. cases were also identified.

Anaphylaxis/hypersensitivity: Seven cases have reported "anaphylaxis" or "anaphylactic shock" (2: one in U.S. and one in China) or hypersensitivity (5). Five of these cases reported that the glutathione was administered via the IV route, one reported the inhalation route and one the oral

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[15] A search of the Cochrane Database of Systematic Reviews on September 13, 2018 (updated February 10, 2022), revealed four reviews with at least one clinical trial that included glutathione as a treatment. The following are the latest publication dates for these four reviews:

a) Nebulized and oral thiol derivatives for pulmonary disease in cystic fibrosis by Tam et al. published July 12, 2013.
b) Antioxidant supplementation for lung disease in cystic fibrosis by Ciofu and Lykkesfeldt published August 7, 2014.
c) Interventions for preventing neuropathy caused by cisplatin and related compounds by Albers et al. published March 31, 2014.
d) Chelation for autism spectrum disorder (ASD) by James et al. published May 11, 2015.

[16] A search of the ClinicalTrials.gov (available at: https://clinicaltrials.gov) database on September 13, 2018 (updated February 10, 2022) revealed 34 different clinical trials that listed the use of glutathione as an intervention. It should be noted that the number of interventions being evaluated in each individual study ranged from one to nine different interventions. If a glutathione study discussed in this review was located on the ClinicalTrials.gov website, the ClinicalTrials.gov study number was provided, ex. NCT00506688.

route. Each case reported a different reason for use (i.e., Alzheimer's disease, Chronic Obstructive Pulmonary Disease (COPD), Lyme disease, prevention of hepatoxicity, pruritus, and rectal cancer). The time to onset for the two anaphylaxis cases were 30 minutes and less than 24 hours. Four cases reported improvement of the adverse events (AEs) after discontinuation of glutathione.

The anaphylactic reaction that occurred in the U.S. was in a 19 year old female patient with a latex sensitivity who received an unknown dose of glutathione IV for the treatment of Lyme disease. She experienced the anaphylactic reaction 30 minutes after starting the infusion and was treated with epinephrine, diphenhydramine IV and steroids IV. The day following the initial anaphylactic reaction to glutathione, the patient experienced anaphylaxis to ceftriaxone IV, despite previously tolerating seven weeks of ceftriaxone. Ten days after the initial anaphylactic episode, the patient was re-challenged with an unknown dose of IV glutathione and she had anaphylaxis that required cardiopulmonary resuscitation. The source of the glutathione was not disclosed.

The anaphylaxis case that occurred in China was in a 55 year old male patient who developed pharyngeal edema, cyanosis and airway "whistling" during his first infusion of glutathione 2400 mg IV for the treatment of rectal cancer. The patient was also started on oral capecitabine 200 mg on the same day (timing related to anaphylaxis event is unclear). Both glutathione and capecitabine were discontinued. The patient received IV saline, dexamethasone 10 mg, calcium gluconate, and aminophylline 250 mg IV infusion. A few hours later the patient's vitals normalized.

Hepatoxicity: One case (a literature case report from Japan; Naito et al. 2010) reported "hepatotoxicity." The patient was receiving glutathione 1200 mg IV/day administered once a week for Parkinson's disease, in addition to three other medications for his Parkinson's disease (e.g., entacapone) and nine additional concomitant medications. After five months of treatment (estimated exposure to glutathione 24,000 mg), the patient reported malaise and anorexia and was hospitalized. The patient had an elevated aspartate aminotransferase (AST) of 1,040 IU/L (normal AST range: 10-34 IU/L) and alanine aminotransferase (ALT) of 890 IU/L (normal ALT range: 10-40 IU/L). Five days after all medications except for L-dopa/DCI 300 mg/day were discontinued, the patient's hepatic enzymes decreased to 241 IU/L (AST) and 595 IU/L (ALT). Hepatic injury resolved within two months. The drug-induced lymphocyte stimulation test (DLST) result for entacapone was negative but equivocally positive for glutathione. It should be noted that the DLST is a test commonly used in Japan to test for drug hypersensitivities; however, false-positive and false-negative results may occur (Saito et al. 2018).

Endotoxin Contamination: In February 2019, FDA warned compounders not to use glutathione L-reduced powder from a certain bulk drug supplier to compound sterile injectable drugs. Excessive levels of endotoxin were found in the bulk drug substance.[17] This event was associated with reports of seven patients who experienced adverse effects; mild nausea and vomiting in six patients and one 90 year old patient who experienced hypotension and difficulty breathing and was sent to an emergency department.

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[17] FDA warning information available at: https://www.fda.gov/drugs/human-drug-compounding/fda-highlights-concerns-using-dietary-ingredient-glutathione-compound-sterile-injectables. Accessed Apr 18, 2022.

Three additional reports of pyrexia following glutathione injections, potentially indicating endotoxin contamination, have been reported. These included a 2021 case in which the patient also experienced tremor and syncope following IM self-injection of a skin lightening product sold online.

Infusion Reactions: One medical clinic reported seven cases of a variety of AEs among nine patients including chest pain, shortness of breath, dizziness, "anaphylaxis," nausea and vomiting, following infusions of a duration of two minutes instead of the intended 10 minutes. These events were reported two years after their occurrence, following a site inspection.

CAERS

CFSAN collects reports of AEs involving food, cosmetics, and dietary supplements in the CAERS. A search of CAERS was conducted in August 2018, updated February 2022, for AEs associated with glutathione based on the term "glutathione." A total of 195 CAERS cases were spontaneously reported that included at least one AE in association with the use of glutathione. However, some cases listed as many as 55 different ingredients in the suspect product. Only one case listed glutathione ("Pure L-Glutathione") as the sole ingredient in the suspected product. It was used to "even skin tone," and the three adverse events reported in this case were rash, scar and skin hyperpigmentation." Thus, 194 CAERS reported case were confounded by concomitant ingredients, and many were also confounded by concomitant medications being taken by the glutathione consumer.

Regarding outcomes, 99 cases reported at least one "Medically Important Event," 77 cases reported "Hospitalization," and 13 cases reported "Life-Threatening" outcomes.

It is not possible to determine whether a causal connection existed between the AEs reported and glutathione because of the large number of other substances in the consumed products or in the concomitant medications.

Food and Drug Administration of the Philippines

AEs reported with IV glutathione injection by the FDA of the Philippines:

- Adverse cutaneous eruptions ranging from skin rashes, to serious and potentially fatal Stevens Johnson Syndrome and toxic epidermal necrolysis
- Thyroid dysfunction
- Kidney dysfunction with potential of development of renal failure; possibly due to high doses of IV glutathione overloading the renal circulation
- Severe abdominal pain in a patient receiving twice-weekly IV glutathione (Sonthalia et al. 2016)

According to the Philippine Dermatological Society, "FDA [of the Philippines] warns against the use of injectable glutathione as a skin whitener because whitening is not an approved use, efficacy is not well-established, and there have been reported side effects."[18]

[18] Philippine Dermatological Society statement available at: https://pds.org.ph/public-advisory-on-glutathione-as-a-skin-whitening-agent/. Accessed Apr 18, 2022.

Thailand
A surge in the use of IV glutathione for skin lightening in Thailand prompted Thai authorities to ban the use of such a modality "for fear of severe adverse reactions, including anaphylaxis" (Arjinpathana and Asawanonda 2012).

b. Clinical trials assessing safety

One clinical trial conducted in healthy volunteers. A 3-arm, randomized, double-blinded, placebo-controlled, 6 month treatment duration study determining the effect of oral glutathione 250 mg/d, glutathione 1000 mg/d and placebo on glutathione levels in blood, erythrocytes, plasma, lymphocyte and exfoliated buccal cells, 61 subjects were randomized, 60 received treatment and data from 54 patients was analyzed (6 patients prematurely discontinued from the trial). "No serious adverse effects were reported by the study participants regardless of arm. All potential AEs reported were minor including colds, stomach virus, lightheadedness, back pain, hot flashes, soft stools, eye twitching, headaches, ear infection, urinary tract infection and constipation. No AEs were attributed to any specific treatment arm. The number of these events reported was similar in each arm of the study (placebo, 20; 250 mg glutathione, 18; and 1,000 mg glutathione, 19). Two individuals dropped out because of allergy-like symptoms, both of which were in the placebo group" (Richie et al. 2015; ClinicalTrials.gov identifier NCT01044277).

A summary of the safety data from glutathione clinical studies and literature reviews is provided below for various uses and routes of administration of glutathione.

IV, Non-U.S.:

- In an FDA search of the scientific literature, one clinical trial of IV glutathione for skin lightening was identified. In this placebo-controlled study that enrolled 50 females aged 27 to 47 years, "GSH Detox forte®" (aqua, glutathione 1200 mg, ascorbic acid, hydrolyzed collagen 35 mg, and sodium chloride) or normal saline was administered intravenously twice weekly for 6 weeks (total 12 injections) for skin tone lightening. Severe adverse effects warranted discontinuation of treatment in nine (eight due to deranged liver function tests and one due to anaphylaxis) of the 25 healthy subjects (36%) who received IV glutathione. All subjects who received glutathione reported side effects
(11 subjects reported feeling of warmth during injection, 10 abdominal cramps, 8 deranged liver functions, 7 feeling of heart sinking, 4 diarrhea, 3 dizziness, 1 anaphylactic shock, and 1 vomiting), while no adverse effect was noted in any patient who received normal saline (Zubair et al. 2016).

- In an open label, uncontrolled study enrolling nine patients with early untreated Parkinson's disease, IV infusions of glutathione (600 mg in 250 mL saline, over 1 hour, twice daily for 30 days) were administered (a total of glutathione 36,000 mg was administered over one month). Two of the nine patients during the 3rd week of IV infusion of glutathione had fever, erythema of the skin, irritation and hardness at injection site, "likely due to infusion thrombophlebitis" (Sechi et al. 1996).

IV, U.S.:

- In a randomized, double-blind, placebo controlled pilot study that enrolled 21 patients (11 received glutathione, 10 received placebo) with Parkinson's disease, whose motor symptoms were not adequately controlled with their current medication regimen, IV administration of glutathione 1,400 mg (diluted in 10 mL of normal saline and administered by IV push over 10 minutes) or placebo was administered three times a week (Monday, Wednesday, Friday) for 4 weeks, in addition to their current medication regimen. A total of glutathione 16,800 mg was administered to each of the 10 patients with Parkinson's disease, who completed the study. There were no early withdrawals because of AEs in either group. The AEs reported by the glutathione patients only who completed the study include nausea, erythema at the infusion site, hair loss, sleep difficulties, sweating increased, upper respiratory infection, and vivid dreaming (Hauser et al. 2009).

- Two MEDLINE searches for studies published up to September 2015 regarding glutathione use for skin lightening (Davids et al. 2016): "In spite of widespread reported use, there are no studies of IV glutathione use for skin lightening or of its safety for chronic use (for any indication). The switch from brown to red melanin production may increase the risk of sun-induced skin cancers in previously protected individuals. Regulatory assessment of systemic glutathione administration for cosmetic use by the Medicines Control Council seems urgently warranted to protect consumers from potential side-effects and from complications of IV infusions. This is especially concerning because of reports of glutathione bought online."

- In a review of studies related to IV treatments for skin lightening, the authors concluded that "it is not suggested to use IV glutathione for skin lightening due to the increased risk of adverse events" (Juhasz and Levin 2018).

Oral/Buccal, Non-U.S.:

- In a randomized, double-blind, placebo-controlled, 2-arm clinical trial enrolling 60 Thai healthy medical students (18-25 years), oral glutathione 500 mg versus placebo was administered once daily for 4 weeks for "total-body skin lightening." Two AEs were reported: flatulence in one glutathione subject and constipation in one placebo subject (Arjinpathana & Asawanonda 2010).

- An open-label, single arm study of the safety and efficacy of a novel glutathione buccal lozenge containing 500 mg of glutathione once daily for 8 weeks was conducted in 30 healthy Filipino women. One subject who dropped out of the study complained of soreness in the gums caused by use of the product. Another subject who dropped out complained that the lozenge was sour and was chalky in texture. There were no serious adverse events (SAEs) and laboratory examination findings remained normal. This report also states that safety continues to be a concern with parenteral administration of glutathione ranging from mild, transient headaches and skin eruptions to severe drug reactions that have required hospitalization, as per the Philippine Dermatological Society's Advisory entitled "Intravenous Glutathione as a Skin Whitening Agent," circulated on June 15, 2011 (Handog et al. 2015).

Oral inhalation, Non-U.S.:

- A randomized, double-blind, placebo-controlled, parallel design "phase 2b," 6 month treatment duration inhalation study conducted in 153 CF patients aged 8 years and older who received doses of 646 mg of glutathione sodium dissolved in 4 mL of water or placebo saline via nebulizer every 12 hours. (Griese et al. 2013). A large number of the subjects prematurely withdrew from the study: 21/73 withdrew from glutathione group (12 due to protocol violations and 9 due to early termination, of which 6 were due to AEs and 3 were due to patient request) and 34/80 withdrew from placebo group (15 due to protocol violation and 19 due to early termination, of which 5 were due to AEs and 14 were due to patient request). Of the 16 subjects (8 glutathione and 8 placebo) who experienced at least one SAE, two SAEs were judged as non-CF related: facial palsy in the glutathione group, which resolved, and chronic IgA nephritis in the placebo group which did not resolve. The remaining 14 subjects who experienced SAEs reported pulmonary exacerbation of CF lung (4 glutathione, 5 placebo), hemoptysis (2 glutathione), abdominal pain and distal intestinal obstruction syndrome (1 glutathione, 2 placebo). Per the authors, the adverse event incidence was "similar" between the glutathione (73 subjects with any adverse event) and placebo groups (77 subjects with any AE), with "somewhat higher" frequencies of pyrexia, abnormal sputum and upper respiratory infection in the glutathione group. It should be noted that when a large percentage of subjects withdraw prematurely (i.e., 36% of the subjects in this study prematurely withdrew), it is difficult to assess the safety of the interventions. A significant number of subjects in this study (11 subjects) withdrew prematurely due to AEs.

- In a randomized, double-blind, crossover, placebo-controlled study of 8 patients with mild asthma, nebulized glutathione 600 mg induced marked bronchoconstriction in one patient [baseline forced expiratory volume at one second (FEV_1) 2.68 L, 90% predicted, which decreased -1.91 L (-69% from baseline) after glutathione treatment, associated with severe wheezing and breathlessness]. In the remaining 7 patients, nebulized glutathione induced cough (4 patients) and breathlessness (2 patients), while no symptoms were reported after receiving nebulized placebo (Marrades et al. 1997).

Oral inhalation, U.S.:

- In a randomized, single-blind, placebo-controlled (inhaled sodium chloride solution 0.9%), 12 month treatment duration trial conducted in 54 adults and 51 pediatric patients (aged 6-45 years) with CF with 97 completing the trial, doses of 10 mg/kg body weight (max 600 mg) twice daily of inhaled glutathione were well tolerated by all patients with AEs reported as single events in some patients and none of them led to study drug discontinuation. No deaths occurred. AEs that occurred only in the glutathione treatment group included distal intestinal obstruction, headache, constipation, pityriasis (rash), and impaired glucose tolerance (Calabrese et al. 2015; NCT01450267).

- A pilot, randomized, double-blind, placebo-controlled, parallel design, 8 week treatment duration, inhalation study was conducted in 19 CF patients aged 6-19 years: 10 treated with active (inhalations of buffered glutathione solution 66 mg/kg of body weight/day) and 9 treated with placebo (inhalations of sodium chloride solution 15 mg/kg of body

weight/day with "hint" of quinine 25-30 µg/kg of body weight/day for blinding), with the daily amount divided into four inhalation sessions/day and spaced 3-4 hours apart. For the first week of the trial, participants were instructed to use one fourth of the recommended total dosage, and in the second week to use one half of the recommended total dosage. After the second week, patients were instructed to use the full daily total dosage. Fourteen subjects (8 active and 6 placebo) completed the study. There was no type of AE (e.g., shortness of breath) that occurred only in the glutathione group and numbers of AEs were comparable (n = 9 placebo and n = 10 glutathione)(Bishop et al. 2005).

Nasal inhalation, U.S.:

- In a 3 month treatment duration, 3-arm (intranasal glutathione 300 mg/day, 600 mg/day, or saline placebo in three divided daily doses), randomized, double-blind, Phase I/IIa study conducted in 30 patients with Parkinson's disease,, one participant withdrew due to an AE "ringing in her head" attributed to the study medication (treatment arm for this patient was not provided) and one withdrew due to schedule conflicts (treatment arm for this patient was not provided). There was one SAE (no further details of treatment arm for this patient or actual side effect were provided; however, it was stated that the Medical Monitor and data monitoring committee determined that this SAE was unrelated). The authors concluded that there were no substantial differences between the groups in the number of AEs reported or observed among all safety measures assessed; however, the AEs "labored breathing" (n=2), "sore throat/redness" (n=2), and "increased thirst" (n=2) were only reported by patients in the 600 mg/day group (Mischley et al. 2015).

- In a 3-month treatment duration, 3-arm (intranasal glutathione 300 mg/day, 600 mg/day, or saline placebo in three divided doses), randomized, Phase IIb study conducted in 45 patients with Parkinson's disease, five subjects prematurely withdrew. One subject in the 600 mg/day glutathione cohort withdrew after developing tachycardia and newly-diagnosed cardiomyopathy (tachycardia resolved after patient stopped the study medicine), one additional subject in the 600 mg/day glutathione cohort withdrew due to logistical problems, two subjects in the 300 mg/day glutathione cohort withdrew (one due to a fall resulting in a broken bone and one due to puffiness under her eyes, attributable to the study intervention) and one subject in the placebo arm withdrew due to chronically-irritated sinuses and headaches, attributable to the study intervention. No additional AE information was described in the publication (Mischley et al. 2017).

c. Pharmacokinetic data

Lomaestro and Malone (1995) noted that "(t)he application of glutathione *per se* as a therapeutic agent is limited because of its low bioavailability due mainly to its low membrane permeability and short half-life in the body." "The apparent half-life of GSH in blood plasma is quite short, 1.6 min" (Homma and Fujii 2015). It is noted that while oral and IV PK data are available, no PK studies associated with oral inhalation or nasal inhalation were found.

IV pharmacokinetics

In a PK study conducted in 10 healthy volunteers, plasma concentrations of glutathione and total glutathione were determined after high-dose IV reduced glutathione (2 grams/m^2) administration. Basal plasma concentrations compared to peak plasma concentrations were glutathione 6.9 ± 2.5 µmol/l to 444 ± 138 µmol/l and total glutathione 17.5 ± 13.4µmol/l to 823 ± 326 µmol/l. It was concluded that high-dose IV glutathione distributes in the extracellular compartment and is cleared from circulation with a half-life of approximately 15 minutes, with plasma cysteine concentration and urinary excretion of total cysteine markedly increased (Aebi et al. 1991).

In a PK study conducted in 7 healthy Korean male volunteers, glutathione 50 mg/kg of body weight was infused over 10 minutes (Hong et al. 2005). Below in Table 6 are the changes in glutathione and GSSG during the observation period, in mean (standard deviation (SD)) concentration (in µM), and the PK parameters.

Table 6. Human pharmacokinetic parameters (Hong et al. 2005)

Time (min)	0	10	20	30	60	120	240
GSH	4.3	154.4	11.1	5.3	4.7	4.0	5.7
	(2.5)	(70.0)	(7.3)	(2.9)	(2.6)	(2.3)	(2.7)
GSSG	9.7	1061.5	144.4	62.3	18.6	11.8	12.0
	(6.8)	(481.2)	(60.0)	(26.8)	(5.3)	(5.3)	(7.4)
Total GSH	14.0	1219.8	155.4	67.6	23.3	15.8	16.8

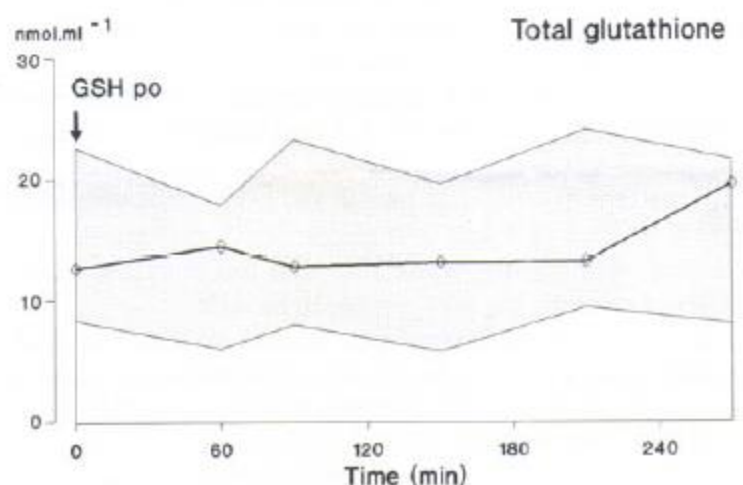
Table 2. Pharmacokinetic parameters of GSH, GSSG and total GSH following high-dosage intravenous administration of GSH (50 mg per kg of body weight) in 7 volunteers

	C_{basal} (µM)	C_{max} (µM)	AUC (µM min)	K (min^{-1})	$t_{1/2}$ (min)	C1 (mL min^{-1} kg^{-1})	Vd (1 kg^{-1})
GSH							
Mean	4.3	150	1242.8	0.07	10.9	309.2	5.528
SD	5.5	234.6	1905.2	0.026	3.3	360.9	6.935
GSSG							
Mean	9.7	1055.8	11148.1	0.066	10.8	10.6	0.164
SD	6.8	480.1	3705	0.012	2	3.7	0.061
Total glutathione							
Mean	14	1205.8	12427	0.066	10.9	9.8	0.152
SD	12.1	621.7	4618.3	0.014	2.2	3.8	0.058

Oral pharmacokinetics

The bioavailability of a single dose of oral glutathione 0.15 mmol per kg dissolved in 200 mL of water was investigated in 7 healthy volunteers who had fasted overnight. Blood samples were drawn every 30 minutes for 270 minutes. The results showed a nonsignificant increase in plasma glutathione after a single dose (as high as 3.0 grams), suggesting negligible systemic availability (See Figure 6 below) for oral glutathione in humans (Witschi et al. 1992).

Figure 6. Human Oral PK (Witschi et al. 1992)



Richie et al. (2015) conducted a randomized, double-blind, placebo-controlled study of oral glutathione administration on “body stores” of glutathione in 54 non-smoking adults. Patients received daily doses of 250 mg glutathione (n = 20), 1000 mg glutathione (n = 20) or placebo (n = 21) for six months. Glutathione levels were measured at baseline and after a six month treatment period in blood, erythrocytes, plasma, lymphocytes and exfoliated buccal mucosal cells. Glutathione levels were found to have increased statistically significantly more than with placebo in the high dose group for erythrocytes, plasma, lymphocytes and buccal cells. In the low dose group, levels increased statistically significantly more than with placebo only in whole blood. Levels returned to baseline following a subsequent one-month washout period. The authors commented that "the extent to which direct absorption may be responsible for the present findings is not known" and propose that "changes in GSH metabolism” may occur with long term supplementation.

It has been suggested that oral absorption may be facilitated by specialized intestinal uptake mechanisms for glutathione such as transmembrane transporters (Vincenzini et al. 1992). However, hydrolysis of the tripeptide in the intestine continues to be considered the primary obstacle to oral glutathione absorption and novel delivery systems are being investigated (Buonocore et al. 2015).

d. Availability of alternative therapies that may be as safe or safer

Multiple drug products are available for many of the proposed uses of glutathione that are discussed in subsequent sections. Given the serious safety concerns associated with the use of glutathione identified above, the existence of approved drug products weigh against placing glutathione on the list.

Conclusions: It is noted that data regarding glutathione safety have been derived from various U.S. and non-U.S. sources. Particularly pertinent to IV use, it remains unclear whether the substance glutathione, or other factors such as components of the IV formulation, treatment

population, or administration procedures, caused particular AEs. Those events that have been reported from multiple countries may be most closely related to the use of the substance glutathione rather than other factors. For instance, anaphylaxis has been reported internationally, but SJS has been identified as a primary safety issue only in the Philippines. This is not a definitive method for establishing causality, but it could help explain the variability in reported AEs.

IV administration of glutathione has resulted in hepatotoxicity and life-threatening anaphylaxis, despite rapid elimination from systemic circulation. One clinical study of the use of nebulized, oral inhalation of glutathione for asthma identified significant safety concerns in this population (Marrades et al. 1997). Oral glutathione is minimally absorbed and appears to be associated primarily with local, gastrointestinal adverse effects. Due to these significant safety concerns, particularly with respect to IV and inhalation formulations, FDA recommends that glutathione not be added to the list of bulk drug substances that can be used in compounding under section 503A of the FD&C Act.

C. Are there concerns about whether a substance is effective for a particular use?

In evaluating effectiveness, FDA considers the available evidence of the substance's effectiveness or lack of effectiveness for a particular use, including reports in peer-reviewed medical literature, if any such evidence exists. This is consistent with the new drug approval process. However, in the new drug approval process, applicants are required to provide substantial evidence that the drug will have the effect it purports or is represented to have under the conditions of use prescribed, recommended, or suggested in the labeling.[19] FDA recognizes that few, if any, of the candidates for the 503A Bulks List will have been studied in adequate and well-controlled investigations sufficient to satisfy this standard. Thus, in its balancing of the relevant criteria, the Agency takes into account whatever relevant evidence concerning effectiveness is available.[20]

The following databases were consulted in the preparation of this section: PubMed, EMBASE, Cochrane Database of Systematic Reviews, and ClinicalTrials.gov.

We evaluated glutathione for 24 proposed uses and considered available data to support its effectiveness.

Information for each use listed below is provided in the following format:

a. Clinical Information - Reports of trials, clinical evidence, and anecdotal reports of effectiveness, or lack of effectiveness, and other information relevant to clinical use of the bulk drug substance with respect to the treatment of a disease or condition.

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[19] See section 505(d) of the FD&C Act. The term "substantial evidence" refers to "evidence consisting of adequate an well-controlled investigations, including clinical investigations, by experts, qualified by scientific training and experience to valuate the effectiveness of the drug involved, on the basis of which it could fairly and responsibly be concluded by such experts that the drug will have the effect it purports or is represented to have under the conditions of use prescribed, recommended, or suggested in the labeling or proposed labeling thereof."
[20] See 21 CFR 216.23(c); see also 81 FR 91,071 (Dec. 16, 2016) and 81 FR 4696, 4701-02 (Mar. 21, 2019).

b. Severity - Whether the product compounded with this bulk drug substance is intended to be used in a serious or life-threatening disease or condition.
c. Alternative Therapies - Whether there are any alternative therapies that may be safer or more effective.
d. Conclusion - Assessment of data related to this use, taking into consideration severity of the disease or condition and availability of alternative therapies.

1. Skin Lightening

Skin lightening refers to the use of depigmenting agents, sometimes as treatment for hyperpigmentation. Skin-lightening (or "skin-bleaching") agents can be important tools in the management of disorders of hyperpigmentation, such as melasma and post-inflammatory hyperpigmentation. However, use of skin-lightening agents, particularly for the purpose of lightening one's natural skin color [to achieve an overall lighter skin color] is a global phenomenon, with the highest rates in Africa, Asia, the Caribbean, and the Middle East, as well as in immigrant populations from these regions in North America and Europe.[21] A variety of substances have been used and been administered via topical, oral or IV routes.

a. Clinical Information

The information provided by the nominator is related to overall skin tone lightening; however, it does not describe a clinical benefit, or otherwise address clinical effects, of glutathione for a specific disease or condition.

In a placebo-controlled study that enrolled 50 females aged 27 to 47 years, “GSH Detox forte” (aqua, glutathione 1200 mg, ascorbic acid, hydrolyzed collagen 35 mg, and sodium chloride), or normal saline, was administered intravenously twice weekly for 6 weeks (total 12 injections) for skin tone lightening. A visual assessment tool using standardized color cards, the Taylor hyperpigmentation scale, was used to measure the skin tone (Taylor et al. 2005).[22] Two body sites, which were non-exposed to sun, were measured with Taylor hyperpigmentation cards (i.e., upper inner arm below the axilla and upper outer thigh). Effectiveness and side effects were assessed at the end of therapy and 2 months, 4 months, and 6 months after cessation of treatment. Final results included only those patients who completed the study, 32 patients (16 treated with glutathione and 16 with placebo). After 12 injections of glutathione, 6 of 16 (37.5%) subjects in the glutathione group showed significant skin lightening, whereas 3 (18.7%) subjects in the placebo group had lighter skin ($p = 0.054$). “After stopping the treatment, this improvement was gradually lost and at six-month posttreatment follow-up only one patient maintained this improvement” (Zubair et al. 2016).

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[21] Topical skin-lightening agents: Complications associated with misuse, https://www.uptodate.com/contents/topical-skin-lightening-agents-complications-associated-with-misuse?search=skin%20lightening&source=search_result&selectedTitle=1~63&usage_type=default&display_rank=1, UpToDate, Accessed Apr 18, 2022.
[22] In a 2005 validation study of the Taylor Pigmentation Scale, skin color was assessed in 30 subjects by 10 investigators. There were statistically significant intraindividual and interindividual variations, although the scale was rated as "easy to use" to document response of hyperpigmentation to therapeutic agents (Taylor et al. 2005). No additional information about the development of the scale was found.

Glutathione is not well absorbed from the gastrointestinal tract and thus, IV administration of glutathione has been used for skin lightening in many countries, especially in southeast Asia. However, a clinical trial of oral glutathione dosing was initiated in Thailand after a surge in the use of IV administration of glutathione for skin lightening resulted in Thai authorities banning the use of IV glutathione "for fear of severe adverse reactions, including anaphylaxis." In this randomized, double-blind, placebo-controlled, 2-arm clinical trial enrolling 60 Thai healthy medical students (18-25 years), oral glutathione 250 mg capsules dosed twice daily versus placebo was administered for 4 weeks for "total-body skin lightening." The primary endpoint was reduction of melanin indices as measured with a Mexameter® that operates based on light absorbance and reflectance.[23] No information was provided regarding a correlation of the melanin indices based on the Mexameter® with, for example, changes in skin color visible to the eye. Melanin indices were measured at six different sites with no statistical correction described for measurement at multiple skin sites. All subjects completed the study. At the end of the trial, the glutathione group showed a statistically significant reduction of melanin indices compared to placebo at two (right side of face and left forearm) of the six measured sites. Two AEs were reported: flatulence in one glutathione subject and constipation in one placebo subject (Arjinpathana & Asawanonda 2012; Alexis & Blackcloud 2013; per NCT01016080, study dates were February 2009 to May 2009).

Following this 4 week study, the same investigator group conducted a 12 week study comparing oral reduced glutathione at doses of 250 mg/d, GSSG at doses of 250 mg/d and placebo for 12 weeks (Weschawalit et al. 2017). The intent of the study was to establish whether the skin lightening seen in the prior study was achievable with a lower dose and maintained the longer study duration. A randomized, double-blind, parallel, three-arm study enrolled 60 healthy volunteers between the ages of 20 and 50. Evaluation of skin color was conducted using the melanin index as measured with the Mexameter® as in the prior study at 6 skin sites and additional objective and subjective assessments of skin properties. The melanin index at all evaluated skin sites for GSSG and glutathione were lower than for placebo but were not statistically significantly different from placebo or between active treatments. The difference in findings from the prior study is primarily attributable by the authors to use of a 250 mg versus the prior 500 mg dose. AEs were reported to have been not serious, but two cases of reversible "transaminitis" were reported without details about enzyme levels, treatment group, etc.

In an open-label, single arm study of the safety and efficacy of a novel glutathione buccal lozenge containing 500 mg of glutathione given once daily for 8 weeks was conducted in 30 healthy Filipino women with Fitzpatrick skin types IV or V, there was a significant ($p<0.0001$) decrease in melanin indices from baseline to endpoint beginning two weeks after initiation of treatment (Handog et al. 2015). However, this outcome appears to be inconsistent with PK data that show that absorption of orally administered glutathione is minimal, and confirmatory trials would be needed.

[23] Measurement was based on Mexameter (Courage-Khazaka Electronic, Koln, Germany) assessments of light absorption/reflection. Information available at: https://www.courage-khazaka.de/images/Downloads/Brochures/Wissenschaftlich/Brochure_Mexameter.pdf. Accessed Apr 18, 2022.

Sonthalia et al. (2018) reviewed the current status of glutathione as a skin lightening agent and conclude that "There is little convincing evidence in favor of glutathione as a therapy for hyperpigmentation at the present time, and there are many unresolved controversies that surround its use. The trials available to date that have evaluated the role of glutathione in skin lightening administered through different modes have numerous limitations. Although the safety of topical and oral GSH seems to be good, their efficacy (especially long-term) remains questionable. The extant evidence to support or discourage use of IV GSH as a therapeutic modality for improving skin tone or pigmentation is minimal and contradictory; notwithstanding the austere concern regarding the potential adverse effects associated with this mode of administration. More evidence in the form of high quality trials with better study design, larger sample size, and long-term follow-up is vital, before our patients are subjected to glutathione-based treatments."

b. Severity

Disorders of hyperpigmentation such as melasma and post-inflammatory hyperpigmentation are not serious conditions.

c. Alternative Therapies

Alternative therapies approved for skin lightening include the FDA approved combination of hydroquinone, tretinoin and fluocinolone acetonide topical cream (Tri-Luma®) for melasma of the face.

d. Conclusion

A review of the literature identified one small IV glutathione clinical study that appears to suggest glutathione lightens the skin. The effect seems to dissipate following glutathione discontinuation. Further, this study did not examine glutathione in lightening the skin for purposes of treating a particular disease or condition. Other studies failed to show a skin lightening effect associated with glutathione or were inadequately designed as a basis for evaluation (i.e., uncontrolled). There are insufficient data to support the effectiveness of oral glutathione for skin lightening. In addition, the nominator has not provided data indicating that any effect of glutathione may have to lighten the skin provides a clinical benefit to address a disease or condition, such as managing disorders of hyperpigmentation.

2. Cystic Fibrosis (CF)

a. Clinical Information

Two Cochrane reviews were identified. First, Cochrane Review (Tam et al. 2013): Nebulized and oral thiol derivatives[24] for pulmonary disease in CF.

Main Results: "Searches identified 23 trials; nine trials (255 participants) are included, of these seven trials are more than 10 years old. Three trials of nebulized thiol derivatives were identified

[24] Drug compounds that contain a thiol (-SH) group, such as glutathione.

(one compared 20% N-acetylcysteine to 2% N-acetylcysteine; another compared sodium-2-mercaptoethane sulphonate to 7% hypertonic saline; and another compared glutathione-SH to 4% hypertonic saline). Although generally well-tolerated with no significant adverse effects, there was no evidence of significant clinical benefit in our primary outcomes in participants receiving these treatments. Oral thiol derivatives were generally well-tolerated with no significant adverse effects, however there was no evidence of significant clinical benefit in our primary outcomes in participants receiving these treatments."

Authors' conclusions: "We found no evidence to recommend the use of either nebulized or oral thiol derivatives in people with CF. There are very few good quality trials investigating the effect of these medications in CF, and further research is required to investigate the potential role of these medications in improving the outcomes of people with CF."

Plain Language Summary: "In summary, the trials included in the review did not provide any evidence that nebulized or oral thiol derivatives were either beneficial or harmful to people with CF. Further research investigating the effects of thiol derivatives in people with CF is required before their use can be recommended" (Tam et al. 2013).

The second Cochrane review was by Ciofu and Lykkesfeldt (2014): Antioxidant supplementation for lung disease in CF. Of 321 nonduplicate study reports of antioxidant supplementation for lung disease in CF, 10 studies met the selection criteria for inclusion in the review. Three of the 10 reviewed studies (Visca et al. 2015, Bishop et al. 2005, Griese et al. 2013) administered glutathione. Only one of the studies reported quality of life data that could be analyzed, but data showed no significant differences between treatment and control. None of the 10 reviewed studies were judged to be free of bias. Oral N-acetylcysteine (a precursor of glutathione) therapy could be continued during the study, but these patients could not be identified in the publication, introducing a confounding factor.

In a double-blind, randomized, placebo-controlled, parallel design, 6 month treatment duration, oral reduced glutathione study conducted in 47 pediatric CF patients aged 18 months to 10 years, 24 were treated with active (oral reduced glutathione 65 mg/kg, divided into 3 doses/day) and 23 were treated with placebo (Visca et al. 2015). There were four primary outcomes (change in weight percentile, body mass index (BMI) percentile, height percentile and fecal calprotectin) and four secondary outcomes (white blood cell count, alanine transaminase, vitamin E level and C-reactive protein).[25] In addition, the study showed a significant improvement in FEV_1 expressed as percent and in forced vital capacity (FVC) after six months from the treatment start, mean difference 17.40 (95% confidence interval [CI] 13.69 to 21.11) and 14.80 (95% CI 9.66 to 19.94) respectively.[26] In this study, the supplementation had a positive effect on the nutritional status (BMI %) of the patients, mean difference 17.20 (95% CI 12.17 to 22.23).

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[25] The primary endpoints in most CF trials include several measures of pulmonary function.

[26] The authors of this Cochrane review obtained additional data from the authors of the Visca et al. 2013 reference and used this data in their analysis. The pulmonary function data provided in the Cochrane review is not included in the Visca et al. 2103 publication; however, an Excel full data spreadsheet containing this information can be accessed at:
https://www.uvicf.org/researchnewsite/glutathionenewsite/ViscaTrial_Data_and_SupplementaryMaterial.html. Accessed Apr 18, 2022.

The glutathione treatment group gained over six months an average of 0.67 standard deviation (SD) in weight-for-age-and sex z score (wfaszs), equal to 19.1 weight percentile points. The placebo group increased significantly less, 0.1 SD in wfaszs (2.1 weight percentile points), $p<0.0001$. Other changes included:

- Fecal calprotectin improved more in the active treatment group (glutathione 52.0 vs placebo 0.5), $p<0.0001$.
- BMI for glutathione improved 0.69 SD BMI-adjusted-for-age-and-sex z score versus placebo 0.22 SD (BMI percentile 21.7 glutathione vs 5.2 placebo), $p<0.0001$.
- Height increased 0.2 SD in height-for-age-and-sex z score (hfaszs) glutathione versus 0.06 SD hfaszs placebo (height percentile 7.0 glutathione vs 2.6 placebo), $p<0.0001$.

It should be noted that there was an unequal distribution of delF508 homozygote subjects (13.6% of glutathione group versus 27.7% of placebo group) which is concerning because delF508 homozygotes usually have a more severe disease than CF patients with other genetic backgrounds. The small sample size is another potential bias.

The other two studies in the 2014 Cochrane review were conducted with inhaled glutathione, Griese et al. (2013), conducted in Germany, and Bishop et al. (2005)[27], conducted in USA. There was an improvement in the FEV_1 expressed as percent predicted after three and six months (n = 153), mean difference 2.57 (95% CI 2.24 to 2.90) and 0.97 (95% CI 0.65 to 1.29) respectively.

Bishop et al. (2005) was a pilot, randomized, double-blind, placebo-controlled, parallel design, 8 week treatment duration, inhalation study conducted in 19 CF patients aged 6-19 years. Ten patients were treated with active (inhalations of buffered glutathione solution 66 mg/kg of body weight/day) and 9 were treated with placebo (inhalations of sodium chloride solution 15 mg/kg of body weight/day with "hint" of quinine 25-30 µg/kg of body weight/day for blinding). Doses were distributed across four inhalation sessions per day and spaced 3-4 hours apart. For the first week of the trial, participants were instructed to use one fourth of the recommended total dosage, and in the second week to use one half of the recommended total dosage. After the second week, patients were instructed to use the full daily total dosage. A total of 14 subjects completed the study (8 active and 6 placebo). There were three premature discontinuations due to hospitalization (1 treated with glutathione and two on placebo) and two due to non-compliance.

Of the four primary endpoints [FEV_1 (% predicted of FEV_1); FVC (% predicted of FVC); forced expiratory flow (FEF) 25−75 (% predicted of FEF25−75 of FVC); peak flow (L/min using flow meter)], three were not statistically different; however, mean change for peak flow was -6.5 L/min for placebo group and +33.7 L/min for glutathione group (p=0.04). There were no significant differences in the secondary endpoints 6-minute walk distance or in sputum amount, viscosity or color between the two groups. The authors stated that an important limitation of the study is that the optimal dose of inhaled glutathione is still unknown and "the study results may be biased due to the small sample size."

[27] Bishop et al. 2005 was also reviewed in a second Cochrane review (Tam et al. 2013).

Bishop et al. (2013 abstract; NCT02029521) in an age-stratified, randomized, placebo-controlled, double-blinded, clinical trial, 44 pediatric CF patients were treated with oral reduced L-glutathione for six months (65 mg/kg, devided into three doses per day). The authors concluded that oral reduced L-glutathione should be considered in pediatric CF patients to improve nutritional status, as well as pulmonary function. The authors also concluded that further study is warranted.

Griese et al. (2013; NCT00506688) was a randomized, double-blind, placebo-controlled, parallel design phase 2b, 6 month treatment duration inhalation study conducted in 153 CF patients aged 8 years and older. Of these, 73 treated with active (inhalations of 646 mg of glutathione-Na powder mixed with 4 mL normal saline every 12 hours via eFlow nebulizer) and 80 treated with placebo (inhalations of 4 mL normal saline every 12 hours via eFlow nebulizer). FEV_1 (absolute values), both as pre-post differences (p=0.180) and as area under the curve (p=0.180) were the primary efficacy endpoints and were not different between the two groups over the 6 month treatment period.

It should be noted that a large number of the subjects prematurely withdrew from the study: 21/73 withdrew from glutathione group (12 due to protocol violations and 9 due to early termination, of which 6 were due to AEs and 3 were due to patient request), and 34/80 withdrew from placebo group (15 due to protocol violation and 19 due to early termination, of which 5 were due to AEs and 14 were due to patient request). Another bias was that oral N-acetylcysteine (a precursor of glutathione) could be continued, and these patients could not be identified in the publication.

Several studies were found in addition to those identified in the Cochrane reviews. In a randomized, single-blind, placebo-controlled (inhaled sodium chloride solution 0.9%), 12 month treatment duration trial conducted in 54 adults and 51 pediatric patients (aged 6-45 years) with CF with 97 completing the trial, inhaled glutathione did not achieve the primary outcome measure of 15% improvement of FEV_1%. The glutathione was administered according to body weight (10 mg/kg) twice daily. The authors noted that most enrolled children had a normal spirometry at baseline with no room of improvement (Calabrese et al. 2015; NCT01450267).

In 2013, the Cystic Fibrosis Foundation and members of the Pulmonary Clinical Practice Guidelines Committee published "Cystic Fibrosis Pulmonary Guidelines: Chronic Medication for Maintenance of Lung Health." One study of glutathione (randomized controlled trial by Bishop 2005) and two studies of N-acetylcysteine for patients with CF were reviewed. Based upon this review, the recommendations were unchanged from previous guidelines, i.e., "For individuals with CF, 6 years of age and older, the CF Foundation concludes that the evidence is insufficient to recommend for or against the chronic use of inhaled or oral *N*-acetylcysteine or inhaled glutathione to improve lung function and quality of life or reduce exacerbations" (Mogayzel et al. 2013).

A multicenter, randomized, placebo-controlled, double-blind Phase II study was found of oral glutathione (n = 30) or placebo (n = 28) in pancreatic insufficient CF patients between the ages of 2 and 10 years. In this 24 week study, glutathione or lactose was given at doses of 65 mg/kg/day in 3 divided doses. No differences were seen between the groups in 6 months: change in weight for age z scores, absolute change in weight, absolute change in BMI, other

anthropometrics, or serum and fecal inflammatory markers (Bozic et al. 2020).

Corti et al. (2017) provided theory on the potential for patient selection criteria based on specific functions. "Conclusive evidence that glutathione inhalation produced protective benefit in CF has never been provided in clinical studies. One reason for this can be the increased activity of gamma-glutamyltransferase (GGT), the enzyme capable of degrading glutathione. GGT can rapidly degrade endogenous glutathione, as well as exogenous glutathione administered by inhalation." Differentiating CF patients with increasing vs. decreasing GGT activation, as measured in sputum, may discriminate subjects more likely profiting from inhaled glutathione, as opposed to those with increasing GGT in which these treatments might even produce aggravation of the damage. "At present, glutathione inhalation cannot be further recommended for usage" (Corti et al. 2017).

b. Severity

CF is a serious or life-threatening disease.

c. Alternative Therapies

Alternative therapies approved for the treatment of CF include bronchodilators such as albuterol and levabuterol; mucus thinners, hypertonic saline and dornase alfa; transmembrane conductance regulator modulator therapies such as ivacaftor, lumacaftor/ivacaftor, tezacaftor/ivacaftor, and elexacaftor/tezacaftor/ivacaftor; and antibiotics such as azithromycin, tobramycin and aztreonam.

d. Conclusion

There is conflicting evidence regarding the clinical effectiveness of antioxidant supplementation including glutathione in CF. Based on the available evidence, glutathione (administered either orally or by inhalation) appears to improve lung function in some cases and decrease oxidative stress; however, due to the very intensive antibiotic treatment and other treatments that CF patients receive, the beneficial effect of glutathione is very difficult to assess in patients with chronic infection without a very large population sample and a long-term (at least six months) study period. There is insufficient information to support the effectiveness of glutathione for the treatment of CF. The existence of approved drugs to treat the disease weigh against including glutathione on the list, particularly in light of CF being a serious or life-threatening disease.

3. Asthma

a. Clinical Information

In a double-blind, 3-arm, crossover, placebo-controlled study, 12 subjects (aged 19-45 years) with mild to moderate bronchial asthma were pretreated with a single dose of nebulized inhaled glutathione (600 mg in 5 mL of saline water), a single dose of sodium cromoglicate (SCG) 20 mg of nebulized solution) or placebo (saline water) with at least 3 days between treatments, followed by a fog (ultrasonically nebulized distilled water) challenge 30 minutes later. All subjects underwent the fog challenge at the same time of day. After placebo pretreatment, the

fog challenge caused a mean 20.41% decrease in FEV^1, after glutathione, a mean 6.04% decrease in FEV^1 and after SCG, a mean 5.99% decrease in FEV^1 (Bagnato et al. 1999).

Other information provided by the nominator explains, “While the specific mechanisms responsible for asthma have yet to be unraveled, oxidative stress from free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) is likely to play a key role in the development and pathogenesis of the disorder.” … “the concept of oxidative stress has evolved considerably since its inception > 25 years ago. In the traditional view, oxidative stress was thought to result from a global disruption in the pro-oxidant and antioxidant balance. In this view, disease was due to an excessive burden of ROS and RNS with an accompanying deficiency of free radical-scavenging antioxidants. This view of oxidative stress ultimately led to several large antioxidant studies that were undertaken in a variety of disease states, but many of these failed to demonstrate clear benefits with regard to clinically relevant health outcomes. These disappointing findings are likely related to a number of factors, including (i) insufficient knowledge of the clinical pharmacology of antioxidants, (ii) insufficient dosing or duration of the antioxidant therapy due to limited dose-response studies, (iii) the method of antioxidant delivery (i.e., oral vs. inhaled), (iv) the composition of the antioxidant agent itself (i.e., solubility, adjuvants, and additives), (v) selection of un-standardized, un-validated or otherwise soft primary outcome indicators, including outcomes of limited relevance to the mechanism of antioxidant action, and (vi) lack of surrogate markers of oxidative stress to accompany functional outcomes” (Fitzpatrick et al. 2012).

b. Severity

Asthma can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies approved for the treatment of asthma include short acting inhaled beta-2 agonists, such as albuterol; long-acting inhaled beta-2 agonists such as salmeterol and formoterol; anticholinergics such as ipratropium bromide; inhaled corticosteroids such as beclomethasone and budesonide nebules; leukotriene receptor antagonists, montelukast and zafirlukast; methylxanthine such as theophylline; mast cell stabilizing medication such as cromolyn and zileuton, and monoclonal antibody immune-modulating drugs such as omalizumab and dupilumab.

d. Conclusion

One single-dose clinical study conducted in only 12 patients was located that suggests the use of glutathione for asthma may have a beneficial effect in asthma, however, this small study provides insufficient information about population, exposure or risk to support use of glutathione. Additional information on the effect of glutathione may have on the structure or function of the body does not provide evidence of any clinical benefit on the use of glutathione in asthma. The existence of approved drugs to treat the disease weigh against including glutathione on the list, particularly in light of asthma being a serious or life-threatening disease.

4. Chronic Obstructive Pulmonary Disease (COPD)

COPD is a lung disease characterized by chronic obstruction of lung airflow that interferes with normal breathing and is not fully reversible. COPD is sometimes called "emphysema" or "chronic bronchitis." Emphysema usually refers to destruction of the tiny air sacs at the end of the airways in the lungs. Chronic bronchitis refers to a chronic cough with the production of phlegm resulting from inflammation in the airways. COPD and asthma share common symptoms (cough, wheeze, and difficulty breathing) and people may have both conditions (World Health Organization).[28]

a. Clinical Information

No clinical studies of glutathione for this use were found.

A case report described the use of nebulized glutathione (2 mL of 60 mg/mL glutathione solution inhaled over a 5-10-minute period) in a 95-year old man with acute respiratory crisis secondary to emphysema and apparent bronchial infection. On return office visit 3 days later, "he showed no signs of respiratory distress, and no adventitious lung sounds were noted on auscultation." He continued daily treatment with glutathione until his death from congestive heart failure two years later. The two authors (both Naturopathic Doctors, "ND") have subsequently prescribed this preparation for 6 patients with emphysema with 5 reporting improved breathing (Lamson and Brignall 2000).

During thoracotomy, researchers obtained a biopsy of the diaphragm from 9 male COPD patients and 7 normal male patients with normal pulmonary function (although both groups had a similar cigarette smoking history), with the expectation that glutathione levels in the COPD samples would be low. However, glutathione levels were similar in diaphragm biopsies from the COPD and control groups (Engelen et al. 2004).

A case control study performed in Tunisia compared venous blood levels of malondialdehyde (MDA), glutathione and protein-cys-SH (PSH) in age-matched and amount of tobacco used-matched male smokers, with and without COPD. The authors prespecified that high levels of MDA and low levels of glutathione and PSH are signs of a significant oxidative stress. Compared to the non-COPD group, the COPD group had significantly lower levels of glutathione and PSH; however, MDA levels were similar for the two groups. This study did not show a change in glutathione concentration in COPD (Moussa et al. 2014).

Inhaled glutathione cannot be recommended as a potential treatment for emphysema since the quality of evidence is currently lacking (Prousky 2008).

A review of pharmacological and dietary antioxidant therapies for COPD did not contain any information pertaining to a clinical trial of glutathione as a therapy for COPD (Biswas et al. 2013).

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[28] Available at: https://www.who.int/news-room/fact-sheets/detail/chronic-obstructive-pulmonary-disease-(copd), Accessed Apr 19, 2022.

b. Severity

COPD can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies approved for the treatment of COPD include short-acting bronchodilators, such as albuterol; long-acting bronchodilators such as formoterol and salmeterol; inhaled steroids such as fluticasone and budesonide; combination bronchodilators and inhaled steroids such as fluticasone/vilanterol, salmeterol/fluticasone, and formoterol/glycopyrrolate); phosphodiesterase-4 inhibitors, roflumilast; and leukotriene modifier, methylxanthines such as theophylline.

d. Conclusion

The above case report is an anecdotal report of effectiveness for COPD, which by itself is insufficient evidence to support the use of glutathione to treat COPD. No clinical studies were located that support the use of glutathione for COPD. Additional information on functional use does not provide evidence of clinical benefit on the use of glutathione in COPD. The existence of approved drugs to treat the disease or condition weigh against including glutathione on the list, particularly in light of COPD being a serious or life-threatening disease.

5. Chronic Lung Disease (CLD)

Neonatal CLD, also referred to as bronchopulmonary dysplasia, is a chronic respiratory disease of infancy that increases the predisposition of patients to develop early onset COPD (McGrath-Morrow and Collaco 2019). It develops in very preterm infants treated with oxygen and mechanical ventilation for respiratory distress syndrome.

a. Clinical Information

No clinical studies of glutathione for this use were found.

Cooke and Drury (2005) stated that: low levels of glutathione are associated with subsequent chronic lung disease in preterm infants, it has been suggested that CLD begins as an inflammatory response to oxygen-derived free radical generation, and the preterm infant may be particularly vulnerable to oxidant injury because endogenous antioxidant activity is deficient at birth. They examined the feasibility of administering a single dose of liposomal glutathione intra-tracheally to 14 ventilated preterm infants (1 mg/kg dose in 5 infants; 10 mg/kg dose in 9 infants). Bronchoalveolar lavage fluid was collected prior to treatment and 12 and 24 hours after dosing for glutathione and malondialdehyde estimation. The authors concluded that within its limitations, this preliminary study shows the feasibility of administering liposomal GSH to preterm infants and some early antioxidant effects. Randomized, blind, controlled studies will be needed to confirm safety issues and longer-term antioxidant activity before clinical trials of liposomal GSH for prophylaxis of CLD can take place. While this reference supports the feasibility of dosing preterm infants with intra-tracheal liposomal glutathione, with a resultant increase in mean glutathione in the bronchoalveolar lavage fluid, it does not provide efficacy and

safety data that demonstrates a benefit for the dosed infants.

b. Severity

CLD can be serious or life-threatening.

c. Alternative Therapies

No therapies are currently FDA-approved for the treatment of CLD.

d. Conclusion

The feasibility of the dosing study described above is insufficient to support the use of glutathione for chronic lung disease. No clinical studies were found that support the use of glutathione for chronic lung disease. Information provided by nominators on the potential effects of the substance on the structure or function of the body does not address any clinical benefits of glutathione for chronic lung disease.

6. Oxidative Stress

Oxidative stress has been defined as the condition when the sum of free radicals in a cell exceeds the antioxidant capacity of the cell (Smeyne & Smeyne 2013). The concept of oxidative stress centers on the imbalance of oxidants against antioxidants beyond physiological limits. The significance of oxidative stress states lies in the resultant oxidative cellular damage and related sequelae that range from cellular dysfunction to cell death (Berk et al. 2008).

a. Clinical Information

In a randomized, double-blind, placebo-controlled clinical trial, 40 healthy adult volunteers without acute or chronic disease were dosed for four weeks with oral reduced glutathione supplementation (500 mg twice daily) or placebo. No significant changes were observed in the measures of oxidative stress used in the study, including glutathione status, i.e., concentrations of reduced, oxidized, total (glutathione + the oxidized sulfide form GSSC) and ratio measures of glutathione status were unchanged by oral supplementation (Allen and Bradley 2011).

Saitoh et al. (2011) proposes the IV administration of glutathione as a strategy to prevent contrast-induced renal oxidative stress. The authors theorize renal oxidative stress may be a cause of contrast-induced nephropathy (CIN), an acute kidney injury that can develop after administration of iodinated contrast material. The exact mechanisms for CIN has yet to be fully evaluated. In a prospective, randomized control study, 21 patients with reduced renal function who underwent elective coronary angiography were administered a control (0.9% saline), oral N-acetylcysteine or IV glutathione for 24 hours to examine the prophylactic effects of IV glutathione for CIN. In all patients, baseline serum and urinary lipid hydroperoxides samples were obtained. The three groups were clinically similar except for the low-density lipoproteins - cholesterol level. CIN occurred in two patients, one in the control and the other in the N-acetylcysteine group. The authors noted study limitations including a small sample size, cystatin

C and liver-fatty acid protein levels, which are known to be the most reliable markers of kidney damage, were not evaluated, some participants were on statin medications which can preclude CIN, and the dose of glutathione necessary to prevent renal oxidative stress by contract medium without adverse effects was not determined. The authors concluded glutathione may be a potential therapeutic strategy against CIN, although this novel therapy should be withheld until larger prospective randomized trials demonstrate its efficacy and safety. The clinical implications of this study are unclear.

b. Severity

No intended treatment population related to this use has been defined as glutathione for oxidative stress is intended to affect the structure or function of the body rather than treat a particular disease or condition; therefore, severity cannot be addressed.

c. Alternative Therapies

No alternative therapies are currently approved to treat oxidative stress.

d. Conclusion

Scientific publications were not found that define a population, dose or risk associated with glutathione for oxidative stress, thus available data do not support the effectiveness of glutathione for oxidative stress. In addition, the nominator has not provided evidence of any clinical benefit associated with the use of glutathione to reduce oxidative stress.

7. *Reduction of the side effects of chemotherapy*

a. Clinical Information

"An ideal chemoprotective agent is one with chemoprotective capacities, without side-effects and which does not reduce anti-tumor efficacy" (Hospers et al. 1999). In alignment with this view, there have been many efforts to identify a chemoprotective agent that will help reduce, in particular, chemotherapy induced peripheral neuropathy. Glutathione has been studied with this intent.

The American Cancer Society (2016) stated:

> Many treatments have been used to try to prevent chemo-induced peripheral neuropathy (CIPN), including glutathione, Vitamin E, calcium and magnesium, some anti-seizure drugs (e.g., Tegretol), and some antidepressants (e.g., Effexor). So far, study results have been mixed for these treatments, and more research is needed.[29]

[29] Available at: https://www.cancer.org/treatment/treatments-and-side-effects/physical-side-effects/peripheral-neuropathy/preventing-cipn.html. Accessed Apr 18, 2022.

The American Society of Clinical Oncology Clinical Practice Guideline for the Prevention and Management of Chemotherapy Induced Peripheral Neuropathy (CIPN) in Survivors of Adult Cancers states:

> Due to a lack of high-quality, consistent evidence, no established agents are recommended for the prevention of CIPN in people with cancer undergoing treatment with neurotoxic agents. Specifically, the following agents should not be offered for prevention of CIPN: acetyl-L-carnitine, amifostine, amitriptyline, CaMg, dietyldithio-carbamate, glutathione, nimodipine, Org 2766, all-trans-retinoic acid, rhuLIF, and vitamin E. (Hershman et al. 2014).

The Cochrane Database of Systemic Reviews contains one review of the evidence about the effect of treatments to prevent or reduce damage to nerves from cisplatin or other platinum-containing chemotherapy. The authors concluded:

> At present, the data are insufficient to conclude that any of the purported chemoprotective agents (acetylcysteine, amifostine, calcium and magnesium, diethyldithiocarbamate, glutathione, Org 2766, oxcarbazepine, retinoic acid or vitamin E) prevent or limit the neurotoxicity of platin drugs among human patients, as determined using quantitative, objective measures of neuropathy.… In summary, the present studies are limited by the small number of participants receiving any particular agent, a lack of objective measures of neuropathy, and differing results among similar trials, which make it impossible to conclude that any of the neuroprotective agents tested prevent or limit the neurotoxicity of platinum drugs (Albers et al. 2014).

When 185 patients undergoing treatment with paclitaxel and carboplatin were randomized to receive either intravenous placebo (n=91; 100 mL of 0.9% normal saline) or 1.5 g/m^2 glutathione (n=94) administered over 15 minutes immediately prior to chemotherapy, there were no statistically significant differences between the two study arms with regard to: 1) peripheral neurotoxicity, assessed utilizing both the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire- Chemotherapy-Induced Peripheral Neuropathy) (p=0.21) and the National Cancer Institute Common Terminology Criteria for Adverse Events scales (p=0.449 for grade 2+ neurotoxicity; p=0.039 for time to development of grade 2+ neuropathy, in favor of the placebo); 2) the degree of the paclitaxel acute pain syndrome (p=0.30 for patients who received every 3–4 week paclitaxel vs. p=0.002, in favor of the placebo, for patients who received weekly paclitaxel); 3) the time to disease progression (p=0.63); or 4) apparent toxicities. Subgroup analysis did not reveal any evidence of benefit in any subgroup (Leal et al. 2014; NCT02311907).

A feasibility study of high-dose cisplatin and 5-fluorouracil with glutathione protection was conducted in 11 untreated patients with measurable metastatic colorectal cancer. Every 4 weeks, the patients received 5-fluorouracil 750 mg/m^2 as a daily continuous infusion on days 1-5 and cisplatin 40 mg/m^2 as a one-hour infusion on days 6-8 with reduced glutathione (glutathione) 2.5 grams administered intravenously prior to each cisplatin infusion. The hydration protocol consisted of 2.5 liters of 5% dextrose with electrolytes daily on days 6-9. Toxicity was minimal and reversible and included nausea/vomiting (11 cases), mild neurotoxicity (4 cases) and leukopenia (2 cases) with 2 patients showing moderate, and transient increased of serum creatinine (< 2 mg/dl) and BUN. Out of 10 evaluable patients, only 2 partial responses were

observed, so the study was closed since the results did not suggest any therapeutic advantage in adding cisplatin to 5-fluorouracil in the present schedule. The authors stated that indirect evidence suggested that these disappointing results were not the consequence of interference of glutathione on the cytotoxic efficacy of cisplatin (Cozzaglio 1990). This study did not provide evidence that glutathione provides protection from chemotherapy.

A total of 35 ovarian carcinoma patients received IV cisplatin 90 mg/m^2 and cyclophosphamide 600 mg/m^2 every 3 weeks. Glutathione 5 g in 200 mL normal saline was intravenously administered to all patients prior to cisplatin to potentially provide chemoprotection from cisplatin adverse effects. The hydration protocol was 1000 mL of fluids without diuretics (the authors stated that conventional hydration for safe cisplatin administration was 2000-3000 mL of fluids). Treatment was well tolerated with no nephrotoxic or neurotoxic effects (Bohm et al. 1991).

When 22 patients with advanced head and neck carcinoma were treated with 5-fluorouracil 400 mg/m^2, folinic acid 500 mg/m^2, cisplatin in escalating doses from 20-40 mg/m^2, and glutathione 1.5 g/m^2, the glutathione appeared to be able to reduce, at least partially, cisplatin-related nephrotoxicity permitting the delivery of higher cisplatin doses. Grade 1 renal toxicity was seen in two of the 22 patients at the cisplatin dose of 30 mg /m^2 per week without forced diuresis. Grade 2 renal toxicity was recorded in the first two patients who received cisplatin 40 mg/m^2 per week with severe impairment of planned dose-intensity. Therefore, no further patients were entered at the cisplatin 40 mg/m^2 dose level (Gebbia et al. 1992).

A total of 79 patients (enrolled 1986-1990) with stage III and IV ovarian cancer (i.e., bulky or extensive residual disease after primary laparotomy or with bulky inoperable tumor masses) were treated with up to five courses of high-dose cisplatin 40 mg/m^2 daily for four days, cyclophosphamide 600 mg/m^2 as an IV bolus on day 4 and glutathione 2.5 grams as a short-term infusion before each administration of cisplatin. The hydration protocol was 2000 mL of fluids without diuretics. Nephrotoxicity was minimal with a transient increase (to < 2 mg/dL) in serum creatinine in 6 patients. Peripheral neurotoxicity and ototoxicity were the most significant long-term toxicities with 3 patients discontinuing therapy (after 4 courses) due to neurotoxicity (Di Re et al. 1993).

In a randomized, double-blind, placebo-controlled, 15 week treatment study, 50 patients with advanced gastric cancer were treated with a weekly cisplatin-based regimen. IV glutathione 1.5 g/m^2 in 100 mL normal saline was also administered to 25 of these patients over a 15-minute period immediately before dosing with once weekly cisplatin 360 mg/m^2, in addition to glutathione 600 mg by IM injection on days 2-5, with saline administered (instead of glutathione) to the 25 placebo patients. After 9 weeks, no patient showed clinically evident neuropathy in the glutathione arm, whereas 16 patients in the placebo arm did. After the 15 weeks, 4 of the 24 assessable patients in the glutathione arm suffered from neurotoxicity versus 16 of 18 in the placebo arm (p=0.0001). The response rate was 76% (20% complete response [CR]) in the glutathione group and 52% (12% CR) in the placebo arm (Cascinu et al. 1995).

Weekly IV glutathione 1.5 grams/m^2 (along with weekly cisplatin 40 mg/m^2, fluorouracil 500 mg/m^2, epi-doxorubicin 35 mg/m^2 and 6S-stereoisomer of leucovorin 250 mg/m^2 and on the other days, filgrastim 5 mg/kg) was administered to 105 patients with advanced gastric cancer to reduce cisplatin-induced neurotoxicity. One cycle consisted of eight 1-week treatments. Patients who showed a response or stable disease received a further 6 weeks of therapy. Mean survival was 11 months, with 2-year survival rate of 5%. Only three subjects complained of neurotoxicity: one WHO grade 1 and two WHO Grade 2 (Cascinu et al. 1997).

Glutathione 2.5 grams was intravenously administered to 50 patients with untreated stage III or IV epithelial ovarian cancer before each cisplatin (40 mg/m^2 once daily on Days 1-4) and carboplatin (160 mg/m^2 once daily on Day 5) administration. Patients also underwent standard IV hydration. The cycle was repeated after 28 days. After 2 courses of induction chemotherapy, the patients underwent surgical reevaluation with debulking, followed by a further 3 cycles of 120 mg/m^2 cisplatin. Toxicity was moderate with lack of significant nephrotoxicity. Neurotoxicity and ototoxicity were acceptable, and no patient discontinued treatment due to toxicity (Bohm et al. 1999).

A total of 52 patients with advanced colorectal cancer received glutathione 1.5 grams/m^2 or normal saline placebo before each bimonthly dose of oxaliplatin. The dose of oxaliplatin was 400 mg/m^2 for the first four cycles of treatment, then 800 mg/m^2 for the next eight cycles of treatment and then 1200 mg/m^2 for the last 12 cycles of treatment. At the fourth cycle, seven patients showed clinically evident neuropathy in the glutathione arm, whereas 11 patients in the placebo arm did. After the eighth cycle, nine of 21 assessable patients in the glutathione arm suffered from neurotoxicity compared with 15 of 19 in the placebo arm. The neurophysiologic investigations (sural sensory nerve conduction) showed a statistically significant reduction of the values in the placebo arm, but not in the glutathione arm. The response rate was 26.9% in the glutathione arm and 23.1% in the placebo arm, showing no reduction in activity of oxaliplatin (Cascinu et al. 2002).

A total of 27 patients were randomized to receive glutathione 1.5 grams/m^2 or saline solution before their oxaliplatin/5-flurouracil/leucovorin (FOLFOX) regimen for colorectal cancer. While the glutathione group showed a statistically significant reduction of neurotoxicity (p=0.0037) compared to the placebo arm, the glutathione group also demonstrated a significantly lower (p=0.0356) oxaliplatin PK parameter "total area under the plasma concentration time curve", i.e., median oxaliplatin AUC_{tot} (ng x h/mL) for the Control group was 166,950 ng x h/mL, while it was only 127,260 ng x h/mL for the glutathione group, and a significantly smaller (p=0.0066) apparent steady-state volume of distribution when glutathione was co-administered and the PK results were evaluated for oxaliplatin in ultrafiltered plasma (Milla et al. 2009).

In summary, results of the studies using glutathione for reduction of side effects of chemotherapy are mixed. Some show potential benefit but they are small studies and lack a control arm. The largest placebo-controlled study showed no benefit of glutathione, and one study showed that glutathione significantly lowered the level of a chemotherapeutic agent which may affect efficacy.

b. Severity

Side (adverse) effects of chemotherapy can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies approved to reduce the side effects of chemotherapy include palifermin injection, amifostine, dexrazoxane, and mesna.

d. Conclusion

Several small studies have been conducted to show glutathione's effects on side effects related to different chemotherapeutic agents for various cancers. Available data are insufficient to support the effectiveness of glutathione for reduction of side effects of chemotherapy. FDA concurs with health professional organizations that there is lack of high-quality and consistent evidence to support the use of certain agents, including glutathione, to prevent chemo-related peripheral neuropathy, and that study results of glutathione to prevent chemo-related peripheral neuropathy have been mixed and more research is needed. In addition, the existence of approved drugs to treat the disease weigh against including glutathione on the list, particularly in light of adverse effects of chemotherapy being serious or life-threatening.

8. Inhibition of Chemical Induced Carcinogenesis

a. Clinical Information

Human clinical trials evaluating whether glutathione use inhibits carcinogenesis were not located; however, 43 respondents to a survey of complementary, alternative and integrative medicine practitioners stated that they used glutathione for cancer (Chen et al. 2011).

"Although not all antioxidants are equal, a variety of cancer chemoprevention clinical trials (with, e.g., selenium or vitamin E supplementation) have uniformly yielded negative results. Such trials are notoriously difficult to design and implement because endpoints require long-term follow-up. Perhaps there are ways to redesign and revisit prevention trials; however, somewhat contentiously there is growing evidence that antioxidants may in fact worsen cancer prognosis and survival. In this regard, animal studies using a genetically engineered mouse model that mimics early non–small cell lung cancer showed that antioxidants were harmful, not beneficial" (Tew et al. 2016).

Glutathione use in cancer must be approached with caution, since some tumors may utilize it intracellularly to resist chemotherapy drugs (Mulder and Ouwerkerk-Mahadevan 1997; No author, Alternative Medicine Review 2001). Glutathione has been implicated in resistance of tumor cells against the chemotherapeutic agent L-phenylalanine mustard (Rothbarth et al. 2002).

The role of glutathione in cancer is a complex one. Because increased levels of glutathione are often associated with increased resistance to cancer chemotherapeutic drugs, selective inhibitors of glutathione synthesis, such as BSO, are being studied with phase 1 trials of BSO in advanced

cancers reporting a relative non-toxic depletion of tumor glutathione with continuous BSO infusions (Balendiran et al. 2004).

It is a concern that IV glutathione may increase resistance to cancer chemotherapeutic drugs.

b. Severity

The chemical induced carcinogenesis to which this use is intended to refer is unclear and its severity cannot be assessed.

c. Alternative Therapies

Alternative therapies approved to inhibit chemical induced carcinogenesis, such as lung cancer, include stopping smoking with smoking cessation treatments such as nicotine replacement therapies (e.g., nicotine polacrilex gum and skin patch) and varenicline.

d. Conclusion

Available data do not support the effectiveness of glutathione in the treatment of chemical induced carcinogenesis. Additional information provided by nominators that address the potential effects of the substance on the structure or function of the body does not address any clinical benefits of the substance for the proposed use.

9. Prevention of radiation injury

a. Clinical Information

Bump and Brown (1990) identified that a strategy for in vivo modification of glutathione levels in healthy tissue to provide radioprotection has not yet been devised. Theoretically, glutathione could provide radioprotection by scavenging radical oxygen species, restoring damaged molecules by hydrogen donation, reducing peroxides and maintaining protein thiols in the reduced state. However, once cancer has occurred, radiation therapy and some forms of chemotherapy rely on ROS toxicity to eradicate tumor cells, which raises the question of whether antioxidants help or hinder the overall outcome of cancer treatment. “At present, with limited available data, most radiation oncologists counsel their patients to refrain from taking antioxidant supplements during radiation therapy.” … “When considering antioxidant supplementation during treatment, it is doubtful whether high doses of radiation given in certain treatments would be rendered less effective if patients took a daily supplement of antioxidants-for example, at RDA levels-yet, we do not know, and more research is needed” (Borek et al. 2004).

In a small, prospective, randomized, placebo-controlled study of 30 women undergoing external-beam radiation for breast cancer, 15 women applied a compounded topical skin gel (“RayGel” containing glutathione and anthocyanins; formulated by a local naturopathic physician) to their breasts 1 to 3 hours prior to radiation therapy compared to 15 women who applied an inert, water-based placebo gel. Severity score was assigned after evaluation of photographs taken every 2 weeks during therapy and at 2 months following completion of the course of radiation.

The "whole breast severity score" was lower in the RayGel group at 93.7 compared to the placebo group at 123. The RayGel group had an average score that was 14% less than the placebo group. The clinicians noted a trend toward the RayGel being less effective as the study progressed and stability tests of the active product did confirm the degradation issue. This resulted in the product being reformulated to a nonaqueous gel with "a high degree of stability." In the discussion section that followed the study report, it was noted that the study had some shortcomings. It was too small to demonstrate statistical significance, patients were not stratified by risk factors associated with radiation damage or by the size of the breast and two patients initially randomized were excluded from the final analysis without seeing what effect removing them would have on the results. In addition, due to the two substances being biologically active and absorbed transdermally, this potentially cell protective compound could get into cancer cells in the vicinity and provide them with protection during irradiation, which would defeat the very purpose of the radiotherapy (Enomoto et al. 2005).[30]

b. Severity

Radiation injury associated with cancer chemotherapy can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies to prevent radiation injury include amifostine injection.

d. Conclusion

Available data do not support the effectiveness of glutathione to prevent radiation injury.

10. Treatment of heavy metal poisoning: cadmium and mercury

a. Clinical Information

No clinical studies of glutathione for the treatment of heavy metal poisoning were found.

While glutathione and N-acetylcysteine have been recommended in the past for the treatment of mercury toxicity, "an examination of available evidence suggests these agents may in fact be counterproductive." Given the inefficiency of elimination of methylmercury via the bile, the known enter-hepatic cycling of methylmercury and the mercury uptake mechanisms of the kidney and brain, "N-acetylcysteine and glutathione would appear to be poor treatment choices for mercury toxicity due to the high risk of redistribution of mercury to those organs" (Rooney 2007).

The substance causing toxicity could modify the potential effect of glutathione treatment. Trivalent inorganic arsenicals, such as arsenite, readily react with glutathione and cysteine and

[30] Per the information provided at https://clinicaltrials.gov for NCT00266331, a larger (i.e., 150 women were enrolled) prospective, blinded, randomized clinical trial of RayGel versus placebo as an alternative for skin care during beam radiation was conducted from October 2004 to May 2011. No scientific publications for this larger RayGel trial were in PubMed.

decrease their bioavailability. Thus, exposure to arsenite is likely to cause depletion of glutathione level (Flora 2009).

In China, administration of glutathione during Ca^{++} EDTA chelation therapy in a 54-year-old patient with chronic cadmium intoxication was associated with a higher blood cadmium level ($p < 0.01$) compared to glutathione alone or EDTA alone and significantly higher urine cadmium excretion, compared to glutathione alone or EDTA alone (Gil et al. 2011) (See Table 7).[31]

Table 7. Cadmium levels (Gil et al. 2011)

	1st session			2nd session		
	EDTA with glutathione	Glutathione	EDTA	EDTA with glutathione	Glutathione	EDTA
Serum cadmium (μg/L)	7.45	5.7	5.65	7.43	5.40	5.40
Urine cadmium (μg/g creatinine)	88.28	17.11	8.75	90.17	19.82	10.98
Urine β_2-microglobulin (ng/mg creatinine)	6353.94	6514.65	5422.95	5594.88	6856.28	5681.31
Urine protein (mg/g creatinine)	123.93	133.18	103.62	110.94	93.84	115.99

However, there was no significant difference between the basal level and the glutathione-alone or EDTA-alone treatments. Six months after the chelation trial ended, the patient still reported similar levels of pain as before the chelation trial (Gil et al. 2011).

While one patient with cadmium poisoning treated with both Ca^{++} EDTA 500 mg and glutathione appeared to have some decline in serum cadmium levels and rise in urinary excretion of cadmium, it is unclear if this was due to treatment sequence, i.e., due to it being the first active treatment. It is concerning that no effect was seen in this one patient during the periods when each treatment was administered as monotherapy and there was no improvement in the symptom of pain.

In a study comparing liver biopsies from six copper-overloaded (Wilson's Disease) patients, hepatic glutathione was markedly lower in five of the six patients compared to nine control group subjects (Summer and Eisenburg 1985). However, no randomized controlled trials evaluating the use of glutathione have yet been conducted in humans with copper overload states (Kidd 1997).

Glutathione may have a role in preventing heavy metal toxicity by acting as a cofactor in detoxication pathways, transporting amino acids and in free radical scavenging. Glutathione can chelate transition metal ions, thereby reducing their toxic potential (Flora et al. 2013). However, the use of glutathione as a chelating agent is limited by its lack of selectivity, which causes the removal of essential metal ions (Jan et al. 2011).

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[31] During Days 1-3, only normal saline 1 L/day was administered. During Days 4-6, Ca^{++} EDTA 500 mg and glutathione 50 mg/kg in 1 L normal saline/day was administered. During Days 7-9, Ca^{++} EDTA 500 mg in 1 L normal saline/day was administered. During Days 10-12, glutathione 50 mg/kg in 1 L normal saline/day was administered. One month later, the same protocol was repeated.

b. Severity

Heavy metal poisoning can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies approved to treat heavy metal poisoning from cadmium or mercury include dimercaprol 10% injection, Calcium Disodium Versenate (edetate calcium disodium injection), and penicillamine. Penicillamine is a chelating agent recommended for the removal of excess copper in patients with Wilson's disease which has been used in the treatment of mercury-induced acrodynia (pain and dusky pink discoloration of hands and feet) and succimer (orally acting, heavy metal chelating agent approved for lead poisoning) which has produced varying degrees of symptomatic improvement in patients with mercury poisoning.

d. Conclusion

There is currently insufficient support for the treatment of heavy metal poisoning with glutathione. While exposure to heavy metals has been associated with a depletion of glutathione levels, this does not support the use of glutathione as a treatment of heavy metal poisoning. The use of glutathione in heavy metal toxicity may harm patients because the lack of glutathione selectivity results in the removal of essential metal ions.

11. Acetaminophen Toxicity

a. Clinical Information

No clinical studies of glutathione in the treatment of acetaminophen toxicity were found.

Acetaminophen and other pharmaceutical or environmental xenobiotics can deplete liver glutathione. The depletion of cellular glutathione leaves the cell particularly vulnerable to oxidative insults following acetaminophen overdose (No author, Alternative Medicine Review 2001).

b. Severity

Acetaminophen toxicity can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies approved for the treatment of acetaminophen toxicity include acetylcysteine (N-acetyl-L-cysteine) solution 20% for oral administration and acetylcysteine 20% solution injection for IV administration.

d. Conclusion

FDA did not identify any data to support the effectiveness of glutathione in the treatment of

acetaminophen toxicity. In addition, the existence of approved drugs to treat the condition weigh against including glutathione on the list, particularly in light of acetaminophen toxicity being a serious or life-threatening condition.

12. Autism Spectrum Disorder (ASD)

a. Clinical Information

A Cochrane review (James et al. 2015) of all randomized controlled trials of pharmaceutical agents compared to placebo in individuals with ASD included one study conducted from May 2005 to April 2007 by the Southwest College of Naturopathic Medicine in two phases (Adams et al. 2009; NCT00811083). During the first part of the study, 77 children with ASD were randomly assigned to received seven days of glutathione lotion or placebo lotion, followed by three days of oral dimerceptosuccinic acid (DMSA). The 49 children who were found to be "high excreters of heavy metals" during phase one continued to phase two to receive three days of oral DMSA or placebo followed by 11 days off, with the cycle repeated up to six times. Per the Cochrane review, no evidence suggested that multiple rounds of oral DMSA had an effect on ASD symptoms measured in children found to be high excreters who had already received three doses of DMSA.

Johns Hopkins University Center for Excellence in Regulatory Science and Innovation (JHU CERSI) conducted an "Evaluation of Bulk Drug Substances Used to Compound Drug Products For Patients with ASD: Phase II, Review of Available Evidence of Safety and Effectiveness And Evaluation of Current and Historical Use" (JHU CERSI 2020). See additional information about the JHU CERSI 2020 report in Section II.D. The JHU CERSI 2020 report identified one study in which 26 children ages 3 to 13 were randomized to receive either transdermal or oral "lipoceutical" glutathione (Kern et al. 2011). The publication did not report efficacy outcomes. Three Key Opinion Leaders, expert practitioners in the ASD field, were interviewed about the potential for therapeutic benefit of various substances. The text below has been extracted from JHU CERSI report regarding glutathione.

> Little was known about glutathione and inositol (another substance under study by JHU for ASD) treatments as they are rarely prescribed or recommended for ASD in clinical practice.

b. Severity

ASD is serious but is not life-threatening.

c. Alternative Therapies

No alternative therapies are currently approved to treat ASD; however, risperidone and aripiprazole are approved to treat the irritability associated with autism.

d. Conclusion

FDA did not identify any data to support the effectiveness of glutathione in the treatment of Autism Spectrum Disorder.

13. Alzheimer's Disease (AD)

a. Clinical Information

No clinical studies were found in which glutathione was used in the treatment of AD.

Glutathione has been shown to decline with aging and in several age-related degenerative diseases, including AD. However, administration of glutathione alone is considered ineffective for increasing intracellular glutathione levels during conditions of acute and chronic oxidative stress. In addition, glutathione does not pass through the blood-brain barrier (Braidy et al. 2015).

Mild cognitive impairment (MCI) is a syndrome considered to represent an at-risk state for dementia with studies suggesting the rate of conversion from MCI to AD is 15% per year. When 54 patients with MCI and 41 healthy control subjects underwent proton magnetic resonance spectroscopy, the concentration of glutathione was measured in the anterior and posterior cingulate and ratios of glutathione were calculated relative to creatine. In comparison with control subjects, patients with MCI had significantly elevated ratios of glutathione in the anterior ($t = -2.2$, $p = 0.03$) and posterior ($t = -2.9$, $p = 0.005$) cingulate. These findings, showing an elevation of glutathione, may be indicative of an early compensatory or neuroprotective response rather than the expected initial disease induced declines (Duffy et al. 2014).

Glutathione concentrations in two key brain regions that are affected by AD pathology [i.e., bilateral hippocampi (HP) in 21 subjects with AD, 22 with mild cognitive impairment (MCI) and 21 healthy old controls; and bilateral frontal cortices (FC) in 19 subjects with AD, 19 with MCI and 28 healthy old controls] were estimated by MEGA-PRESS (MEscher-GArwood-PRESS) in vivo proton magnetic resonance spectroscopy (MRS). AD-dependent reduction of glutathione was observed in both HP and FC ($p < 0.001$). The authors concluded that glutathione levels estimated with MRS in the HP and FC regions constitute clinically relevant biomarkers for AD and MCI (Mandal et al. 2015).

Intracellular levels of glutathione have been measured in lymphoblast lines from 12 patients with familial AD [with presenilins (PS) or amyloid precursor protein (APP) gene mutations], 5 patients with sporadic AD, and 9 age-matched controls. Levels of glutathione and total glutathione in cells from patients with sporadic AD were very similar to those in control cells. However, lymphoblasts carrying PS and APP gene mutations showed significantly decreased glutathione content with respect to controls (Cecchi et al. 1999).

b. Severity

Alzheimer's disease can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies approved for the treatment of Alzheimer's disease include cholinesterase inhibitors such as donepezil, rivastigmine, and galantamine; glutamate regulators such as memantine; cholinesterase inhibitor + glutamate regulator such as donepezil, and memantine; and orexin receptor antagonist such as suvorexant and aducanumab.

d. Conclusion

FDA did not identify any data to support the effectiveness of glutathione in the treatment of Alzheimer's disease. Additional information provided by the nominator addressing use of glutathione to affect the structure or function of the body does not provide evidence of clinical benefit on the use of glutathione in Alzheimer's disease. In addition, the existence of approved drugs to treat the disease weigh against including glutathione on the list, particularly in light of Alzheimer's disease being a serious or life-threatening disease.

14. Parkinson's Disease

a. Clinical Information

In an open label, uncontrolled study enrolling 9 patients with early untreated Parkinson's disease, IV infusions of glutathione (600 mg in 250 mL saline, over 1 hour, twice daily for 30 days) were administered with results showing a "42% decline in disability"[32] after one month of treatment. This effect lasted 2–4 months after discontinuation of the infusions. A total of glutathione 36,000 mg was administered over the one month. Two of the 9 patients during week 3 of IV infusion of glutathione had fever, erythema of the skin, irritation and hardness at injection site, "likely due to infusion thrombophlebitis" (Sechi et al.1996).

In a review article on the use of antioxidants and other supplements for the prevention and treatment of Parkinson's disease, the authors concluded at present, antioxidants and supplements appear to have a limited role in the prevention or treatment of Parkinson's disease (Weber and Ernst 2006).

In a randomized, double-blind, placebo controlled pilot, Phase 2 study that enrolled 21 patients (11 received glutathione, 10 received placebo) with Parkinson's disease whose motor symptoms were not adequately controlled with their current medication regimen, the addition of IV administration of glutathione 1,400 mg (diluted in 10 mL of normal saline and administered by IV push over 10 minutes) three times a week (Monday, Wednesday, Friday) for 4 weeks to their current medication regimen produced no significant differences from placebo (p=0.32) in the Unified Parkinson's Disease Rate Scale (UPDRS) ADL + Motor scores. A total of glutathione

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[32] Mean % improvement from baseline total score (i.e., baseline total score was 27 ± 10) in the Modified Columbia University Rating Scale (CURS) Total Scores for these 9 subjects were 42% ± 16% after one month of twice daily infusions. After a 2-4 month wash-out period, these same 9 subjects returned to approximately the same mean baseline Modified CURS Total Score (i.e., 27 ± 8) and were then treated with oral levodopa 375 mg/day + carbidopa 37.5 mg/day with mean % improvement from baseline total score in the Modified CURS Total Scores for the 8 subjects who returned for follow-up being 44.5% ± 19% after one month of oral levodopa and carbidopa treatment.

16,800 mg was administered over the one month (Hauser et al. 2009; per NCT01177319).

A 3-month treatment duration, 3-arm (intranasal glutathione 300 mg/day, 600 mg/day or saline placebo in three divided daily doses), randomized, double-blind, Phase I/IIa study was conducted in 30 patients with Parkinson's disease, with 28 patients completing the study. A mild clinical improvement in UPDRS symptoms was noted in both treatment groups (Mischley et al. 2015; NCT01398748).

A 3-month treatment duration, 3-arm (intranasal glutathione 300 mg/day, 600 mg/day or saline placebo in three divided doses), randomized, Phase IIb study was conducted in 45 patients with Parkinson's disease. Primary endpoint was change in UPDRS. All cohorts, including placebo, improved over the intervention period, and neither treatment group was superior to placebo (Mischley et al. 2017; NCT02424708).

In a Letter to Editor regarding the Hauser et al. 2009 study, described in detail in the following clinical studies section, it was stated: "One of the most commonly asked patient-driven questions about Parkinson's disease therapy has been whether to use glutathione. Glutathione acts as an antioxidant and has been found reduced by as much as 50% in the brains of patients with Parkinson's disease, other parkinsonian disorders and even incidental Lewy body disease and thus is not specific for Parkinson's disease. A small group of physicians have been promoting this therapy and charging a fee for infusion of glutathione service in their office practices. Despite the lack of evidence for efficacy, the lack of data that glutathione crosses the blood brain barrier, the requirement for the placement of an IV line, and the need for patients to pay out of pocket, many medical practices have persisted in offering the therapy." The authors concluded "this glutathione study failed to meet a minimum clinical efficacy criteria that would allow doctors to offer, promote, and charge for the therapy in their office practices. On the basis of these results, patients with Parkinson's disease should be encouraged to say no to an IV placed in their arm for the false hope of symptomatic glutathione treatment" (Okun and Lang 2010).

Although there is a general reduction of glutathione levels in the brain during aging, this reduction is accelerated in Parkinson patients (Smeyne & Smeyne 2013). In Parkinson's disease, mitochondrial failure has been specifically implicated with the substantia nigra becoming greatly depleted of glutathione (No author, Alternative Medicine Review 2001). However, no effective therapies have yet been identified that can enhance glutathione levels in affected brain regions (Ballatori et al. 2009). The threshold of glutathione depletion, below which the cell will usually die, is 70-80 percent. The mitochondria, with their high oxygen radical flux, are particularly vulnerable. Glutathione cannot easily penetrate the blood brain barrier due to the presence of the cysteine SH group and is not efficiently absorbed into neuronal cells in the brain (Bharath et al. 2002). Glutathione itself does not penetrate neurons since they do not possess a glutathione transporter (Martin and Treismann 2009). Thus, the benefits of glutathione treatment for Parkinson's disease, patients remain unclear and will require further studies.

b. Severity

Parkinson's disease can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies approved for the treatment of Parkinson's disease include levodopa/carbidopa; catechol o-methyltransferase (COMT) inhibitors such as entacapone and tolcapone; dopamine agonists such as pramipexole and ropinirole; and Monoamine Oxidase Type B (MAO-B) inhibitors such as selegiline and rasagiline; and anticholinergics such as benztropine and trihexyphenidyl.

d. Conclusion

Available data do not support the effectiveness of glutathione in the treatment of Parkinson's disease. Additional information provided by the nominator on the use of glutathione to affect the structure or function of the body does not provide evidence of clinical benefit on the use of glutathione in Parkinson's disease. In addition, the existence of approved drugs to treat the disease weigh against including glutathione on the list, particularly in light of Parkinson's disease being a serious or life-threatening disease.

15. Major Depressive Disorder (MDD)

a. Clinical Information

No clinical studies of gluthathione in the treatment of MDD were found.

Several factors are believed to enhance the brain's susceptibility to oxidative damage (Berk et al. 2008):

- High oxygen utilization (thus, generating higher amounts of free radical by-products)
- Biochemical environment conducive to oxidation (high lipid content; reducing potential of some neurotransmitters and presence of redox-catalytic metals, for example iron and copper)
- Relatively limited antioxidant defenses (such as albumin and bilirubin)
- Generation of secondary oxidative cellular insults through the neurotoxic effects of released excitatory amines (particularly dopamine and glutamate) and secondary inflammatory responses

While the glutathione system has the most favorable theoretical foundation as a novel therapeutic target for oxidative stress, there are few published clinical trials of antioxidants in primary psychiatric disorders and fewer with a randomized, placebo-controlled design.

When fibroblast cell cultures from 16 patients with MDD and 16 controls were compared, there was no significant difference in glutathione levels between groups; therefore, the findings do not support a role for either glutathione or glutathione reductase in the increased oxidative stress in MDD (Gibson et al. 2012).

b. Severity

MDD can be a serious disease.

c. Alternative Therapies

Alternative therapies approved for the treatment of MDD include selective serotonin reuptake inhibitors such as fluoxetine hydrochloride, and sertraline; serotonin-norepinephrine reuptake inhibitors such as duloxetine and venlafaxine; atypical antidepressants such as bupropion and vortioxetine; tricyclic antidepressants such as imipramine and nortriptyline; and monoamine oxidase inhibitors such as tranylcypromine and phenelzine.

d. Conclusion

FDA did not identify any data to support the effectiveness of glutathione in MDD. Additional information provided by the nominator addressing the effect of glutathione may have on the structure or function of the body does not provide evidence of clinical benefit on the use of glutathione in major depressive disorder. In addition, the existence of approved drugs to treat the disease weigh against including glutathione on the list, particularly in light of MDD being a serious disease.

16. Schizophrenia

a. Clinical Information

No clinical studies of glutathione use in schizophrenic patients were located.

It has been proposed that schizophrenia is associated with atypical levels of glutathione, particularly in the anterior cingulate cortex (ACC). However, in a meta-analysis comparing 261 patients with schizophrenia versus 185 healthy controls, there were no significant differences in ACC glutathione (Dey et al. 2018).[33]

Twenty schizophrenia patients and 16 age-and gender-matched normal controls underwent testing to determine the levels of glutathione in the posterior medial frontal cortex by using 3T SIGNA EXCITE H-MRS with the spectral editing technique, MEGA-PRESS. The glutathione levels in this area of the brain in schizophrenia patients were not different from those of normal controls. An unexpected correlation was noted between lower glutathione levels in the posterior medial frontal cortex and greater severity of negative symptoms in the schizophrenia patients (Matsuzawa et al. 2008).

While total glutathione levels may be decreased in the peripheral blood (plasma) of patients with schizophrenia (Nucifora et al. 2017), no scientific literature was located that demonstrated clinical efficacy after the administration of glutathione to patients with schizophrenia. While some schizophrenia clinical trials have evaluated the use of a glutathione precursor, i.e., N-acetyl cysteine (Dean et al. 2009), no clinical trials of glutathione administered to schizophrenic patients were located.

[33] Abstract available at https://academic.oup.com/schizophreniabulletin/article-abstract/44/suppl_1/S221/4957604. Accessed Apr 18, 2022.

b. Severity

Schizophrenia can be a serious disease.

c. Alternative Therapies

Alternative therapies approved for the treatment of schizophrenia include first-generation antipsychotics such as chlorpromazine and fluphenazine; second-generation antipsychotics such aripiprazole and asenapine; and long-acting injectable antipsychotics such as fluphenazine decanoate and paliperidone.

d. Conclusion

FDA did not identify any data to support the effectiveness of glutathione in schizophrenia. In addition, the existence of approved drugs to treat the disease weigh against including glutathione on the list, particularly in light of schizophrenia being a serious disease.

17. Helicobacter pylori (H. pylori) Infection

a. Clinical Information

No clinical studies of glutathione use to treat *H. pylori* were found.

Gastric mucosa of aged subjects can have low glutathione, as can patients with gastritis and/or duodenal ulcer linked to *H. pylori* infection (No author, Alternative Medicine Review 2001).

"*H. pylori* is known to feed on macrophages and neutrophils abundant at the site of inflammation caused by the ulcer. As glutathione can improve the numbers and activity of macrophages, peptic ulcers may be exacerbated" (Sonthalia et al. 2016).

b. Severity

H. plylori infections can result in serious symptoms.

c. Alternative Therapies

Alternative therapies approved for the treatment of *H. pylori* infection include triple therapy consisting of a combination administration of a proton pump inhibitor such as lansoprazole, esomeprazole, pantoprazole or rabeproazole; antibiotics such as clarithromycin, and amoxicillin; and bismuth.

d. Conclusion

FDA did not identify any data to support the effectiveness of glutathione in *H. pylori* infection.

18. Human Immunodeficiency Virus (HIV) Infection

a. Clinical Information

HIV infection and sequelae feature systemic glutathione depletion. The cachexia and wasting of AIDS may be amenable to glutathione repletion. HIV depletion of lung epithelial lining fluid (ELF) glutathione may predispose to opportunistic infections, and the ELF may be repleted using aerosolized glutathione (No author, Alternative Medicine Review 2001).

"Glutathione levels were determined in freshly isolated T cells from 13 healthy subjects and 13 individuals with HIV infection. We observed that glutathione concentrations were significantly lower in T cells isolated from individuals with HIV infection compared to T cells from healthy subjects" (Guerra et al. 2011).

While it has been noted that there is a significant decrease in the levels of glutathione in peripheral blood mononuclear cells (PBMCs) and red blood cells (RBCs) isolated from individuals with HIV infection (Morris et al. 2014b), research has not yet shown that treatment with glutathione is effective treatment for HIV infection.

Purified glutathione 600 mg in 4 mL saline was delivered over 25 minutes by aerosol twice daily for 3 days to 14 HIV seropositive individuals. Glutathione levels in lung epithelial fluid were compared before and at 1, 2, and 3 hours after aerosol administration. No symptoms referable to the aerosol administration of glutathione were noted. While glutathione levels in the epithelial lining fluid increased after administration, the levels returned to baseline by 3 hours after administration. Glutathione levels in venous plasma did not change significantly during the study period. The authors noted that this study does not show clinical efficacy such as a reduction in the incidence of pulmonary opportunistic infections or evaluate the exact pattern of deposition of the glutathione in the lungs (Holroyd et al. 1993).

In a double-blind, placebo-controlled study 30 HIV-infected individuals with CD4+ T cell counts below 350 cells/mm^3 were given either placebo or "liposomal glutathione" (L-glutathione) over a 3-month period. 15 of the HIV-infected individuals resulted in an increase in the levels of IL-12, IL-2 and IFN-γ and a decrease in levels of IL-6, IL-10 and free radicals and no change in the levels of TGF-β, IL-1 and IL-17 when compared to their placebo counterparts. Those in the placebo group showed no significant difference throughout the study. The authors concluded, "supplementation with L-GSH in HIV-infected individuals with CD4+ T cell counts below 350 cells/mm^3 can help restore redox homeostasis and cytokine balance, therefore aiding the immune system to control opportunistic infections" (Valdivia et al. 2017).

b. Severity

HIV infection can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies approved for the treatment of HIV include bictegravir/emtricitabine/

tenofovir alafenamide, ibalizumab-uiyk, dolutegravir and ripivirine, emtricitabine and tenofovir alafenamide, darunavir and cobicistat, and crofelemer.

d. Conclusion

While glutathione levels may be decreased in patients with HIV infection, no scientific literature was located that supported clinical efficacy after the administration of glutathione to patients with HIV infection. Additional information provided by the nominator on the effect glutathione may have on the structure or function of the body does not provide evidence of clinical benefit on the use of glutathione in HIV infection. In addition, the existence of approved drugs to treat the disease weigh against including glutathione on the list, particularly in light of HIV infection being serious or life-threatening.

19. Tuberculosis (TB)

a. Clinical Information

No clinical studies of glutathione use to treat TB were found.

Upward trends in the global incidence of TB are largely the result of the emergence of multidrug resistant *Mycobacterium tuberculosis* (MDR-*M. tb*) strains and an increasing number of immunocompromised individuals due to the AIDS pandemic. "We anticipate that research in the coming years will find a likely role for glutathione as a highly promising immune-adjunctive component of treatment protocols in MDR-TB, particularly in immunocompromised individuals suffering from HIV" (Allen et al. 2015).

It has been noted that there is a significant decrease in the levels of glutathione in PBMCs and RBCs isolated from individuals with active TB. The authors concluded that research has not yet shown that treatment with glutathione is effective treatment for tuberculosis (Morris et al. 2013d).

b. Severity

Tuberculosis can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies approved for the treatment of tuberculosis include bedaquiline (for treatment of multi-drug resistant tuberculosis), rifapentine, rifampin, isoniazid, ethambutol, and pyrazinamide.

d. Conclusion

FDA did not identify any data to support the effectiveness of glutathione in tuberculosis.
In addition, the existence of approved drugs to treat the disease weigh against including glutathione on the list, particularly in light of tuberculosis being a serious or life-threatening

disease.

20. Otitis Media

a. Clinical Information

A randomized, placebo-controlled clinical trial enrolled 60 children 3 to 12 years of age with a middle ear effusion that had persisted for 3 months or longer (30 treated with glutathione 600 mg/day in 4 mL saline by nasal aerosol subdivided into 2 minute administrations every 3 or 4 waking hours for a total of 5 daily administrations for 2 weeks and 30 receiving only 4 mL saline, with same procedures as the glutathione group). Improvement was assessed by otoscopy (to determine discolored tympanic membrane (TM), TM opacity or air-fluid level), tympanometry (to determine tympanometry type), and audiometry (to assess hearing loss). Treatment of otitis media with effusion with nasal aerosol administration of reduced glutathione suggested improvement in two-thirds of the patients with otitis media, i.e., at one-month follow-up, 20 glutathione-treated patients and 5 placebo-treated patients showed improvement. Only patients who had improved in one or both ears at the first follow-up were to return 2 months later. At three months after treatment, improvement had occurred in 18 glutathione-treated patients and 3 placebo-treated patients. The author identified the complexity of otitis media, having "multifactorial" causes (e.g., bacterial, inflammatory) and therefore treatment aspects (Testa et al. 2001).

b. Severity

Otitis media is generally not a serious condition.

c. Alternative Therapies

Alternative therapies approved for the treatment of the bacterial infection in otitis media include antibiotics such as amoxicillin, azithromycin, and ceftriaxone; and ear drops such as floxin otic and ciprofloxacin otic suspension.

d. Conclusion

The minimal data indicating effectiveness for some study participants are insufficient to support effectiveness of glutathione in treating otitis media.

21. Peripheral Obstructive Arterial Disease

a. Clinical Information

A randomized, double-blind, placebo-controlled trial of glutathione was conducted in 40 patients with Fontaine stage II peripheral artery disease (i.e., intermittent claudication; walking-induced leg muscle pain relieved by rest). Twice daily for 5 days, 20 patients received IV glutathione 0.646 gram in 250 mL of 0.9% saline and 20 received 250 mL of IV 0.9% saline. At the end of the infusion period, only the glutathione group showed a statistically significant increase in

plethysmographic values (measuring blood flow in the leg) after treadmill testing compared to rest measurements. This value was higher ($p<0.02$) than that in the saline group at the same state of the study (Arosio et al. 2002). Pain free walking distance (PFWD) increased in the glutathione group from 113±11meters to 221±15 meters during the study and increased in the placebo group from 119±18 meters to 159±26 meters. No statistical comparison of the two treatment groups PFWD changes was provided.

b. Severity

Peripheral obstructive arterial disease can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies approved for the treatment of peripheral obstructive arterial disease include daily aspirin (with or without dipyridamole), clopidogrel, cilostazol, rivaroxaban, and pentoxifylline.

d. Conclusion

The minimal data indicating effectiveness for some study participants is insufficient to support effectiveness of glutathione in treating peripheral obstructive arterial disease. The existence of approved drugs to treat the disease weigh against including glutathione on the list, particularly in light of peripheral obstructive arterial disease being a serious or life-threatening disease.

22. Anemia

a. Clinical Information

In a double-blind, randomized, placebo-controlled study of 20 glucose-6-phosphate dehydrogenase deficient patients with acute hemolytic crisis and anemia, 10 were administered glutathione (at admission, an IV bolus of 30 mg/kg body weight, followed by slow infusion at 30 mg/kg body weight at 6, 12, 18, and 24 hours) and 10 were given placebo. Treatment with glutathione did not modify erythrocytes, platelets, or hemoglobin during the hemolytic crisis and white blood cells and the level of Heinz bodies remained unchanged. The fact that after glutathione administration there was no clinical evidence of its effect on erythrocytes or hemoglobin, may be attributed, per the author, to various factors (drug dose, type of disorder, choice of hemochromocytometric indices and parameters) as well as the poor efficacy of glutathione on erythrocytes with marked congenital enzymopathy (Corbucci 1990).

A total of 28 patients with chronic renal failure were all treated with glutathione 1200 mg IV at the end of each dialysis session for at least 9 months to treat anemia. Of the 28 patients, 14 had been receiving IV or subcutaneous erythropoietin for at least 12 months prior to initiation of this study and the erythropoietin (EPO) treatment was continued in these patients during the study. After 3 months of treatment with glutathione, 17 patients (eight also on EPO and nine not) had significantly improved RBC, hemoglobin, hematocrit, and reticulocytes; however, 11 patients (six also on EPO and five not) did not respond and their red blood cell parameters were

unmodified during glutathione treatment. No comparison to placebo or other treatment is available from this uncontrolled study. The authors had no explanation for why glutathione was ineffective in 40% of the patients, except that perhaps non-responders had a more severe deficiency of other antioxidants, such as vitamin E, so that glutathione alone was unable to improve their anemia (Usberti et al. 1997).

Patients suffering from chronic renal failure (CRF) show normocytic and normochromic anemia. In a double-blind, randomized, placebo-controlled, 120-day treatment duration study of 20 CRF patients, 10 received glutathione 1200 mg and 10 received an equal volume of saline solution, each delivered intravenously three times per week, at the end of each hemodialytic session (Costagliola et al. 1992). While hematocrit and hemoglobin in the glutathione group showed only a minor improvement from Day 0-90, the glutathione group had a significant ($p<0.05$) increase in both hematocrit and hemoglobin on the last treatment day, Day 120 (See Table 8). It is unclear why an abrupt increase occurred between days 90 and 120, when increases during the prior intervals were minimal. Assessment on Days 150 and 180 reflect a decline in parameters following cessation of treatment in the glutathione group.

Table 8. Assessment of anemia (Costagliola et al. 1992**)**

Time days	Hematocrit %	Reticulocytes %	Hemoglobin g/100 ml
Placebo treated			
0	26.06 ± 1.11	1.49 ± 0.01	8.11 ± 0.61
30	25.16 ± 1.03	1.50 ± 0.02	8.15 ± 0.61
60	25.10 ± 0.69	1.30 ± 0.01	8.11 ± 0.69
90	25.00 ± 1.07	1.40 ± 0.02	8.15 ± 0.70
120	25.10 ± 1.10	1.60 ± 0.01	8.18 ± 0.74
150	25.12 ± 0.90	1.50 ± 0.02	8.10 ± 0.65
180	25.15 ± 1.11	1.55 ± 0.01	8.15 ± 0.79
Glutathione treated			
0	27.06 ± 1.02	1.58 ± 0.01	8.18 ± 0.82
30	28.32 ± 1.12	1.55 ± 0.02	8.23 ± 0.82
60	28.45 ± 1.10	1.30 ± 0.01	8.40 ± 0.85
90	28.48 ± 1.10	1.40 ± 0.02	8.57 ± 0.80
120	33.23 ± 1.11*	1.00 ± 0.01*	10.07 ± 0.92*
150	27.92 ± 0.90	1.40 ± 0.02	8.80 ± 0.79
180	26.32 ± 1.00	1.80 ± 0.01	8.10 ± 0.76

Values are mean ± SD; *p < 0.05; **p < 0.01; ***p < 0.005 (St

In four chronic hemodialysis patients, 1200 mg IV glutathione, was administered at the end of each hemodialytic session with a reduction in 2 patients of the need for erythropoietin (Zachee et al. 1995).

b. Severity

Anemia can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies approved for the treatment of anemia include iron (for iron deficiency anemia), ferric carboxymaltose injection (for iron deficiency anemia), hydroxyurea (for sickle cell anemia), peginesatide (for treatment of anemia due to chronic kidney disease), epoetin alfa and epoetin alfa-epbx (for treatment of anemia caused by chronic kidney disease, chemotherapy or use of zidovudine in patients with HIV infection), and taliglucerase alfa (for treatment of Gaucher disease).

d. Conclusion

The minimal data indicating effectiveness for some study participants are insufficient to support effectiveness of glutathione in treating anemia. While a few patients appeared to have less of a need for erythropoietin when a small total number of chronic hemodialysis patients were administered IV glutathione, it appears that the treatment effect from IV glutathione was insufficient to support the continued evaluation of glutathione in patients undergoing chronic hemodialysis. The current standard of care for the treatment of anemia of chronic renal failure includes erythropoiesis-stimulating agents, iron agents and red cell transfusions.[34] The existence of approved drugs to treat the disease weigh against including glutathione on the list, particularly in light of anemia being a serious or life-threatening disease.

23. Diabetes

a. Clinical Information

No clinical studies of the use of glutathione to provide therapeutic effect in patients with diabetes were found.

Twenty subjects (10 healthy controls and 10 patients with Type 2 diabetes mellitus (T2DM) who were not taking metformin or glitazones) underwent an assay of glutathione levels in plasma, RBCs and monocytes. Compared to healthy subjects, there was a two-fold decrease in the levels of total glutathione in RBCs and a three-fold decrease in the levels of total glutathione in monocytes isolated from individuals with T2DM and a two-fold decrease in total glutathione in plasma samples from individuals with T2DM (Lagman et al. 2015).

In diabetics, erythrocytes and platelets can be low in glutathione. Mild to moderate exercise can help normalize glutathione status in diabetics, although strenuous exercise can deplete glutathione (No author, Alternative Medicine Review 2001).

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[34] Kidney Disease Improving Global Outcomes (KDIGO) Clinical Practice Guidelines for Anemia in Chronic Kidney Disease. *Kidney International Supplements.* Aug 2012; 2(4): 279-335. Available at: https://kdigo.org/wp-content/uploads/2016/10/KDIGO-2012-Anemia-Guideline-English.pdf.

When a 1-hour infusion of glutathione ($1.35g \cdot m^2 \cdot min^{-1}$) was administered to 10 non-insulin dependent diabetes mellitus (NIDDM) patients and 10 healthy controls, there was no change in total glucose uptake at 1 or at 2 hours after the start of the infusion in either group; however, there was a statistically significant increase in total glucose uptake in both groups from baseline to final testing point, i.e., at 3 hours after initiation of the infusion (De Mattia et al. 1998).

When 10 elderly subjects with normal glucose tolerance and 10 elderly patients with impaired glucose tolerance (IGT) underwent glutathione infusion (10 mg/min), the glutathione infusion significantly potentiated glucose-induced insulin secretion only in the patients with IGT (Paolissa et al. 1992).

Ten patients with NIDDM (average duration 12.6 years) were administered glutathione 600 mg IM at 6 pm for 10 days to evaluate the effect on PAI-1, a plasminogen activator inhibitor (which is believed to be a biological risk factor for coronary heart disease when it is elevated). Testing for plasma glucose, red blood cell glutathione concentration, platelet aggregation, PAI-1 antigen and tissue plasminogen activator (t-PA) plasma concentrations was performed the day before glutathione treatment was begun and after the 10 days of glutathione administration. Their lab results were compared to 14 normal controls, who had testing for basal plasma values of PAI-1 antigen, t-PA, and RBC glutathione concentration. Because the plasma PAI-1 levels in the NIDDM patients decreased (80.1 ± 5.2 vs 68.4 ± 5.9 ng/mL, $p<0.2$) and the RBC glutathione concentration increased (1.53 ± 0.2 vs 1.99 ± 0.1 $\mu mol/10^{10}$ RBC, $p<0.02$), the authors concluded that their data suggested that glutathione may be useful to improve the fibrinolytic state in NIDDM. Plasma t-PA, platelet aggregation, and basal plasma glucose levels did not change from basal values (Martina et al. 1996).

Fifteen patients with NIDDM (average duration 11.4 years) were administered glutathione 600 mg IM at 6 pm for 10 days to evaluate the effect on platelet constitutive nitric oxide synthase (cNOS). Testing for platelet cNOS, plasma glucose, RBC glutathione concentration, and PAI-1 antigen concentrations was performed the day before glutathione treatment was begun and after the 10 days of glutathione administration. Compared to basal values, RBC glutathione concentration increased (1.4 ± 0.1 *vs* 1.9 ± 0.1 $\mu mol/10^{10}$ RBC, $p<0.001$), platelet cNOS activity increased (0.7 ± 0.1 *vs* 2.9 ± 0.2 $fmol \cdot min\text{-}1 \cdot 10^{-9}$ PLTs, $p<0.001$) and the plasma PAI-1 levels diminished (81.4 ± 3.7 *vs* 68.7 ± 4.0 ng/mL, $p<0.002$). The authors concluded that these data suggest that the administration of glutathione, in patients with T2DM, improves platelet cNOS activity together with a reduction of PAI-1 (Martina et al. 2001).

The functional effects of oral glutathione in 18 patients with T2DM have been evaluated. To et al. (2021) conducted a randomized, double-blind, placebo controlled, parallel group study comparing oral liposomal glutathione (15 mL glutathione in liposomes suspended in liquid for a daily dose of 1260 mg liposomal glutathione, with no glutathione equivalent provided) to a placebo liposome formulation for 3 months. Levels of the proinflammatory cytokine IL-6 and MDA declined with glutathione administration, and glutathione levels increased compared to placebo without statistical significance. In a three week, double-blind, placebo-controlled study of oral doses of 1000 mg glutathione versus placebo in obese patients with and without T2DM (n = 20) whole body insulin sensitivity increased in the glutathione group as measured by a hyperinsulinemic-euglycemic clamp and muscle biopsy. Analysis of variance analysis for

glucose tolerance parameters (e.g., fasting glucose, fasting insulin) did not find that the “group” factor (i.e., placebo or glutathione) was statistically significant as a main effect or interaction (Sondergard et al. 2021, NCT02948673).

b. Severity

Diabetes can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies approved for the oral treatment of diabetes include sulfonylureas such as glipizide and glyburide; biguanides such as metformin; thiazolidinediones such as pioglitozone and rosiglitazone; alpha-glucosidase inhibitors such as acarbose and miglitol; meglitinide repaglinide and nateglinide; DPP-4 inhibitors such as sitagliptin and linagliptin; SGLT2 inhibitors such as canagliflozin and dapagliflozin; and bile acid sequestrant such as colesevelam.

d. Conclusion

There is insufficient evidence to establish any clinical benefit associated with the use of glutathione in diabetes. Additional information provided by the nominator on the effect glutathione may have on the structure or function of the body does not provide evidence of any clinical benefit on the use of glutathione in diabetes. The existence of approved drugs to treat the disease weigh against including glutathione on the list, particularly in light of diabetes being a serious or life-threatening disease.

24. Septic Shock

a. Clinical Information

One group of Italian researchers has evaluated the effect of glutathione on peroxidative indexes in patients with septic shock (Ortolani et al. 1992; Ortolani et al. 2000). In 1992, 130 patients with septic shock were divided into 3 randomized groups: Group A (50 patients) received a standard therapy including parenteral nutrition, antibiotics and other drugs eventually needed due to the patient’s pathology; Group B (50 patients) received standard treatment plus 1200 mg/day of glutathione by continuous infusion; Group C (30 patients) received standard therapy plus 4800 mg/day glutathione by continuous infusion. The following five tests were performed prior to first glutathione administration (Baseline; Day 0) and then every third day until Day 15: 1) gas chromatographic evaluation of ethane in expirate, 2) plasmatic concentration of MDA by HPLC, 3) activated complement C5 fragment by leukocyte aggregation test, 4) AFp by immuno-enzymatic kit, and 5) erythrocyte membrane deformability. When the 75 post baseline test results were compared to the 15 baseline test results, 12 of the Group B or C post-baseline test results (with 6 of the 12 occurring at the 12 day and 15 day blood draws) were statistically significantly different from the control Group A post-baseline test results. Based on these results, albeit limited without additional clinical outcomes, the investigators concluded that the higher 4800 mg/day glutathione infusion limited the peroxidative stress of septic shock patients within 6-9 days, while the lower 1200 mg/day glutathione infusion had comparable effects in

12-15 days (Ortolani et al. 1992). No confirmation of the findings from the two studies described above by Ortolani et al. have been found and it is unclear whether the laboratory endpoints selected by Ortolani et al. were the appropriate tests to adequately determine glutathione's use to change function or the disease course.

In 2000, 30 patients with septic shock were randomized into three groups within 24 hours of diagnosis. All 30 patients received septic shock therapy, including parenteral nutrition, antibiotics, and volume-expanding and inotropic agents. The 10 patients in Group A were the controls. The 10 patients in Group B also received 70 mg/kg/d of IV glutathione. The 10 patients in Group C also received 70 mg/kg/d of IV glutathione and 75 mg/kg/d of N-acetyl-L-cysteine. Protection against oxygen free radicals was evaluated with plasma concentrations of glutathione and GSSG and indirectly evaluated by measuring expired ethane, plasma malondialdehyde, erythrocyte membrane stiffness, activation of complement factor 5, and clinical scores at admission and on Days 3 and 5 of treatment. While both Group B and Group C demonstrated a statistically significant reduction in the indirect markers on Day 5 and a statistically significant improvement in the Apache II and Organ Failure LOD clinical scores on Day 10, the decrease was more marked in Group C (Ortolani et al. 2000).

b. Severity

Septic shock can be serious or life-threatening.

c. Alternative Therapies

Alternative therapies approved for the treatment of septic shock include antibiotics; vasopressors such as norepinephrine; and IV crystalloid solutions.

d. Conclusion

The minimal data indicating effectiveness for some study participants are insufficient to support effectiveness in treating septic shock. Additional information provided by the nominator on the effect glutathione may have on the structure or function of the body does not provide evidence of clinical benefit on the use of glutathione in septic shock. In addition, the existence of approved drugs to treat the disease weigh against including glutathione on the list, particularly in light of Septic shock's being a serious or life-threatening disease.

D. Has the substance been used historically as a drug in compounding?

Databases searched for information on glutathione in regard to Section II.D. of this evaluation included PubMed, Natural Medicines, compoundingtoday.com, European Pharmacopoeia, British Pharmacopoeia, Japanese Pharmacopoeia, and Google.

FDA also obtained information on the current and historical use of glutathione in compounded drugs from a research study conducted by JHU CERSI on Bulk Drug Substances Used to Compound Drugs for Patients with Autism Spectrum Disorder (JHU CERSI, 2020).

1. Length of time the substance has been used in pharmacy compounding

The nominators did not provide historical use data. Glutathione was named by Sir Frederick Gowland Hopkins after he isolated the substance from yeast and animal tissues in 1921 (Hopkins and Dixon 1922 in Simoni et al. 2002). A literature search revealed that glutathione has been used since at least 1965 when researchers compared oral and IM glutathione to placebo for the treatment of hepatic cirrhosis (Cook and Sherlock 1965). Study participants in the oral treatment group received two 50 mg glutathione tablets three times a day for 28 days and the IM treatment group received glutathione 200 mg daily for 14 days. The study authors noted that the glutathione preparations used were supplied by Jouillié Laboratories in France.

The IV use of glutathione is documented as early as 1980 when a study was published assessing glutathione as an adjuvant to chemotherapy treatment for stomach cancer (Fujimoto et al. 1980). The induction treatment group received glutathione 1,500 mg daily intravenously to "induce hepatic drug-metabolizing enzymes."

Compounding with glutathione can be traced back to at least November 2010 when the International Journal of Pharmaceutical Compounding published an article describing the formulation for Glutathione 25% in Pluronic Lecithin Organogel (Loyd 2010). Additionally, an Australian article from 2015 describes a cluster of AEs related to endotoxin-contaminated glutathione compounded sterile preparations (Johnstone et al. 2015). We are aware of various compounded drug products containing glutathione compounded from a bulk drug substance by an outsourcing facility since at least January 2017.[35]

2. The medical condition(s) it has been used to treat

According to the Natural Medicines database, glutathione is used to treat cisplatin-induced neurotoxicity (Natural Medicine Comprehensive Database 2021); however, it is unclear whether patients are obtaining compounded glutathione for this condition. Results from a Google search using the terms *glutathione compounding* indicate that many compounding pharmacy websites[36] describe glutathione as the "most powerful, most versatile and most important part of the body's self-generated antioxidants (endogenous)," the "master antioxidant," and the "mother of all antioxidants." The websites assert that glutathione can be used in the treatment and prevention of alcoholism, aging, heart disease, liver disease, lung disease, chronic otitis media, chronic rhinitis, cataracts, glaucoma, cancer, osteoarthritis and "diseases that break down the body's

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[35] The Drug Quality and Security Act, signed into law on November 27, 2013, created a new section 503B in the Federal Food, Drug, and Cosmetic Act. Under section 503B, a compounder can become an outsourcing facility. Outsourcing facilities are required to provide FDA with a list of drugs they compounded during the previous six-month period upon initial registration and in June and December each year. This retrospective information does not identify drugs that outsourcing facilities intend to produce in the future.

[36] See https://www.pharmacompoundia.com/topical-glutathione-for-skin-lightening/, https://www.pavilioncompounding.com/glutathione-and-acetyl-glutathione/, https://www.keycompounding.com/glutathione-need-more/, https://wellnesspharmacy.com/products/glutathione/, https://www.olympiapharmacy.com/product/l-glutathione/, https://www.americanintegrative.com/what-is-glutathione/, https://www.belmarpharmasolutions.com/clinicians/treatment-options/acetyl-glutathione/, https://www.realhealthmedical.com/if-you-like-to-breathe-or-want-to-breathe-better-this-treatment-is-for-you; https://www.lemonwaterwellness.ca/blogs/resources/nebulizer-glutathione. All accessed Apr 18, 2022.

immune system," may improve neurological conditions such as Parkinson's disease, MS, amyotrophic lateral sclerosis (ALS), AD, and multiple chemical sensitivity disorder; inhibits melanin production and lightens skin tone, and may alleviate autism, attention deficit disorder (ADD), and macular degeneration in deficient patients. Glutathione is/has been compounded in numerous delivery forms including oral, inhalation, nebulized, nasal spray, transdermal, topical, IV, IM, and rectal formulations.

3. How widespread its use has been

The JHU-CERSI report evaluated the current and historical use of six bulk drug substances (inositol, 2,3-Dimercapto-1-propanesulfonic acid, glutathione, melatonin, oxytocin and methylcobalamin) for use in ASD (JHU CERSI 2020). The report drew on three distinct data resources for estimates of use: a clinical sample, population sample, and a national sample, supplemented with interviews of key opinion leaders in research and practice.

Use of Glutathione in a Clinical Sample: In a clinical sample of 1,788 children with ASD under 17 years of age that receive care at Kennedy Krieger Institute Center for Autism and Related Disorders, <1% of parents used glutathione for their child with ASD, all administered as an injection.

Use of Glutathione in a Population Sample: In a population of 1,487 parents of children under 18 years of age from the Simons Foundation Powering Autism Research through Knowledge initiative, an online registry of self-referred parents/caregivers of individuals with autism, use of glutathione was rare and endorsed in <2% of responses.

Use of Glutathione in a National Sample: Evaluation of Medicaid claims data from the years 2010-2014 for children with ASD revealed that of the medications assessed, use of glutathione was <1% among children with and without ASD.

Key Opinion Leaders (KOL): Phone interviews with three KOLs, composed of currently practicing physicians and researchers with expertise in ASD and complementary and alternative medicine, were conducted to obtain a qualitative understanding of the patterns of use and knowledge of the compounded drug substances of interest for ASD in mainstream clinical practice. Little was known about glutathione treatment as it is rarely prescribed or recommended for ASD in clinical practice.

Per JHU-CERSI, some limitations of the study include recall bias with self-reporting, Center for Medicare and Medicaid Services data not capturing drug utilization paid for by non-Medicaid means and limited number of KOLs interviewed. The JHU-CERSI study is one source of information that we considered among many.

There are several drug products not approved by FDA containing glutathione with national drug codes (NDCs) that are marketed for human use.[37] According to outsourcing facility reports submitted to FDA, several outsourcing facilities prepared single active ingredient drug products compounded in injection, suppository, cream, and capsule dosage forms containing glutathione.

[37] Available at: NDC Ingredient Name (fda.gov).

Additionally, outsourcing facilities reported preparing injection products containing glutathione and other drugs.

Glutathione has been used for skin lightening in many regions throughout the world including North America, South America, Asia, the Middle East, and Africa (Pollock et al. 2020).

A Google search indicates that use of IV glutathione for skin lightening is prevalent in the U.S. Medical spas across the country offer glutathione injection and infusion skin lightening treatments.[38] One company offers skin whitening "Moon Face" IV glutathione infusions administered in patients' homes and private jets. The website[39] includes pictures and videos of celebrity endorsements. Most spas usually offer treatment in combination with IV Vitamin C. Doses can range from 400 grams to 1200 grams. According to one website, "patients typically see a shade decrease in skin color and results can be evident as soon as four weeks."[40] Another website states, "one round of treatment is 10 IV's that will be performed twice a week for 5 weeks. Typically one round of treatment (10 IV's) can lighten the skin from 1 to 3 shades lighter."[41] Many of the websites recommend maintenance treatments for better results. Of the websites reviewed, the majority failed to mention the risks associated with IV glutathione. Only two websites included information about the risks of IV glutathione, albeit minimized. One website states, "Most patients do not experience any adverse side effects; however, due to the detoxification reaction, some patients may experience mild headaches or nausea."[42] Another website states, "Possible side effects may include abdominal cramps, bloating, trouble breathing due to bronchial constriction, allergic reactions, such as rash."[43] An "online wellness provider" in the U.S. offers prescriptions for IM glutathione through telemedicine visits.[44] The clinic website promotes that glutathione injections can be prescribed for self-administration and doses can range from 1,500 mg to 4,000 mg depending on the indication and administration frequency. Additionally, IV glutathione injections are prevalent in South Korea and are promoted by plastic surgery clinics providing services to the local population and tourists.[45]

Authorities in the Philippines and Ghana have issued warnings against the use of IV glutathione for skin lightening citing lack of safety and efficacy data and side effect concerns.[46] Additionally, on February 1, 2019, FDA issued a Compounding Risk Alert for glutathione powder, distributed by Letco Medical, due to potentially high levels of endotoxins and reported

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[38] See https://revivifymedicalspa.com/body/glutathione-skin-lightening/; https://oskinmedspa.com/service/iv-glutathione-injections/; https://ivvitamintherapylosangeles.com/services/iv-glutathione-for-skin-whitening/; https://www.dermatology-treatment-center.com/wayne/nj/glutathione-skin-treatment.html; https://pureharmonymedspa.com/skin-lightening/ All accessed on Apr 18, 2022.

[39] See https://leaa.io/glutathione.html Accessed Apr 18, 2022.

[40] See https://ivvitamintherapylosangeles.com/services/iv-glutathione-for-skin-whitening/ Accessed Apr 18, 2022.

[41] See https://pureharmonymedspa.com/skin-lightening/ Accessed Apr 18, 2022.

[42] See https://ivvitamintherapylosangeles.com/services/iv-glutathione-for-skin-whitening/ Accessed Apr 18, 2022.

[43] See https://pureharmonymedspa.com/skin-lightening/ Accessed Apr 18, 2022.

[44] See https://www.invigormedical.com/lifestyle/glutathione-injection-cost/ Accessed Apr 18, 2022.

[45] See https://cindyhospital.com/pages/oneday/oneday06.php; https://seoulguidemedical.com/whitening-injection-in-korea/; https://beauty.jivaka.care/blogs/blog/skin-whitening-injections-in-korea All accessed on Apr 18, 2022.

[46] See https://www.fda.gov.ph/fda-advisory-no-2019-182-unsafe-use-of-glutathione-as-skin-lightening-agent/; https://www.bbc.com/pidgin/46616992 All accessed Apr 18, 2022.

AEs.[47] Authorities in Thailand banned IV glutathione "for fear of severe adverse reactions, including anaphylaxis" (Arjinpathana and Asawanonda 2012).

The International Journal of Pharmaceutical Compounding (IJPC) has published compounding formulations for glutathione 25% topical gel[48], glutathione 40% transdermal cream[49], glutathione 60 mg/mL inhalation solution[50], glutathione 250 mg gelatin troche[51], and glutathione 250 mg PEG troche[52]. As previously noted, glutathione inhalation preparations pose safety concerns. As such, we further assessed the IJPC formula for glutathione inhalation solution and noted that it contains L-glutathione, ascorbic acid, sodium bicarbonate, sodium bicarbonate solution, and sterile water for inhalation.

4. Recognition of the substance in other countries or foreign pharmacopeias

Glutathione is listed in the Japanese Pharmacopoeia (17th Edition)[53] and the European Pharmacopoeia (10th Edition, 10.3)[54]. A search of the British Pharmacopoeia (BP 2020)[55] did not show any listings for glutathione.

Conclusions: Available literature indicates that glutathione has been used since at least 1965. Compounding with glutathione can be traced back to at least 2010. Based on internet searches, it appears that compounders have been preparing glutathione as oral, inhalation, nebulized, nasal spray, transdermal, topical, IV, IM, subcutaneous, and rectal formulations. Glutathione is used in many regions around the world, and certain authorities have issued warnings against IV glutathione. According to the JHU CERSI report that evaluated six substances used for ASD, glutathione is rarely used as a treatment for ASD. Glutathione is listed in the Japanese and European Pharmacopoeia.

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[47] See https://www.fda.gov/drugs/human-drug-compounding/fda-highlights-concerns-using-dietary-ingredient-glutathione-compound-sterile-injectables Accessed Apr 18, 2022.
[48] Available at: https://compoundingtoday.com/Formulation/FormulaPDF.cfm?FormulaID=2495 Subscription required; accessed Oct 19, 2021.
[49] Available at: https://compoundingtoday.com/Formulation/DownloadNamedPDF.cfm?ID=4208 Subscription required; accessed Oct 19, 2021.
[50] Available at: https://compoundingtoday.com/Formulation/FormulaPDF.cfm?FormulaID=1537 Subscription required; accessed Oct 19, 2021.
[51] Available at: https://compoundingtoday.com/Formulation/DownloadNamedPDF.cfm?ID=3825; https://compoundingtoday.com/Formulation/DownloadNamedPDF.cfm?ID=4210 Subscription required; accessed Oct 19, 2021.
[52] Available at: https://compoundingtoday.com/Formulation/DownloadNamedPDF.cfm?ID=4209 Subscription required; accessed Oct 19, 2021.
[53] Available at: https://www.mhlw.go.jp/file/06-Seisakujouhou-11120000-Iyakushokuhinkyoku/JP17_REV_1.pdf Accessed Apr 18, 2022.
[54] Available at: https://pheur.edqm.eu/app/10-3/search/ Subscription required; accessed Apr 18, 2022.
[55] Available at: https://www.pharmacopoeia.com/ Accessed Apr 18, 2022.

III. RECOMMENDATION

We have balanced the criteria described in section II above to evaluate glutathione for the 503A Bulks List. After considering the information currently available, a balancing of the criteria ***weighs against*** glutathione being placed on that list based on the following:

1. Glutathione is well-characterized. The solid dosage forms must be protected from oxygen. In addition to protection from oxygen, appropriate storage temperature and pH controls will be needed for liquid/semi-solid dosage forms. The need for protection from oxygen for liquid/semi-solid dosage forms should be carefully evaluated because storage without protection from air can further reduce the shelf-life of such formulations.

2. The safety profile of glutathione includes serious safety issues (e.g., anaphylaxis/hypersensitivity, hepatoxicity, severe wheezing, and breathlessness). Thus, glutathione injections (IV, IM) and glutathione inhalation preparations, which provide rapid, irreversible exposure, are not recommended for addition to the 503A list due to safety concerns.

3. There is either no available information, or insufficient evidence of effectiveness of glutathione in association with any of the nominated uses. Some evidence may support the use of glutathione in skin lightening but no specific population or dose exposure has been established. FDA has not found data indicating that any effect of glutathione may have to lighten the skin provides a clinical benefit to address a disease or condition, such as managing disorders of hyperpigmentation. The bioavailability of oral dosage forms is minimal and systemic exposure from injectable formulations is associated with rapid metabolism. In addition, the existence of approved drugs to treat several of the proposed serious or life-threatening conditions weigh against including glutathione on the list. Therefore, no dosage form of glutathione is recommended for addition to the 503A list based on effectiveness.

4. Available literature indicates that glutathione has been used since at least 1965. Compounding with glutathione can be traced back to at least 2010. Based on internet searches and outsourcing facility product reporting data, it appears that compounders have been preparing glutathione as oral, inhalation, nebulized, nasal spray, transdermal, topical, IV, IM, subcutaneous, and rectal formulations. Glutathione is used in many regions around the world, and certain authorities have issued warnings against IV glutathione. According to the JHU CERSI report that evaluated six substances used for ASD, glutathione is rarely used as a treatment for ASD. Glutathione is listed in the Japanese and European Pharmacopoeia.

Based on the information the Agency has considered in balancing the four evaluation factors, the lack of effectiveness data and safety data for use of products in patients ***weighs against*** glutathione being added to the 503A Bulks List.

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Tab 3

Ammonium Tetrathiomolybdate

Tab 3a

Ammonium Tetrathiomolybdate Nominations

January 27, 2021

Department of Health and Human Services Food
and Drug Administration
Division of Dockets Management (HFA-305) 5630
Fishers Lane, Room 1061
Rockville, MD 20852

Re: Pharmacy Solutions Petition to Docket FDA-2015-N-3534

"Bulk Drug Substances That May Be Used to Compound Drug Products in Accordance With Section 503A of the Federal Food, Drug, and Cosmetic Act; Revised Request for Nominations"

To Whom it may concern:

Pharmacy Solutions respectfully submits for consideration this Nomination of the bulk drug substance, Ammonium Tetrathiomolybdate to the list of bulk drug substances that may be used for compounding under Section 503A. Please reference Appendix 1 and Attachment 3.

This Petitioner formally requests, under Title 21 of the Code of Federal Regulations and the provisions of the Federal Food, Drug, and Cosmetic Act, that the Commissioner of Food and Drugs either amend or otherwise withhold action on the proposed Amendments to the Bulk List of Drug Substances That Can Be Used to Compound Drug Products. This action would be in Accordance with Section 503A of the Federal Food, Drug, and Cosmetic Act (the "Amendment") to the extent set forth below.

This active ingredient does not appear on an FDA-published list of drugs that present demonstrable difficulties for compounding. In addition, it is not a component of a drug product that has been withdrawn or removed from the market because the drug or components of the drug have been found to be unsafe or not effective. Furthermore, there is not an FDA approved entity on the market with similar mechanism of action and safety profile as a copper chelating agent.

Many trials of efficacy and safety have been completed and are provided for your review in the attached docket (see attachments 1&2). Please note that major medical institutions around the united states use this life saving treatment modality as a copper chelator in various cancer treatment in patients refractory to available treatment options and in Wilson's disease. Some of the centers have published data which is also provided for your review.

Ammonium Tetrathiomolybdate is approved in Europe as an orphan designation (See attachments 5&6) and an NDA and orphan status designation in the US were applied for in the past also. Please see attachment 7 for development timeline.

This life saving therapy and letters of support from providers and patients were provided to the FDA also and this treatment modality has been used by hundreds of patients across the country with phenomenal results. These patients attribute their treatment success and increased survival to Ammonium Tetrathiomolybdate. Many providers believe it is one of the best agents for copper chelation and decreased angiogenesis in various cancers when other modalities fail. These patients are monitored very carefully by providers during treatment and they believe is it much better tolerated and safer than other copper chelators. (See Attachment 4: provider attestation letter from Dr. Linda Vahdat, MD, MBA)

We urge the FDA to consider and add this bulk substance to the 503A list as we do not want to cause disruption in access and patient care to this life saving treatment.

Appendix 1: General Substance Information
Attachment 1: Supporting Clinical Data (1/2)
Attachment 2: Supporting Clinical Data (2/2)
Attachment 3: Independent Analytical results
Attachment 4: Provider Support Letter- Dr. Linda Vahdat, MD, MBA
Attachment 5: European Medicines Agency Orphan Designation
Attachment 6: Martindale Drug Information-The Royal Pharmaceutical Society of Great Britain 2020
Attachment 7: Ammonium Tetrathiomolybdate Development Timeline

Thank you for your prompt consideration of this request. If you need further information or clarification, we will be happy to provide additional letters of provider and patient support for this request.

Professionally Yours;

Sahar Swidan, Pharm.D., FAARFM, ABAAHP, FACA
CEO, Pharmacy Solutions

Column A—What information is requested?	Column B—Put data specific to the nominated substance	Response	
What is the name of the nominated ingredient?	Provide the ingredient name.	Ammonium Tetrathiomolybdate	
Is the ingredient an active ingredient that meets the definition of “bulk drug substance” in § 207.3(a)(4)?	Provide an explanation for why it is considered an active ingredient when it is used in specific compounded drug products, and provide citations to specific sources that describe its active properties.	It is a bulk drug substance and studies below illucidate its mechanism of action further as copper chelator and further investigation is ongoing.	
Is the ingredient listed in any of the three sections of the Orange Book?	Confirm whether the ingredient is a component of an FDA-approved product.	No	
Were any drug monographs for the ingredient found in the USP or NF monographs?	Confirm whether the ingredient is the subject of an applicable USP or NF monograph.	No	
What is the chemical name of the substance?	Chemical name.	diazanium;bis(sulfanylidene)molybdenum;sulfanide	
What is the common name of the substance?	Common name.	Ammonium Tetrathiomolybdate; Tiomolibdate Diammonium; ATTM	
Does the substance have a UNII code?	UNII code.	4V6I63LW1E	
What is the chemical grade of the substance?	Provide the chemical grade.	See C of A file attached	
What is the strength, quality, stability, and purity of the ingredient?	Provide the strength, quality, stability, and purity information and attach a certificate of analysis.	See attached file for API purity tested by third party lab and also potency testing of final compounded product	
		Assay	Percentage of product (%)
		Ammonium Tetrathiomolybdate	99
		Aluminum	0.0001
		Calcium	0.0001
		Chromium	0.0001
		Copper	<0.0005
		Iron	<0.0003
		Lead	0.0001
		Magnesium	0.0001
		Nickel	0.0001
		Silicon	0.0001
		Tin	0.0001
		Titanium	0.0001

How is the ingredient supplied?	Describe how the ingredient is supplied (e.g., powder, liquid).	Powder
Is the substance recognized in foreign pharmacopeias or registered in other countries?	List the foreign pharmacopeias or other countries in which it is registered.	Orphan Drug Designation (EU/3/08/539) given by the European Medicines Agency on April 1 2008 for the treatment of Wilson's disease
Has information been submitted about the substance to the USP for consideration of drug monograph development?	Put yes, no, or unknown. If yes, state the status of the monograph, if known.	Unknown
What dosage form(s) will be compounded using the bulk drug substance?	State the dosage form(s).	Oral dosage forms as requested by prescriber
What strength(s) will be compounded from the nominated substance?	List the strength(s) of the drug product(s) that will be compounded from the nominated substance, or a range of strengths, if known.	20mg-60mg capsules
What are the anticipated route(s) of administration of the compounded drug product(s)?	List the route(s) of administration of the compounded drug product(s).	Oral
Has the bulk drug substance been used previously to compound drug product(s)?	Describe past uses of the bulk drug substance in compounding.	Yes, 20-60mg capsules and 20-40mg oil filled capsules
What is the proposed use for the drug product(s) to be compounded with the nominated substance?	Provide information on the proposed use of the compounded drug product.	Treatment of Wilson's Disease and Copper Chelation Therapy
What is the reason for use of a compounded drug product rather than an FDA-approved product?	Provide a rationale for the use of a compounded drug product.	No FDA-approved product available
Is there any other relevant information?	Provide any other information you would like FDA to consider in evaluating the nomination.	Please see clinical trials, provider letters of support due to lack of availability of other treatment options and patient letter documents attached

Are there safety and efficacy data on compounded drugs using the nominated substance?	Provide a bibliography of safety and efficacy data for the drug compounded using the nominated substance, if available, including any relevant peer-reviewed medical literature.

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Advances in Brief

Treatment of Metastatic Cancer with Tetrathiomolybdate, an Anticopper, Antiangiogenic Agent: Phase I Study[1]

George J. Brewer, Robert D. Dick, Damanjit K. Grover, Virginia LeClaire, Michael Tseng, Max Wicha, Kenneth Pienta, Bruce G. Redman, Thierry Jahan, Vernon K. Sondak, Myla Strawderman, Gerald LeCarpentier, and Sofia D. Merajver[2]

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Abstract

Preclinical and *in vitro* studies have determined that copper is an important cofactor for angiogenesis. Tetrathiomolybdate (TM) was developed as an effective anticopper therapy for the initial treatment of Wilson's disease, an autosomal recessive disorder that leads to abnormal copper accumulation. Given the potency and uniqueness of the anticopper action of TM and its lack of toxicity, we hypothesized that TM would be a suitable agent to achieve and maintain mild copper deficiency to impair neovascularization in metastatic solid tumors. Following preclinical work that showed efficacy for this anticopper approach in mouse tumor models, we carried out a Phase I clinical trial in 18 patients with metastatic cancer who were enrolled at three dose levels of oral TM (90, 105, and 120 mg/day) administered in six divided doses with and in-between meals. Serum ceruloplasmin (Cp) was used as a surrogate marker for total body copper. Because anemia is the first clinical sign of copper deficiency, the goal of the study was to reduce Cp to 20% of baseline value without reducing hematocrit below 80% of baseline. Cp is a reliable and sensitive measure of copper status, and TM was nontoxic when Cp was reduced to 15–20% of baseline. The level III dose of TM (120 mg/day) was effective in reaching the target Cp without added toxicity. TM-induced mild copper deficiency achieved stable disease in five of six patients who were copper deficient at the target range for at least 90 days.

Introduction

The concept of antiangiogenic treatment for solid tumors, which was pioneered by Folkman (1–3), has a firm rationale and shows efficacy in animal tumor models (4–12). Compounds that interfere with critical steps in the angiogenesis cascade are reaching the clinic (13). The steps required for successful tumor angiogenesis at the primary and metastatic sites are diverse, and they depend on an imbalance between angiogenesis activators (14–15) such as vascular endothelial growth factor and basic fibroblast growth factor and inhibitors such as thrombospondin 1 (16–20), angiostatin (21–23), and endostatin (10). The relative importance of the different angiogenesis-modulating molecules in different tissues may determine the relative potency of antiangiogenic compounds to elicit a response at both the primary and metastatic sites. Therefore, it would be very desirable to develop an antiangiogenic strategy that would affect multiple activators of angiogenesis in order for it to be generally applicable to human tumors. Because copper is a required cofactor for the function of many key mediators of angiogenesis, such as basic fibroblast growth factor (24–27), vascular endothelial growth factor, and angiogenin (28), we have developed an antiangiogenic strategy for the treatment of cancer based on the modulation of total body copper status. The underlying hypothesis of this work is that a window of copper deficiency exists in which angiogenesis is impaired, but other copper-dependent cellular processes are not affected enough to cause clinical toxicity.

It has been amply demonstrated that copper is required for angiogenesis (29–31), and several years ago, some promising animal tumor model studies were carried out using an anticopper approach (32–33). The chelator penicillamine and a low-copper diet were used to lower copper levels in rats and rabbits with implanted intracerebral tumors. However, although they showed reduced tumor size, the animals treated with the low-copper regimen did not show improved survival over untreated controls.

For the past 20 years, we have developed new anticopper therapies for Wilson's disease, an autosomal recessive disease of copper transport that results in abnormal copper accumulation and toxicity. One of the drugs currently being used, TM,[3] shows unique and desirable properties of fast action, copper specificity, and low toxicity (34–36), as well as a unique mechanism of

Received 7/19/99; revised 11/1/99; accepted 11/9/99.

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[1] Supported by NIH Grants RO3-CA-77122 (to S. D. M.), P30-CA46592 (to M. W.), and MO1-RR00042 (to the General Clinical Research Center); the Tracy Starr Award from the Cleveland Foundation (to S. D. M.); a grant from the Tempting Tables Organization (to S. D. M.); and donors to the University of Michigan Comprehensive Cancer Center.

[2] To whom requests for reprints should be addressed, at University of Michigan Comprehensive Cancer Center, 7217 CCGC, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0948.

[3] The abbreviations used are: TM, tetrathiomolybdate; Cp, ceruloplasmin; Hct, hematocrit; GI, gastrointestinal; CAT, computer-assisted tomography.

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action. TM forms a stable tripartite complex with copper and protein. If given with food, it complexes food copper with food protein and prevents absorption of copper from the GI tract. There is endogenous secretion of copper in saliva and gastric secretions associated with food intake, and this copper is also complexed by TM when it is taken with meals, thereby preventing copper reabsorption. Thus, patients are placed in a negative copper balance immediately when TM is given with food. If TM is given between meals, it is absorbed into the blood stream, where it complexes either free or loosely bound copper with serum albumin. This TM-bound copper fraction is no longer available for cellular uptake, has no known biological activity, and is slowly cleared in bile and urine.

The underlying hypothesis of an anticopper, antiangiogenic approach to cancer therapy is that the level of copper required for angiogenesis is higher than that required for essential copper-dependent cellular functions, such as heme synthesis, cytochrome function, and incorporation of copper into enzymes and other proteins. Because of the unique and favorable characteristics of TM as an anticopper agent compared with other anticopper drugs, we evaluated it in animal tumor models for toxicity and efficacy as an anticopper, antiangiogenic therapy. These studies showed efficacy in impairing the development of *de novo* mammary tumors in Her2-neu transgenic mice (12), and TM showed no clinically overt toxicity as copper levels were decreased to 10% of baseline. Here we report the first human trial of an anticopper approach to antiangiogenesis therapy based on the use of TM in patients with metastatic cancer. This Phase I trial of TM yielded information on dose, dose response, evaluation of copper status in patients, and toxicity (37). Although the study was not designed to definitively answer efficacy questions, we report preliminary observations on efficacy and novel approaches to following disease status in trials of antiangiogenic compounds.

Patients and Methods

Patients. Eighteen adults with metastatic solid tumors exhibiting measurable disease, life expectancy of 3 or more months, and at least 60% Karnofsky performance status were enrolled. We excluded patients with effusions or bone marrow involvement as the only manifestations of disease and those who had severe intercurrent illness requiring intensive management or were transfusion dependent. Patients had to have recovered from previous toxicities and had to meet the following requirements for laboratory parameters: (*a*) WBC $\geq$ 3,000/mm^3; (*b*) absolute neutrophil count $\geq$ 1,200/mm^3; (*c*) Hct $\geq$ 27%; (*d*) hemoglobin $\geq$ 8.0 g/dl; (*e*) platelet count $\geq$ 80,000/mm^3; (*f*) bilirubin $\leq$ 2.0 mg/dl; (*g*) aspartate aminotransferase and alanine aminotransferase $\leq$ 4 times the upper limit of institutional norm; (*h*) serum creatinine $<$ 1.8 mg/dl or calculated creatinine clearance $\geq$ 55 ml/min; (*i*) calcium $<$ 11.0; (*j*) albumin $\geq$ 2.5 g/dl; (*k*) prothrombin time $\leq$ 13 s; and (*l*) partial thromboplastin time $\leq$ 35 s. Other requirements were demonstrable progression of disease in the previous 3 months after standard treatments such as surgery, chemotherapy, radiotherapy, and/or immunotherapy or progressive disease after declining conventional treatment modalities.

Treatment Schema: Doses and Escalation. Three dose regimens were evaluated. All dose levels consisted of 20 mg of TM given three times daily with meals plus an escalating (levels I, II, and III) in-between meals dose given three times daily for a total of six doses/day. Loading dose levels I, II, and III provided TM at 10, 15, and 20 mg, three times daily between meals, respectively, in addition to the three doses of 20 mg each given with meals at all dose levels.

Baseline Cp was taken as the nearest Cp measurement to day 1 of treatment (including day 1) because blood was drawn before TM treatment from all patients. The target Cp reduction was defined as 20% of baseline Cp. Due to Cp assay variability of approximately 2% at this institution, a change of Cp to 22% of baseline was considered as achieving the desired reduction of copper. In addition, if the absolute Cp was less than 5 mg/dl, then the patient was considered as having reached the target Cp. No patient reached the 5 mg/dl target without also being at least 78% reduced from baseline. After reaching the target copper-deficient state, TM doses were individually tailored to maintain Cp within a target window of 70–90% reduction from baseline.

Six patients were to be enrolled at each dose level. After four patients were enrolled at level I, if one patient experienced dose-limiting toxicity (defined as Hct $<$ 80% of baseline), two more patients were enrolled at level I. If no dose-limiting toxicity was observed, patients were enrolled at the next dose level. Treatment was allowed to continue beyond induction of target copper deficiency if the patients experienced a partial or complete clinical response or achieved clinical stable disease by the following definitions. Complete response is the disappearance of all clinical and laboratory signs and symptoms of active disease; partial response is a 50% or greater reduction in the size of measurable lesions defined by the sum of the products of the longest perpendicular diameters of the lesions, with no new lesions or lesions increasing in size. Minor response is a 25–49% reduction in the sum of the products of the longest perpendicular diameters of one or more measurable lesions, no increase in size of any lesions, and no new lesions; stable disease is any change in tumor measurements not represented by the criteria for response or progressive disease; progressive disease is an increase of 25% or more in the sum of the products of the longest perpendicular diameters of any measurable indicator lesions compared with the smallest previous measurement or appearance of a new lesion. Because copper deficiency is not a cytotoxic treatment modality, the patients who provide information about the efficacy of TM for long-term therapy in this population of patients with advanced cancer are primarily those who remained within the target Cp window of 20 $\pm$ 10% of baseline for over 90 days without disease progression.

Monitoring of Copper Status. A method was required to monitor copper status easily and reliably, so that the TM dose could be adjusted appropriately during this trial. With TM administration, serum copper is not a useful measure of total body copper because the TM-copper-albumin complex is not rapidly cleared, and the total serum copper (including the fraction bound to the TM-protein complex) actually increases during TM therapy (34–36). The serum Cp level obtained weekly was used as a surrogate measure of total body copper status. Cp was measured by the oxidase method; the Cp measurements were made by nethelometry (differential light scattering from a

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colored or turbid case solution with respect to a control solution) using an automated system and reagents available commercially (Beckman Instruments, Inc., Fullerton, CA). The serum Cp level is controlled by Cp synthesis by the liver, which, in turn, is determined by copper availability to the liver (38). Thus, as total body copper is reduced, the serum Cp level is proportionately reduced. The serum Cp level is in the range of 20–35 and 30–65 mg/dl for normal controls and cancer patients, respectively. Our objective was to reduce Cp to ≤ 20% of baseline and to maintain this level, within a window spanned by 20 ± 10% of baseline Cp, with typical Cp values in the range of 7–12 mg/dl. Because there appears to be no untoward clinical effects from this degree of copper reduction, we have termed this level of copper deficiency "chemical copper deficiency." The first indication of true clinical copper deficiency is a reduction in blood cell counts, primarily anemia, because copper is required for heme synthesis as well as cellular proliferation (36). Thus, the copper deficiency objective of this trial was to reduce the Cp to ≤ 20% of baseline without decreasing the patient's Hct or WBC to below 80% of baseline value at entry.

Toxicity, Follow-Up, and Disease Evaluation. Complete blood counts, liver and renal function tests, urinalyses, and Cp level were performed weekly for 16 weeks and then performed biweekly at the clinical laboratories of the University of Michigan Health System or at other affiliated certified laboratories. Physical examinations and evaluations of toxicity were carried out every 2 weeks for 8 weeks and then performed every 4 weeks for the duration of therapy. Toxicity was evaluated using the National Cancer Institute Common Toxicity Criteria. Extent of disease was evaluated at entry, at the point of achievement of copper deficiency (defined as Cp ≤ 20% of baseline), and every 10–12 weeks thereafter. CAT or magnetic resonance imaging was used as appropriate for conventional measurement of disease at all known sites and for evaluation of any potential new sites of disease. Angiogenesis-sensitive ultrasound with three-dimensional Doppler analyses was used in select cases as an adjunct to conventional imaging to evaluate blood flow to the tumors at different time points.

TM Preparation and Storage. TM was purchased in bulk lots suitable for human administration (Aldrich Chemical Company, Milwaukee, WI). Because TM is slowly degraded when exposed to air (oxygen replaces the sulfur in the molecule, rendering it inactive; Refs. 34–36), it was stored in 100-g lots under argon. At the time a prescription was written, the appropriate dose of TM was placed in gelatin capsules by research pharmacists at the University of Michigan Health System. Previously, we had shown that TM dispensed in such capsules retained at least 90% of its potency for 8 weeks (34). Thus, TM was dispensed to each patient in 8-week installments throughout the trial.

Measurement of Blood Flow. Blood flow was measured by ultrasound in select patients with accessible lesions at the time they became copper deficient and at variable intervals of 8–16 weeks thereafter. Three-dimensional scanning was performed on a GE Logiq 700 ultrasound system, with the 739 L, 7.5 MHz linear array scanhead. The scanning and vascularity quantification techniques were as described previously by the authors (39, 40).

Results

Patient Characteristics

Eighteen eligible patients (10 males and 8 females) with 11 different types of metastatic cancer who had progressed through or (in one case) declined other treatment options were enrolled in the trial in the order in which they were referred. Six, five, and seven patients were enrolled at the 90, 105, and 120 mg/day drug levels, respectively, following the protocol dose escalation schema. One patient originally assigned to the 105 mg/day level was removed early to pursue cytotoxic chemotherapy, due to rapid progression of disease. This same patient was later retreated at the 120 mg/day level for a longer duration; thus, he is counted only at the 120 mg/day level for the analyses. The average age was 59 years; the average baseline Cp was 47.8 mg/dl, which is elevated with respect to the normal level, reflecting the patients' disease status. Table 1 summarizes the patient characteristics for each dose level.

Toxicity

There were no cardiac, pulmonary, GI, renal, hepatic, hematological, infectious, skin, mucosal, or neurological toxicities observed for Cp levels at or above 20% of baseline. Mild (>80% of baseline Hct) reversible anemia was observed in four patients with Cp levels between 10–20% of baseline. Two of these patients had been treated with cytotoxic chemotherapy, and two patients had evidence of extensive bone marrow involvement with their disease at the time of entry into the trial. Although in the latter two cases, the anemia was most likely due to causes other than treatment, TM was temporarily discontinued until Hct was restored to acceptable levels with a transfusion of 2 units of packed RBCs. In one patient, it is very likely that the copper deficiency caused by TM produced the anemia. Stopping administration of the drug allowed the Hct to recover within 5–7 days without the need for transfusion; at the patient's request, TM was restarted at a lower dose, without further complications of anemia. Several patients experienced transient, occasional sulfur-smelling burping, within 30 min of TM ingestion. No additional toxicities of any type were observed with long-term maintenance of mild clinical copper deficiency over 8–15 months. Of note, no evidence of GI or other mucosal bleeding or impaired healing of minor trauma were observed with long-term therapy. One premenopausal patient with extensive metastatic renal cancer experienced normal menstrual periods during TM therapy, including 2.5 months of observation while she was copper deficient with Cp < 20% of baseline.

Cp as a Surrogate Measure of Copper Status

Fig. 1 shows the response of Cp as a function of time on TM therapy, expressed as the ratio of Cp at time *t* to baseline Cp level for each patient enrolled at the 90, 105, and 120 mg/day dose levels. Increasing the in-between meals dose from 10 mg three times daily to 15 or 20 mg three times daily had no significant effect on the rate of decrease of the Cp level, reaching a level of 50% baseline at a mean of 30 days (median = 28 days). The response of Cp to TM therapy as a function of time exhibited only minor fluctuations; when TM was discontinued, a rapid rise in Cp was observed within 48 h.

Four patients were removed from study due to progression

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Table 1 Patient characteristics

	Assigned TM Dose (mg/day)			
	90	105	120	Total
No. of patients	6	5	7	18
Sex (M/F)	3/3	1/4	6/1	10/8
Mean age (SD) (yrs)	64 (12)	60 (12)	53 (17)	59 (14)
Primary Tumor				
Breast	2	2	0	4
Colon	0	1	0	1
Lung	1	0	0	1
Melanoma	0	0	1	1
Pancreas	0	1	0	1
Prostate	2	0	0	2
Angiosarcoma	0	0	2	2
Chondrosarcoma	0	1	0	1
Nasopharyngeal tumor	0	0	1	1
Hemangioendothelioma	0	0	1	1
Renal tumor	1	0	2	3
Baseline				
Baseline Cp mean	52.6	49.3	42.7	47.8
Baseline Cp range	36.6–74.1	38.1–65.0	31.9–52.7	31.9–74.1
Baseline Hct mean	31.9	37.2	41.9	37.3
Baseline Hct range	26.6–35.6	33.8–42.5	35.4–45.7	26.6–45.7

of disease before achieving the target Cp of 20% of baseline, whereas the remaining 14 patients achieved the target Cp level. Because all 14 patients who achieved the target Cp level wished to remain on study, they were allowed to do so, according to the protocol, as long as they did not exhibit disease progression or toxicity. The TM doses were adjusted in these patients to maintain the Cp level between 10–20% of baseline. These patients provide the preliminary evidence of the efficacy and long-term tolerance of this approach.

Dose Adjustments to Maintain Target Cp

TM doses were adjusted to maintain a Cp target level of 20% of baseline and to prevent absolute Cp values < 5 mg/dl. Due to the routine 7-day turn-around for the Cp test at our laboratory, these dose changes were made approximately 7–10 days after the blood for the Cp measurement was taken. After achieving the target Cp, the in-between meals dose was typically decreased by 20 mg. Further decreases of 15–30 mg were necessary during long-term therapy. A patient with metastatic chondrosarcoma secondary to radiation treatment for breast cancer on long-term therapy has stable disease after 12 months of copper deficiency, with stable quality of life. One biopsy-proven metastatic nodule on her third digit is easily measurable and has been stable. Other sites of suspected disease in the chest also remain stable. Interestingly, this patient has required only a minor adjustment to her TM dose from the initial loading dose level to maintain the target Cp throughout this relatively long period. Fig. 2, *A* and *B*, illustrates the Cp response to dose adjustments required for two more representative patients over approximately 100 days of therapy. Thus far, the patient in Fig. 2*A* has required only decreases in dose 60 days apart. Most patients have required both an increase and a decrease in dose during long-term therapy. For example, as shown in Fig. 2*B*, the TM dose was increased after day 100 to respond to an increase in Cp outside the target range. Overall, there was considerable individual variability in the dose adjustments required. In conclusion, the Cp response to TM therapy evaluated weekly is not brittle or subject to wide fluctuations.

Measurement of Response of Metastatic Cancer to TM

Clinical Evaluation. Although the patients received different initial loading doses of TM, the Cp maintenance window of 20 ± 10% of baseline was used in all groups, regardless of the loading dose. Patients who maintained this degree of copper deficiency through tailored adjustments of the TM dose for over 90 days are likely to reflect the antiangiogenic activity of TM against their tumors. The period of 90 days is selected for two main reasons. First, TM is not cytotoxic to either cancer or endothelial cells and mainly impairs endothelial cell function and proangiogenic factor production. This mechanism of action is expected to have a very slow effect on the size of tumor masses. Second, as tumors sequester copper, the microenvironment of the tumor is expected to take a longer time to be rendered copper deficient. Table 2 summarizes the clinical course of the 18 patients.

Fourteen patients achieved the target copper deficiency before disease progression or other disease complications. Of these, eight patients either progressed within 30 days of achieving copper deficiency or have had stable disease for <90 days; it is unlikely that most of these tumors experienced an antiangiogenic environment long enough to evaluate clinical response to this type of therapy. In all patients removed from the protocol due to disease progression or choice and in one patient removed from the protocol due to the need for abdominal surgery to relieve a small bowel obstruction, much more rapid rates of progression of disease were noted clinically after discontinuation of TM therapy.

The remaining six patients experienced stable disease (five of six patients) or progression of disease at one site, with stable disease elsewhere (one of six patients). Two patients who have

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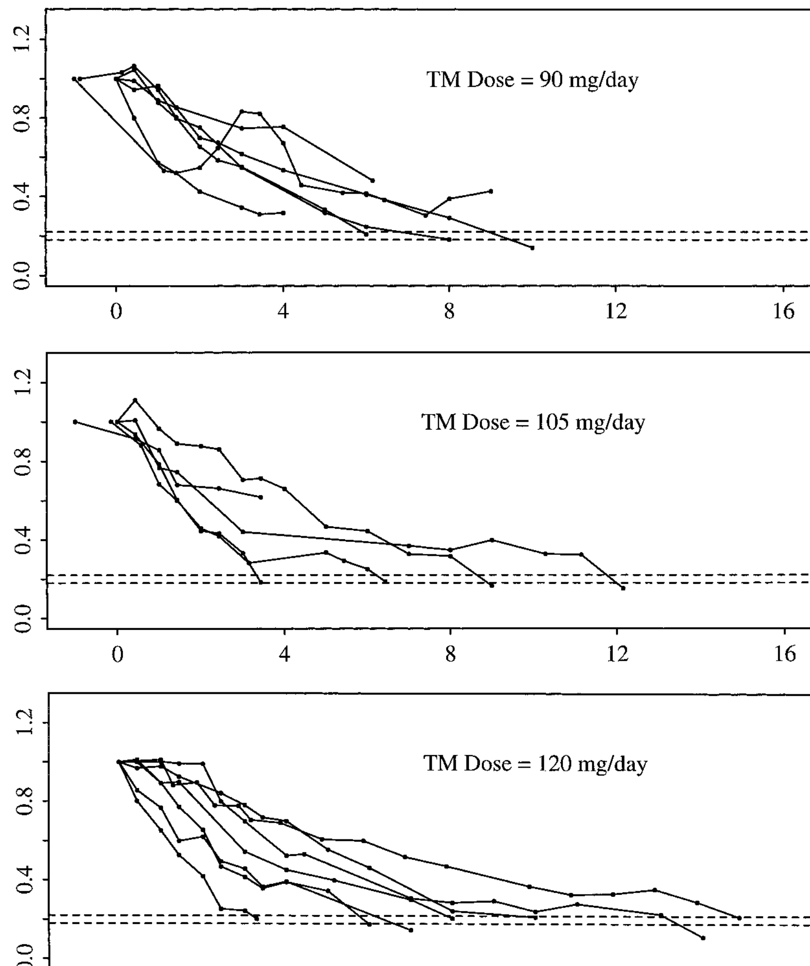

Fig. 1 Cp as a surrogate marker of total body copper status. Rates of decrease of the ratio of Cp at time *t* to baseline Cp as a function of days on TM therapy are depicted for dose levels I, II, and III. The average time to 50% reduction of Cp is 30 days.

stable disease by standard criteria also experienced complete disappearance of some lung lesions and a decrease in the size of other lung lesions during observation periods at target Cp of 120 and 49 days. The five patients on long-term (>90 days) maintenance therapy with stable disease have been copper deficient for 120–413 days at the time of this analysis.

Radiological Evaluation. Serial evaluations of tumor masses by conventional imaging with CAT scan or magnetic resonance imaging revealed that the radiographic appearance of certain masses changed significantly over time. In particular, areas of presumed central necrosis (corresponding to lower attenuation of the X-ray signal) were observed in a variety of tumor types, most notably renal cell cancer, angiosarcoma, and breast cancer. Seeking to evaluate the blood flow to the tumors as a function of time during copper deficiency on long-term TM therapy, lesions accessible to ultrasound were imaged with color flow three-dimensional ultrasound at the onset of copper deficiency and at 2–4-month intervals thereafter.

A representative example of the comparison between conventional CAT scan images and blood flow-sensitive three-dimensional ultrasound is depicted in Fig. 3. Here, a rib metastasis from renal cell carcinoma is depicted when the patient reached target copper deficiency (Fig. 3, *A* and *C*) and 8 weeks later (Fig. 3, *B* and *D*) by these two complementary imaging modalities. Fig. 3, *A* and *B*, shows stable size of this lesion by CAT scan over time, although a more distinct region of probable central necrosis is observed in Fig. 3*B*. In comparison, the color pixel density shown in Fig. 3, *C* and *D*, is the fraction of image voxels within the margins of the mass filled with color flow signals. There has been a 4.4-fold decrease in blood flow to this mass over a period of approximately 8 weeks. In addition to the mass depicted in Fig. 3, this patient had extensive disease in the chest, pelvis, and femurs.

TM in Combination with Other Treatment Modalities

During the long-term maintenance of copper deficiency, additional treatment modalities were added to TM as deemed appropriate for the optimal management of the patients. A patient with previously untreated metastatic breast cancer is doing well with a good-to-excellent quality of life after 12 months of treatment. She had metastases in the paratracheal, posterior cervical, and retroperitoneal lymph node chains but had declined all cytotoxic therapy. The patient had stable disease for more than 6 months on TM treatment, when, due to a

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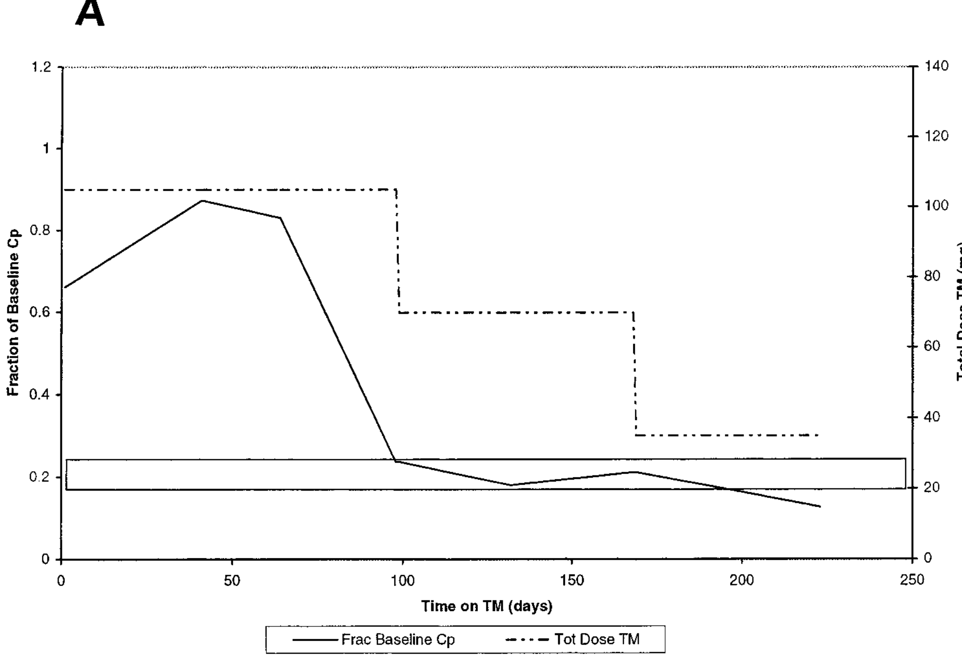

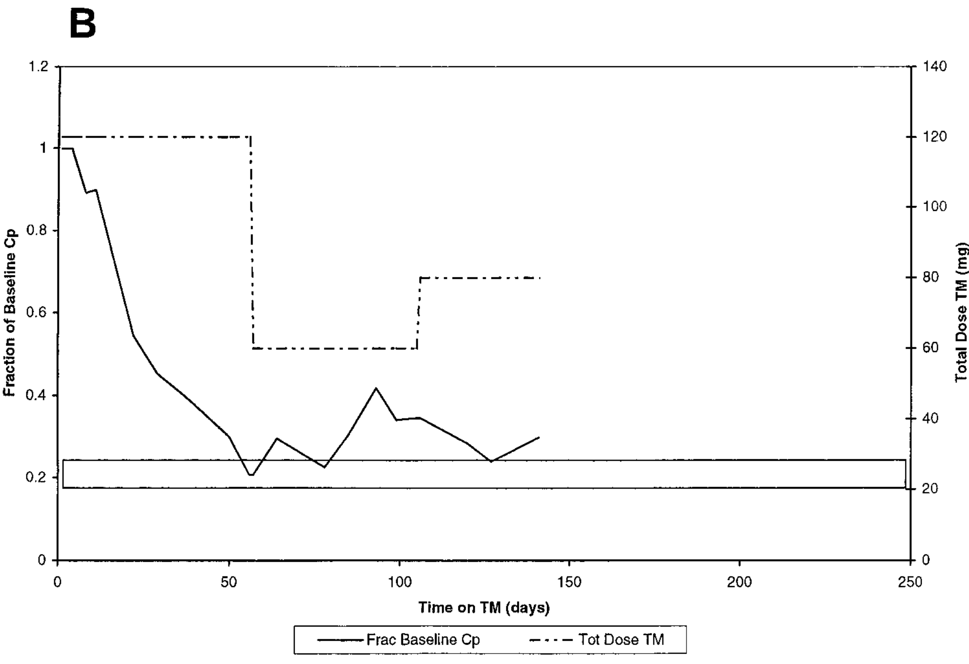

Fig. 2 Management of long-term therapy with TM to maintain a Cp target of 20% of baseline in two patients. *A*, patient required a decrease in dose at two time points that were 60 days apart. To prevent his Cp from falling below 5 mg/dl, this patient will likely require a decrease in TM dose in the future. *B*, TM dose was increased after day 100 to prevent a drifting of the Cp above the target range. Heterogeneity of diet and tumor behavior (such as tumor cell lysis) may account for the individual variability in dose adjustment needs.

slight increase (less than 25% of baseline) in the bidimensional size of the paratracheal and retroperitoneal nodes, she began concurrent trastuzumab (Herceptin; Genentech) therapy after this drug became commercially available. This patient showed a rapid response to trastuzumab at all sites of disease: after one cycle, there was a clinical complete response in the neck; and after three cycles of trastuzumab, there was radiological confirmation of complete response at all previous sites of disease. The patient remains on TM, but the trastuzumab was discontinued after six doses. She continues to maintain her status as a complete responder on TM alone for more than 6 months after discontinuation of trastuzumab therapy. Because the complete response was achieved after the addition of trastuzumab therapy, this patient is classified as having only stable disease on TM on Table 2.

Two patients with extensive angiosarcoma of the face and scalp achieved stable disease on TM. In one patient with severe chronic bleeding from an ocular lesion that threatened the orbit, IFN-α2 was added to TM to attempt to enhance tumor response. Given the suggestion that, based on studies of progressing

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Table 2 Summary of type and length of response to TM therapy

	No. of patients (Total = 18)	Duration in days of copper deficiency (average)
Type of response		
Did not achieve target Cp	4	
Achieved target Cp	14	
Target Cp <90 days	8/14	
Disease progression	7	
Stable disease with partial regression of lung lesions	1	49[a]
Target Cp >90 days	6/14	
Stable disease with partial regression of lung lesions	1/6	120[a]
Stable disease	4/6	159[b], 329[a], 351[a], 413[a] (313[a])
Disease progression at one site, stable elsewhere	1/6	120[a]

[a] On therapy.
[b] Patient discontinued therapy.

hemangiomas, the use of low-dose IFN may be efficacious for the treatment of hemangioma (41), IFN-α was administered to both of these patients at a dose of 500,000 units s.c. twice a day. Radiotherapy was also given to these two patients while on TM to attempt to control actively bleeding (but not progressing) lesions. Both patients had disease stabilization for >60 days, with one of these patients remaining with stable disease for over 5 months before discontinuation of therapy due to patient choice. No exacerbation of toxicity was observed by the addition of any of these treatment modalities to TM.

Discussion

This is the first human trial of induction and maintenance of copper deficiency with TM as an antiangiogenic therapy for cancer. In a group of patients with advanced cancer, we have demonstrated that TM is remarkably nontoxic when Cp is lowered to 10–20% of baseline levels for up to 17 months of treatment. The only drug-related toxicity observed was mild anemia, which was easily reversible with adjustment of the TM dose to bring the Cp level to the desired target. Despite the diverse roles that copper plays in essential biological processes including heme synthesis and superoxide dismutase and cytochrome function, no lasting significant adverse effects were observed on reduction of Cp to approximately 20% of baseline or to a range between 5 and 15 mg/dl. From our data, we surmise that this level of copper reduction constitutes the lower limit of chemical copper deficiency and the beginning of mild clinical copper deficiency, the first manifestation of which is mild anemia. Table 3 summarizes the stages of copper deficiency in humans and their clinical characteristics. This information was derived from studies of patients with Wilson's disease, from occasional patients with chemical and clinical copper deficiency, and from copper-deficient small rodents. Note that as Cp is reduced below 5 mg/dl, it becomes an insensitive marker of the degree of copper deficiency. However, based on observations in humans with normal copper metabolism from this trial, we find that Cp is a sensitive and valid marker of copper status for levels above 5 mg/dl. This key finding allows the targeting of the antiangiogenic window of copper deficiency that appears to be required to slow or arrest tumor growth.

The Cp response to TM-induced copper deficiency is monotonic and exhibits little intersubject variability; therefore, there is essentially no risk of sudden changes or unpredictable fluctuations that might make dose management difficult. Following Cp levels once every 1–2 weeks is adequate to monitor copper status early in therapy. As a corollary, overtreatment is easily detectable and correctable. Using the six times/day dose regimen borrowed from our Wilson's disease work and initial TM doses ranging from 90–120 mg/day, the serum Cp was reliably lowered to 50% of baseline in 17 of 18 patients and to 20% of baseline in 14 of 18 patients. Reduction to 50% of baseline was achieved, on average, in 30 days, with further reduction to Cp levels of 5–10 mg/dl taking 20–30 days. Although this rate of decrease in Cp is reasonable for the initial treatment of early malignant lesions or in the adjuvant setting, in widely metastatic advanced cancer, this rate of decrease will not be sufficiently rapid to prevent some disease progression during induction of copper deficiency in a significant number of patients. Because loading dose variations of 90–120 mg/day do not appear to affect the rate of Cp reduction, and given the typical daily intake of copper with food, we conclude that higher doses in-between meals will likely be required to accelerate the rate of induction of copper deficiency. A follow-up trial is under way to test this hypothesis.

As a result of this study, it is apparent that with our present TM dose regimens, there is considerable lag between the initiation of TM therapy and the reduction of copper levels in tumors to a likely antiangiogenic level. Further retarding the ability to reach antiangiogenic levels of copper deficiency is the likelihood that most tumors sequester copper (42–45). Thus, it is reasonable to hypothesize that additional time may be required to deplete the tumor microenvironment to an effectively low level of copper, which is defined as a level low enough to inhibit angiogenesis. It is difficult to estimate this time accurately from our study. Thus, patients with very rapidly progressive large tumors may be relatively poor candidates for this approach to antiangiogenesis therapy as a single modality.

Another level of complexity is added by the fact that in bulky disease, initially effective antiangiogenesis may cause brisk tumor necrosis, as was documented in the mass shown in Fig. 3. Tumor lysis may result in the release of additional copper from the dying cells. In the case of the patient whose mass is shown in Fig. 3, a transient rise in Cp was observed at approx-

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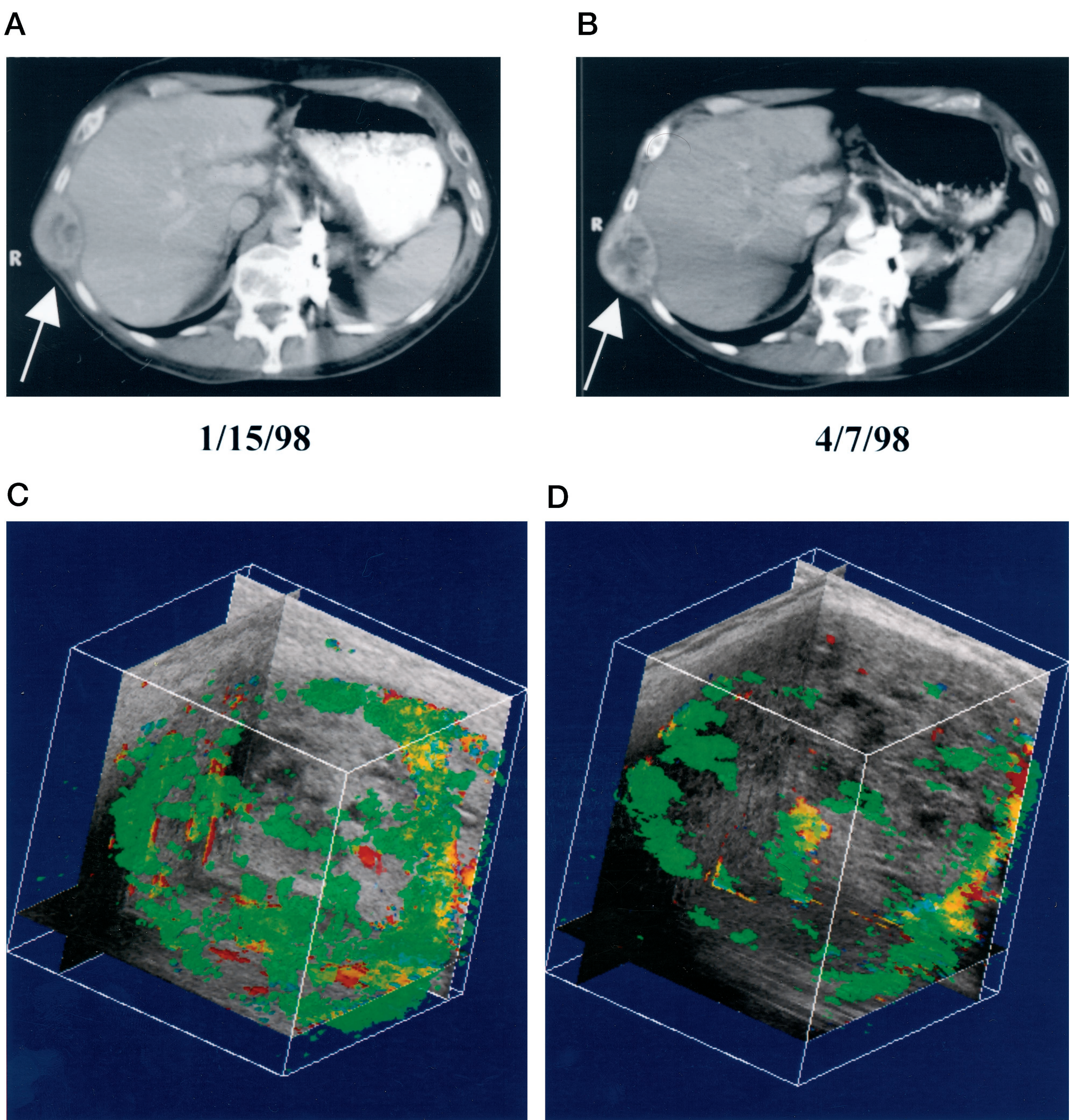

Fig. 3 Evaluation of antiangiogenic response to copper deficiency. CAT scans of the chest of a 59-year-old male with metastatic renal cell carcinoma shortly after achievement of target Cp (*A*) and 8 weeks later (*B*) are shown. Comparable frequency-shift color Doppler image volumes from the superficial renal cell carcinoma rib metastasis were evaluated at the same time points (*C* and *D*). Three-dimensional rendering of the vascularity is shown in *green* superimposed on three orthogonal image planes extracted from the reconstructed grayscale volume. The back plane shown exemplifies one of the frequency-shift color Doppler image planes acquired during the patient scan. Vascularity at initiation of TM therapy (*C*) is markedly greater than that seen 8 weeks later (*D*). Quantitatively, color pixel density is 4.4 times greater in the tumor volume scanned at the first time point; the mean flow velocities are equivalent at both times.

imately the same time as the ultrasound suggested that the large tumor mass might be undergoing central necrosis due to a significant decrease in blood flow. For these reasons, we conclude that a period of 60–90 days of Cp at the target level of 20% of baseline is a reasonable starting point for evaluation of response to anticopper therapy in future trials in patients with measurable disease. In the two patients who exhibited partial regression of lung lesions, tumor control may have begun ear-

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Table 3 Stages of copper deficiency and its clinical effects in humans

Type of copper deficiency	Cp level[a] % Baseline Cp	Absolute Cp level (mg/dl)	Clinical manifestations
Chemical	10–30 (target range)	5–15 (target range)	None[b] Probable inhibition of tumor angiogenesis
Clinical			
Mild	<10%	<5	Mild anemia, Hct ~80% of baseline Mild neutropenia
Moderate	<10%	<5	Moderate anemia, leukopenia, possibly symptomatic
Severe	<10%	<5	Severe bone marrow depression, diarrhea, cardiac arrhythmias may occur rarely, peripheral neuropathy[c] In children, inhibition of epiphyseal bone growth

[a] Normal serum Cp levels are 20–35 mg/dl. Cp levels in cancer patients are elevated (20–75 mg/dl).
[b] Bone marrow effects such as anemia and/or leukopenia may occur if the induction of copper deficiency is very rapid, as with high doses of TM, at higher levels of Cp than shown here.
[c] In general, signs and symptoms other than bone marrow depression require severe copper deficiency to have been present for weeks to months.

lier. It is also interesting to note that in both of these patients, the lung parenchymal metastases were the sites of tumor regression. It is possible that mild clinical copper deficiency impairs superoxide dismutase function (46) so that under conditions of high oxidant stress, such as those present in the lung, the metastatic foci are more susceptible to oxidative damage.

Despite individual differences, the use of three-dimensional ultrasound to determine the total blood flow to a given mass demonstrates that maintenance of mild copper reduction to 20% of baseline induced for at least 8 weeks appears sufficient to alter tumor blood flow. Due to the relative insensitivity of CAT to the blood flow or metabolic status of the lesions, parallel imaging modalities, as demonstrated here for three-dimensional ultrasound, will be required to assess functional response in addition to tumor size.

In light of the data presented above, we advance the preliminary conclusion that the size of solid tumors of a variety of types may be stabilized or decreased by TM, given sufficient time in a state of mild clinical copper deficiency represented by a decrease in Cp to or below 20% of baseline, as defined by this study. Among the patients maintained at the target Cp level for more than 90 days, a significant proportion of cases (five of six) were stabilized, with no detriment to their quality of life. However, in this population of patients with advanced disease, only 39% of those treated were able to be maintained at the target Cp for this duration.

The pattern and speed of progression observed in these patients have also provided useful preliminary information. One patient achieved stable disease at all sites but one and has chosen to remain on TM therapy due to disease stabilization at the more life-threatening sites of disease (bowel and paratracheal lymph nodes; the site of progression in this patient with melanoma is a large adrenal metastasis. This and other observations in this trial suggest that whereas copper deficiency may be generally inhibitory of angiogenesis, heterogeneity of tumor type and the specific location of metastases may modulate the response to this therapeutic modality. The small number of patients in this study and the design of this study preclude more detailed conclusions regarding efficacy at specific metastatic sites. Because it appears that lesions progress at a much faster rate on copper repletion than while on TM therapy, future trials may formally incorporate the use of adjunct modalities, either systemically or loco-regionally, to address the specific sites of progression while allowing the patients to remain in a copper-deficient state.

We report preliminary observations of combination therapies of TM with radiotherapy, trastuzumab, and IFN-α without apparent exacerbation of toxicity of the added modality. Taken as a whole, the safety and preliminary efficacy data derived from this trial support the conduct of additional studies designed to test the specific efficacy of TM alone or in combination for the treatment of early metastatic disease, minimal disease, and in adjuvant high-risk clinical settings, including chemoprevention.

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Clinical Cancer Research

Treatment of Metastatic Cancer with Tetrathiomolybdate, an Anticopper, Antiangiogenic Agent: Phase I Study

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Clin Cancer Res 2000;6:1-10.

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Phase II Trial of Tetrathiomolybdate in Patients with Advanced Kidney Cancer[1]

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ABSTRACT

Purpose: **Tetrathiomolybdate (TM), a copper-lowering agent, has been shown in preclinical murine tumor models to be antiangiogenic. We evaluated the antitumor activity of TM in patients with advanced kidney cancer in a Phase II trial.**

Experimental Design: **Fifteen patients with advanced kidney cancer were eligible to participate in this trial. TM was initiated p.o. at 40 mg three times a day with meals and 60 mg at bedtime to deplete copper. A target serum ceruloplasmin (CP) level of 5–15 mg/dl was defined as copper depletion. Doses of TM were reduced for grade 3–4 toxicity and to maintain a CP level in the target range. Once copper depletion was attained, patients underwent baseline tumor measurements and then again every 12 weeks for response assessment. Patients not exhibiting progressive disease at 12 weeks after copper depletion continued on treatment. Serum levels of Interleukin (IL)-6, IL-8, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were assayed pretreatment and at various time points on treatment. Dynamic contrast enhanced-magnetic resonance imaging (DCE-MRI) was performed on selected patients in an attempt to assess changes in tumor vascularity.**

Results: **All of the patients rapidly became copper depleted. Thirteen patients were evaluable for response. No patient had a complete response or PR. Four patients (31%) had stable disease for at least 6 months during copper depletion (median, 34.5 weeks). TM was well tolerated, with dose reductions most commonly occurring for grade 3–4 granulocytopenia of short duration not associated with febrile episodes. Serum levels of IL-6, IL-8, VEGF, and bFGF did not correlate with clinical activity. Serial DCE-MRI was performed only in four patients, and a decrease in vascularity seemed to correlate with necrosis of a tumor mass associated with tumor growth.**

Conclusions: **TM is well tolerated and consistently depletes copper as measured by the serum CP level. Clinical activity was limited to stable disease for a median of 34.5 weeks in this Phase II trial in patients with advanced kidney cancer. Serum levels of proangiogenic factors IL-6, IL-8, VEGF, and bFGF may correlate with copper depletion but not with disease stability in this small cohort. TM may have a role in the treatment of kidney cancer in combination with other antiangiogenic therapies.**

INTRODUCTION

Approximately 31,800 new cases of kidney cancer occur annually in the United States with an associated 11,600 deaths (1). The incidence and death rate from kidney cancer have increased over the last 2 decades, although the reason for this increase is unknown (2). One-third of the patients who receive a diagnosis of kidney cancer present with metastatic disease. Of the patients who present with local disease and are considered for surgery with curative intent, approximately one-third will go on to develop metastatic disease. Metastatic kidney cancer is resistant to all "standard" forms of radiation therapy, chemotherapy, and hormonal therapies used in the treatment of other kinds of carcinomas.

In the United States, IL-2[3] is the only Food and Drug Administration-approved systemic treatment for metastatic kidney cancer. High-dose bolus IL-2 has resulted in an overall response rate of 15% in patients with metastatic kidney cancer. Approximately 7% of patients achieve a CR, with 80% of these CRs maintained beyond 7 years.[4] Despite these encouraging results of durable CRs, the vast majority of patients with advanced kidney cancer are either not eligible to receive, or do not derive benefit from, IL-2. A critical goal, therefore, in the

Received 10/10/02; revised 12/30/02; accepted 12/30/02.

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[1] Supported by NIH Grant MO1-RR00042 (to the General Clinical Research Center), NIH Grant RO1-CA-77612 (to S. D. M.), and United States Army Breast Cancer Research Program Postdoctoral Fellowship (to Q. P.). S. D. M. and G. J. B. are consultants and have a financial interest in Attenuon, Limited Liability Corporation, which has licensed tetrathiomolybdate as an anticancer compound from the University of Michigan.

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[3] The abbreviations used are: IL, interleukin; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; TGF-α, transforming growth factor α; TM, tetrathiomolybdate; CP; ceruloplasmin; MR, magnetic resonance; DCE, dynamic contrast enhanced; DCE-MRI, DCE-MR imaging; CR, complete response; PR, partial response; VHL, von Hippel-Lindau; AUC, area under the curve; IAUC, initial area under the enhancement curve.

[4] Proleukin: Update of Product License Application, 1996 (on file, Food and Drug Administration).

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treatment of advanced kidney cancer is the development and evaluation of new therapies.

Angiogenesis is an essential process necessary for the growth and progression of tumors and, therefore, a potential target for the treatment of cancer. Several proangiogenic molecules are known from experimental models of tumor angiogenesis such as VEGF, bFGF, IL-8, and TGF-α. These factors are increased in the serum of some patients with cancer and may even have prognostic significance (3).

Kidney cancer is a tumor that is characterized by a high degree of vascularity. Kidney cancer is associated with VHL hereditary syndrome, and the majority of sporadic kidney cancers have loss of heterozygosity at chromosome 3p (spanning the region of the *VHL* gene) or exhibit mutations of the *VHL* gene itself (4, 5). Inactivation of the *VHL* gene is associated with a loss of VEGF suppression and may be one potential explanation for the highly vascular nature of kidney cancer (6). Serum levels of proangiogenic molecules such as VEGF, bFGF, and IL-6 are elevated in patients with advanced kidney cancer, although no causal relationship between these factors and the malignant phenotype has been established (7, 8).

We have been investigating the role of copper as an essential trace element that is required for angiogenesis. It has been demonstrated that copper is required for angiogenesis and tumor growth in several murine tumor models (9–11). Recently, we have evaluated the role of TM in angiogenesis. TM is a copper-lowering agent that has been evaluated extensively in the treatment of Wilson's disease (12–14). TM forms a stable tripartite complex with copper and protein. When given with food, it complexes food copper with food protein and prevents absorption of copper. When TM is administered in the nonfed state, it is absorbed into the blood, in which it complexes either free or loosely bound copper with serum albumin. This TM-bound copper fraction is no longer available for cellular uptake, has no known biological activity, and is slowly cleared in bile and urine. TM has been shown to decrease serum copper levels as well as decrease angiogenesis and tumor growth in two different murine tumor models (15, 16).

In a Phase I trial of TM, we were able to consistently reduce copper levels to 20% of pretreatment baseline, as determined by the surrogate marker serum CP (17). We have subsequently found that higher initial doses than those reported in our Phase I trial result in a more rapid decline in serum CP levels.[5] On the basis of the results of our Phase I trial, we have evaluated TM in a Phase II trial for treatment of advanced kidney cancer.

A potential difficulty of evaluating antiangiogenic agents in the treatment of cancer is that the standard criterion for antitumor activity, tumor shrinkage, may not be applicable. Antiangiogenic agents may prevent tumor growth but not result in a measurable decrease in tumor size. This becomes especially difficult to judge in patients with advanced kidney cancer, a disease with a highly variable natural history. One-third of patients with advanced kidney cancer will meet standard criteria for stable disease while on observation (18). A minority of patients will exhibit stable disease out to 12 months (19).

[5] Unpublished observations.

To assist in the evaluation of TM therapy in patients with advanced kidney cancer, we also evaluated potential surrogate markers of antiangiogenic activity. DCE-MRI is emerging as a powerful noninvasive method of imaging the process relevant to the tumor microcirculation. Recent advances in MR technology provide the enhanced spatial and temporal resolution that allows the application of this methodology to the management of cancer patients. Several studies have shown that DCE-MRI measures correlate well with tumor angiogenesis and are sensitive to tumor physiology and to the pharmacokinetics of the contrast agent in individual tumors (20–24). The high diagnostic/prognostic value of DCE-MRI originates from its ability to provide high-resolution images that depict the perfusion and permeability of small vessels, especially the capillary network. Furthermore, MR studies are reproducible and can be used to monitor tumors longitudinally to detect changes in vascularity induced by treatment. As a result, DCE-MRI measurements offer the potential for a more accurate prediction of tumor response to therapy than do traditional tumor volume measurements.

Multiple potential proangiogenic molecules have been described. One of the mechanisms of the antiangiogenic effect of TM may be by decreasing levels of proangiogenic molecules such as IL-6, IL-8, bFGF, and VEGF secreted by the tumor and inflammatory cells (16). Serum levels of these molecules were monitored serially as part of this trial.

PATIENTS AND METHODS

Eligibility Criteria. Patients must have had documented metastatic kidney cancer who either had not responded to IL-2 or were not eligible to receive IL-2. Patients may also have received forms of therapy other than IL-2, and all prior therapy must have been completed at least 4 weeks before trial entry. Patients had to have a life expectancy of at least 5 months and a performance status 0 or 1. All of the patients had to have adequate organ function defined by: creatinine ≤20 mg/dl, total bilirubin ≤1.5 mg/dl, aspartate aminotransferase ≤2.5 times the institutional upper limit of normal, platelet count ≥100,000/ mm^3, absolute granulocyte count >1000/mm^3, and hematocrit ≥29% (patients may be transfused to this level). Patients were excluded if they had an active infection, were pregnant or had active brain metastases requiring corticosteroid therapy for symptom control. Patients with a treated brain metastasis that had been stable for at least 2 months on imaging were allowed on trial. This study was approved by the University of Michigan Institutional Review Board (Medicine), and all of the patients signed an approved informed consent. This trial was also approved by the University of Michigan General Clinical Research Center.

Study Design. Before treatment, all of the patients had a complete history and physical and baseline laboratory evaluation including: complete blood count with differential and platelet count, serum chemistry profile to include creatinine, aspartate aminotransferase, and total bilirubin, β HCG in women of childbearing potential, and baseline CP level. Pretreatment serum levels of VEGF, bFGF, IL-6, and IL-8 were obtained. Patients underwent studies as indicated to determine the extent and sites of metastatic kidney cancer within 6 weeks of the start

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of treatment (to include imaging of the brain). Those patients who were thought to have an appropriate lesion for DCE-MRI also underwent this procedure. Appropriate lesions for DCE-MRI were nonpulmonary, nonosseous lesions.

Patients initially received TM at 40 mg three times a day with meals and 60 mg at bedtime. Patients kept a medication log to document compliance. A CP level was obtained weekly for 8 weeks, then every 2 weeks. The target CP level was 5–15 mg/dl. Complete blood count was checked every 2 weeks and chemistry profile every month. Patients were evaluated with a history and physical at least monthly. Standard Common Toxicity Criteria version 2.0 was used for toxicity monitoring. If a patient had a decline in hematocrit to <80% of baseline or any other grade 3 or 4 toxicity, TM was held for 5 days. TM was then restarted at 40 mg p.o. with meals twice a day (omitting one with-meal dose) with the once daily 60 mg dose at bedtime maintained. After a TM dose adjustment, CP levels were again monitored weekly until a CP level of 5–15 mg/dl was achieved. If an additional dose reduction was required, then the bedtime dose was reduced to 40 mg once daily. Subsequent dose adjustments were made for toxicity in an attempt to maintain CP levels at 5–15 mg/dl. Dose adjustments were not to occur more frequently than every 2 weeks. Patients were not to be taking multivitamins or nutritional supplements that contain minerals and were asked to avoid organ meats in their diet.

When the CP level reached 5–15 mg/dl (defined as the onset of copper deficiency) patients underwent evaluation for baseline tumor measurement with appropriate radiological studies. Response Evaluation Criteria in Solid Tumors (RECIST) were used for response assessment. Patient were then evaluated for response at 12 weeks from this baseline evaluation, at which time they also underwent DCE-MRI of the same lesion that was evaluated pretreatment. Patients who did not exhibit progressive disease could remain on trial. In addition to CR and PR, stable disease for 6 months was also considered a beneficial response. For patients who remained on study, response assessments continued at 12-week intervals. Serum levels of VEGF, bFGF, IL-6, and IL-8 were obtained at the same time points as imaging studies.

Criteria for Discontinuing Protocol Treatment. Protocol treatment was discontinued for patient preference, unacceptable toxicity, or progressive disease. In this protocol, the first time point for progressive disease was 12 weeks after reaching copper deficiency. Evidence of tumor growth on radiological evaluation at the time of attaining copper deficiency compared with pretreatment was to be expected and was not accepted as treatment failure.

Assays for VEGF, bFGF, IL-6, and IL-8. Blood from patients and normal volunteers was collected in a serum separator tube and was allowed to clot for 30 min before centrifugation at 1000 × *g* for 10 min. Serum was immediately frozen (−70 C) in aliquots of 0.75 ml in microcentrifuge tubes. Human VEGF and human bFGF ELISAs were performed as directed by the manufacturer (R&D Systems, Minneapolis, MN). Briefly, serum (100 μl) was pipetted in triplicate onto the wells precoated for a monoclonal antibody specific for each factor and incubated for 2 h. After 3 washes to remove unbound substances, an enzyme-linked monoclonal antibody specific for each factor was added to the wells and incubated for 2 h. After a wash to remove unbound antibody-enzyme reagent, a substrate solution was added onto the wells and allowed to incubate for 30 min. Optical intensity of each well was measured using a microplate reader. ELISAs for IL-6 and IL-8 were performed by the University of Maryland Cytokine Core Laboratory (Baltimore, MD). For the analysis of cytokine levels, *t* tests were used to compare normal controls with the baseline levels for cases, after performing a log transformation on the levels to approach normality. For comparisons of posttreatment values with baseline levels, paired *t* tests were used.

MR Imaging and Analysis. The MR studies were performed on a 1.5T magnet (Signa; GE Medical System, Milwaukee, WI) with a torso phased-array coil. After a routine (noncontrast) MR exam, DCE-MRI was performed using a three-dimensional spoiled gradient-echo sequence: (TR, 5.2–7.5 ms; TE, 1–2 ms; flip angle, 30°; section thickness, 10–22 mm with zero interpolation yielding an effective slice thickness of 5–11 mm; sections, 12, spectral fat suppression, FOV, 38–42 cm; phase FOV, 0.75; matrix, 224 × 128, NEX 0.5; acquisition time, 6–8 s per temporal phase). Imaging plane was the sagittal or sagittal oblique to encompass the entire tumor and aorta, and to reduce the in- and out-of-plane effect of respiratory motion on the tumor. After a pre-contrast acquisition, DCE imaging was performed after a bolus injection of 0.1 mmol/kg gadolinium chelate (Gadopentetate Dimeglumine-Magnevist; Berlex Laboratories, Wayne, NJ) at a rate of 2 ml/sec via a power injector (Spectris, Medrad, Pittsburgh PA). Automated contrast-bolus detection (SmartPrep) was used to time the dynamic studies. The three-dimensional acquisitions were obtained in groups of three phases, each group requiring an 18–24-s breath-hold. Twenty-five acquisitions were obtained over 4 min. Two additional delayed phases were obtained at 6 min.

Quantitative analyses of the DCE-MRI data were performed using two analysis methods: the AUC, described by Evelhoch (25) with slight modification (26) using the aorta instead of muscle (as was initially described by Evelhoch) as the reference tissue; and two-compartment analysis (27, 28). The dynamic acquisitions were coregistered manually to minimize the effect of motion on the tumor volume. Tumor volume was manually defined by drawing volume of interest (VOI) around the tumor on all 12 slices. This VOI was also applied to calculate tumor statistics for each DCE-MRI parameter. For the AUC, regions of interest were defined over the aorta to provide reference tissue. Gadolinium concentration curves were generated using assumed T_1 values. The reference tissue concentration curves were then used to normalize the tumor AUC. The normalized AUC for each tumor; a hypothesized measure of tumor vascularity, was assessed at 30, 60, and 90 s after contrast injection, also called the IAUC (25).

For the two-compartment analysis, standard measures including the rate constant (κ_{ep}) and the transfer constant (K^{trans}) were assessed (28). These parameters are also hypothesized to be related to tissue vascularity. The aorta was used to provide the arterial input function (AIF).

When multiple tumor nodules were present, the most vascular tumor nodule was chosen for the analysis. The same nodule was analyzed at all time points.

Percentages of change in DCE-MRI parameters were calculated relative to pretreatment values using the following formula:

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Table 1 Patient characteristics

Characteristic	*n*	%
Total	15	
Male	11	73
Female	4	27
Age (yr)		
Median	59	
Range	46–78	
Performance status		
0	13	86
1	2	14
Prior treatment		
IL-2	11	73
Chemotherapy	3	20
None	3	20
Nephrectomy	13	87
Dominant sites of disease		
Lung (lung only)	10 (5)	66
Liver	5	33
Bone	2	13
Primary	2	13
Adrenal	1	7
Time from diagnosis of stage 4 to study entry (mo)		
Median	21	
Range	5–34	

$$\mathit{Posttreatment\ vascularity} - \frac{\mathit{Pretreatment\ vascularity}}{\mathit{Pretreatment\ vascularity}} \times 100$$

Statistical Design. The original design for this study was a Minimax two-stage Phase II study design (29). This design suggested an initial accrual 13 eligible patients. If none of those 13 attained a positive response, then the trial would be stopped. Positive response was defined as CR, PR, or stable disease. If one or more patients had a positive response, then an additional 14 patients would be entered. According to this design, if the response rate was as low as 5%, there would be a 51% chance of stopping the trial after just 13 eligible patients and only a 4.2% chance of going on to a larger study (false positive error). However, if the true response rate was 20% for TM in renal cell cancer patients, there would be only a 5% chance of stopping the study early and a 19.9% chance of rejecting the therapy for further study (false negative). The average sample size, if the same design were used over and over, is 19.8 patients if the true response rate is 5%.

RESULTS

Fifteen patients were enrolled between October 2000 and February 2001. Patient characteristics are shown in Table 1. Most of the patients had received at least one prior therapy for metastatic kidney cancer (usually high-dose IL-2), and 13 had undergone nephrectomy. As expected for this population, the predominant site of metastases was the lung with six patients having only lung disease. Five patients had predominant liver metastases and two had bulky primaries in place (one with bilateral kidney cancer). The median time from initial diagnosis to metastasis was 2 years with a median time from metastasis to study enrollment of 21 months. The study population was healthy with only 2 patients exhibiting any symptoms of their disease and 13 patients asymptomatic (performance status, 0).

All of the patients reached copper deficiency as defined for this study. The median time to reach a CP level $\leq$15 mg/dl was 5 weeks, with a range of 1–15 weeks. The one patient who did not achieve copper deficiency until 15 weeks on study was taking multiple nutritional supplements, which were not reported to the investigators during this time. Once these supplements were discontinued, the patient rapidly reached the target CP level. When this patient was excluded from analysis, the median time to copper deficiency was 4.5 weeks.

Thirteen patients were evaluable for response. Two patients who were not evaluable discontinued therapy early for reasons other than progressive disease. Both patients discontinued protocol treatment at 9 weeks on study, 4 weeks after achieving copper deficiency. One discontinued therapy secondary to toxicity (fatigue) and the other for an inability to adhere to protocol therapy. As defined in this study, the time point for the first response evaluation was when a patient was copper deficient for 12 weeks. Eight of the 13 patients met this criterion. Five patients had evidence of progressive disease before 12 weeks (median, 7.5 weeks) while they were copper deficient.

Eight patients maintained copper deficiency for 12 weeks. Of these eight patients, four exhibited progressive disease on scheduled evaluations at 12 weeks and four had stable disease. The 4 patients with stable disease continued on therapy and had a total duration of copper deficiency of 28–45 weeks (median, 34.5 weeks). All of them ultimately had progressive disease, one in sites of known disease and three with new sites of disease. Overall, the 6-month progression-free survival was 31%, and the median time to progression was 13 weeks (range, 3–45 weeks).

The patients with stable disease differed from the rest of the study population in time from first metastasis to study entry. These four patients had a median time from metastasis to study enrollment of 27 weeks compared with the nine patients who progressed more rapidly and who had a median of 13.5 weeks ($P = 0.0018$).

All of the patients were evaluable for toxicity. TM was relatively well tolerated. Only one patient discontinued therapy secondary to toxicity (fatigue). Grade 1–2 fatigue was reported by almost all of the patients, as was the occurrence of sulfurous eructation after taking TM. Other toxicities reported that did not require a dose reduction were as follows: four patients had grade 1 nausea; three patients had grade 1–2 diarrhea; four patients complained of occasional episodes of feeling dizzy without documented blood pressure changes; and two patients developed a self-limiting macular rash on their trunk.

Eleven patients had dose reductions in TM. The most frequent cause for a dose reduction of TM was grade 3 or 4 granulocytopenia. Four patients had one reduction; two patients had two dose level reductions; and one patient each, had three and four dose level reductions. After stopping TM, the granulocytopenia decreased to grade 1 or 2 by 5 days. There were no episodes of febrile neutropenia. Granulocytopenia was not seen before 12 weeks on study. Despite dose reductions in TM, the CP level remained within the study-specified target range. One patient had a single dose level reduction for anemia. Two

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patients had one dose reduction of TM for a CP level that was below the range defined by the study.

Serum levels of VEGF, bFGF, IL-6, and IL-8. Serum levels of VEGF, bFGF, IL-6, and IL-8 were determined pretreatment, at the time of reaching copper deficiency, and after 3 and 6 months of copper deficiency (Table 2). For control, we used the serum levels from 29 healthy adults obtained at two different time points. The pretreatment levels of VEGF, IL-6, and IL-8 were significantly higher then control levels ($P \leq 0.0003$). Pretreatment levels of bFGF were not significantly different from unaffected controls ($P = 0.10$).

At the time of reaching copper deficiency, the levels of all four of the factors were significantly reduced from pretreatment levels ($P \leq 0.035$). At this time point, only the serum level of IL-6 remained significantly greater then control levels. At 3 months of copper deficiency, the levels of VEGF, bFGF, and IL-8 were not significantly different from pretreatment levels. Only IL-6 at 3 months remained significantly reduced from pretreatment levels.

DCE-MRI Imaging. Four patients had tumor nodules accessible for DCE-MRI and were on treatment long enough for posttreatment evaluations. The results of the two methods of analysis are shown in Table 3. There was a significant increase (>25%, WHO criteria) in the bi-product measurements of the tumor mass in all four of the patients (mean, 77%; range, 26–147%). Tumor vascularity, as assessed by the IAUC/aorta, had increased (5 and 73%) in two patients, and reduced (−5 and −22%) in two patients. Likewise, K^{trans} was increased in one patient (63%) and reduced in three patients (−11, −13, and −21%). In the three patients with increase in tumor size and reduction (or minimal increase) in tumor vascularity, there was extensive tumor necrosis. There was no necrosis in the tumor that increased in both size and vascularity.

DISCUSSION

We have evaluated TM, a novel antiangiogenic molecule in patients with advanced kidney cancer. Although the process of angiogenesis is a multifactorial process that has not been fully elucidated, copper appears to be an essential requirement of this process. TM has been shown in a Phase I cancer clinical trial and in Wilson's disease to consistently deplete copper. In this Phase II trial, we have once again confirmed the ability of p.o. administered TM to rapidly deplete copper as evaluated by serum CP levels.

TM has been relatively well tolerated. All of the patients experienced some degree of fatigue, which was generally mild, with only one patient discontinuing therapy because of this symptom. The other side effect seen in most patients was sulfurous eructation that occurred after taking TM. The toxicity that required a dose reduction of TM was granulocytopenia. However, this was never associated with a febrile episode and was rapidly reversible with holding TM for 5 days. It is difficult to state the exact etiology of the granulocytopenia, but we have made some potentially useful observations. It is not an acute toxicity, because it did not occur before 12 weeks on study. Also it seems to exhibit a complex relationship to copper deficiency as assessed by CP levels because, after dose reductions for granulocytopenia, the CP level remained in the deficient range

Table 2 Serum levels

Patient	VEGF (pg/ml)					bFGF (pg/ml)					IL-6 (pg/ml)					IL-8 (pg/ml)				
	Base	Cu def	3 mo	6 mo	9 mo	Base	Cu def	3 mo	6 mo	9 mo	Base	Cu def	3 mo	6 mo	9 mo	Base	Cu def	3 mo	6 mo	9 mo
1	261.61	16.16	294.54			12.07	6.76	6.25			42.17	28.71	36.24			45.87	21.54	51.38		
2	267.82	222.2	222.09	220.45		5.02	3.28	3.43	6.12		4.46	0	2.13	3.86		37.54	6.39	14.36	16.24	
3	424.21	294.82	273.8			6.56	2.26	2.35			29.8	9.51	15.69			30.76	3.99	4.1		
4	344.47	356.65	398.6			3.18	4.07	4.67			7.17	8.96	7.96			13.86	14.09	15.63		
5	401.25	138.32	274.43	463.23		8.11	4.71	4.5	7.43		42.85	2.84	14.63	30.57		50.03	40.53	36.57	46.2	
6	297.92	283.78	234.05			23.47	13.81	15.63			6.45	3.78	3.36			13.22	8.97	9.68		
7	925.35	766.01	252.89			2.97	1.49	6.32			12.15	9.54	11.63			15.02	7.95	9.48		
8	211.97	218.51	206.09	NA	648.87	5.33	2.49	2.83	NA	8.54	6.78	6.57	3.87	NA	7.63	63.88	72.37	63.45	NA	86.31
9	474.17	354.03				2.26	ND				6.12	4.13				6.46	1.08			
10	646.08	240.12				6.98	4.41				20.67	20.54				9.02	8.25			
11	448.42	432.04				1.05	8.58				45.5	41.63				10.84	11.67			
12	204.64	163.77	256.84			3.13	2.13	4.06			14.05	11.73	16.43			36.85	33.24	37.5		
13	128.5	130.15	132.86			4.29	4.78	4.63			26.48	21.65	19.67			9.31	11.78	11.28		
14	127.8	111.98	109.89	128.76		6	4.35	4.3	6.19		9.67	11.31	10.53	9.46		15.34	12.97	14.63	13.01	
15	345.62	304.69	368.24			5.46	6.36	6.59			33.86	24.63	30.62			42.62	35.64	40.12		

[a] Base, baseline; Cu def, copper deficient; ND, not determinable; NA, no sample available.

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Table 3 DCE-MRI results

Patient no.	Pretreatment vascularity	Posttreatment-1[a] vascularity	% change	% change in biproduct diameter	Necrosis
Analysis 1, IAUC/aorta					
5	750.0582	787.4162	4.98	147	++
6	869.5683	827.1792	−4.87	26	+
8	426.5111	331.5037	−22.28	51	+++
13	231.9096	400.8148	72.83	84	No
Analysis 2, two-compartment model (K^{trans})					
5	295.4349	258.3588	−12.55	147	++
6	297.7969	236.1423	−20.70	26	+
8	114.4119	102.3782	−10.52	51	+++
13	69.0811	111.7936	61.83	84	No

[a] Posttreatment-1 vascularity, 1st on treatment evaluation at 12 weeks of copper deficiency: biproduct diameter, product of two bidimensional measurement; +, minimal; ++, moderate; +++, large.

(≤15 mg/dl) and granulocytopenia did not always recur. Two case reports of pancytopenia (30), occurring in patients who had Wilson's disease and were receiving TM, show a clinical pattern of leukopenia similar to the one that we report. These two cases, after chronic TM exposure (2 and 5 months) at high doses, also exhibited leukopenia that was rapidly reversible with the discontinuation of TM (several days). Bone marrow biopsies performed on these patients were thought to be consistent with a myeloid maturation arrest. However, these two cases, as well as two other cases associated with TM use in Wilson's disease reported in the literature (31), exhibited pancytopenia, which was not seen in our patients. The mechanism of this toxicity warrants further investigation in patients with normal copper homeostasis.

Using standard criteria of CR and PR, TM alone has shown no efficacy in patients with advanced kidney cancer. However, the standard response criteria may have no value in evaluating a drug that is not known to be cytotoxic but rather may be cytostatic. Stable disease also has its pitfalls in evaluating efficacy in a Phase II trial. Using criteria of stable disease for at least 6 months after copper depletion as defined in this trial, we found that our overall response rate was 31%. Interestingly, the four responding patients differed markedly from the rest of the study population. The median time of having metastatic kidney cancer before entering this trial was significantly greater for these responders (median of 27 weeks *versus* 13.5 weeks). In retrospect, this group of responding patients might have been expected to have a longer progression-free survival, based on the natural history of their disease before study entry. Nonetheless, we cannot say for sure that they would have; therefore, by our entry criteria and the more inclusive definition of response that includes stable disease, 31% of patients in this cohort were responders. In the future, methods will need to be developed to facilitate the clinical development of cytostatic agents and, at the same time, appropriately use patient numbers. One method is to better define the population before study entry so that it is more uniform.

Prognostic subgroups of patients with advanced kidney cancer have been defined (32). However, this method has been seen as more suited as a means to stratify patients for larger randomized Phase III trials than for Phase II exploratory trials. Another method that may be more appropriate in the Phase II setting has been used before in a trial of IFN in advanced kidney cancer (19). In this type of trial, patients are entered into the first phase, which consists of monitoring time off therapy for evidence of disease progression. Once the patients have disease progression, they are then begun on the investigational therapy. The time to disease progression on observation *versus* treatment can then be compared in each individual patient with each patient being used as his or her own control. This method may be best suited for kidney cancer trials because of the variable natural history of this disease.

Another method of evaluating cytostatic agents in small Phase II trials would be to use surrogate end points of efficacy. In antiangiogenic trials, there is no consensus as to what these surrogate end points should be (33). In our trial, we evaluated serum levels of several factors that have been associated with angiogenesis, and we also used specialized imaging to assess the vascularity of tumors. The serum levels of VEGF, IL-6, and IL-8 were significantly reduced at the time of reaching copper deficiency as compared with pretreatment levels, indicating that the attainment of copper deficiency, at least initially, correlates with a decrease in these mediators, as predicted from the laboratory. However, this effect did not persist at a later study time point. For individual patients, there was no direct correlation between these levels and response, but the trial, with only 13 patients evaluable for response, was not powered to evaluate this relationship.

We also attempted to analyze tumor vascularity by using DCE-MRI. In the four patients in whom we were able to image, a decrease in vascularity as measured by DCE-MRI did not correlate with a decrease or stability in the size of the tumor. In the few examples from this study, it appears that DCE-MRI vascularity correlates better with the degree of necrosis than with actual tumor growth as measured by tumor mass size.

The factors responsible for the neovascularity associated with malignant growth are not well delineated in all tumor types. Copper seems to be one of many necessary substrates required for this process. TM is a relatively well-tolerated copper-depleting agent that, as a single agent, achieved stable disease in 31% of patients with advanced kidney cancer. Given the natural history of this disease, this percent of stable disease may not be significantly different from that observed without treatment. Because of the multiple pathways involved in angiogenesis, TM

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may be of benefit in combination with other antiangiogenic therapies.

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Clinical Cancer Research

Phase II Trial of Tetrathiomolybdate in Patients with Advanced Kidney Cancer

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Clin Cancer Res 2003;9:1666-1672.

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Published OnlineFirst October 21, 2016; DOI: 10.1158/1078-0432.CCR-16-1326

Cancer Therapy: Clinical

Clinical Cancer Research

Influencing the Tumor Microenvironment: A Phase II Study of Copper Depletion Using Tetrathiomolybdate in Patients with Breast Cancer at High Risk for Recurrence and in Preclinical Models of Lung Metastases

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Abstract

Purpose: Bone marrow–derived progenitor cells, including VEGFR2$^+$ endothelial progenitor cells (EPCs) and copper-dependent pathways, model the tumor microenvironment. We hypothesized that copper depletion using tetrathiomolybdate would reduce EPCs in high risk for patients with breast cancer who have relapsed. We investigated the effect of tetrathiomolybdate on the tumor microenvironment in preclinical models.

Experimental Design: Patients with stage II triple-negative breast cancer (TNBC), stage III and stage IV without any evidence of disease (NED), received oral tetrathiomolybdate to maintain ceruloplasmin (Cp) between 8 and 17 mg/dL for 2 years or until relapse. Endpoints were effect on EPCs and other biomarkers, safety, event-free (EFS), and overall survival (OS). For laboratory studies, MDA-LM2-luciferase cells were implanted into CB17-SCID mice and treated with tetrathiomolybdate or water. Tumor progression was quantified by bioluminescence imaging (BLI), copper depletion status by Cp oxidase levels, lysyl oxidase (LOX) activity by ELISA, and collagen deposition.

Results: Seventy-five patients enrolled; 51 patients completed 2 years (1,396 cycles). Most common grade 3/4 toxicity was neutropenia (3.7%). Lower Cp levels correlated with reduced EPCs ($P = 0.002$) and LOXL-2 ($P < 0.001$). Two-year EFS for patients with stage II–III and stage IV NED was 91% and 67%, respectively. For patients with TNBC, EFS was 90% (adjuvant patients) and 69% (stage IV NED patients) at a median follow-up of 6.3 years, respectively. In preclinical models, tetrathiomolybdate decreased metastases to lungs ($P = 0.04$), LOX activity ($P = 0.03$), and collagen crosslinking ($P = 0.012$).

Conclusions: Tetrathiomolybdate is safe, well tolerated, and affects copper-dependent components of the tumor microenvironment. Biomarker-driven clinical trials in high risk for patients with recurrent breast cancer are warranted. *Clin Cancer Res; 23(3); 666–76. ©2016 AACR.*

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Corrected online 9/2/2020.

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doi: 10.1158/1078-0432.CCR-16-1326

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Introduction

The tumor microenvironment has emerged as a critical target influencing cancer progression. It is well established in preclinical mouse models of breast cancer progression that the premetastatic niche depends on bone marrow–derived cells to create a hospitable microenvironment that supports tumor progression (1–5). We and others have shown that this includes bookmarking of the premetastatic niche by hematopoietic progenitor cells (HPCs; CD45$^+$, CD34$^+$, VEGFR1$^+$) and activation of the angiogenic "switch" by VEGFR2$^+$ endothelial progenitor cells (EPCs; CD45^{dim}, CD133$^+$, VEGFR2$^+$) and CD11b$^+$ myeloid progenitor cells (6–9). Furthermore, we have demonstrated, in a large cohort of patients with breast cancer, that this model of metastatic progression recapitulates the preclinical models (10).

Copper has emerged as an essential component of the metastatic process, and multiple preclinical studies have demonstrated that

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Translational Relevance

This study targets copper-dependent processes within the tumor microenvironment known to be critical for establishment of the premetastatic niche in a cohort of patients with breast cancer at high risk of relapse. The premetastatic niche constitutes a local microenvironment that provides optimal infrastructure for disseminated tumor cells to colonize and grow. Using a copper depletion strategy that targets copper-dependent processes critical for creating this niche, we show a markedly reduced infrastructure critical for tumor progression (LOX- and bone marrow–derived progenitor cells called VEGFR2$^+$ EPCs), and this is possibly most relevant in triple-negative breast cancer. Breast cancer recurrence in this high-risk cohort of patients is quite low with relapses rare after 2 years, even in the stage IV patients with NED. Translational studies are underway to understand the clinical observations and identify those patients who are potentially best suited to this strategy.

copper depletion decreases proliferation, blood vessel formation, tumor growth, and motility (11–15). Copper depletion may also reverse epithelial–mesenchymal transition (EMT) and downregulate expression of EMT-related genes, such as vimentin and fibronectin (16). Furthermore, copper is a key component of several enzymes critical to remodeling the tumor microenvironment and lung premetastatic niche, including lysyl oxidase (LOX), superoxide dismutase-1, and vascular adhesion protein-1 (17–21).

Specifically LOX, secreted by the primary tumor, is a copper-dependent amine oxidase that accumulates at premetastatic sites, where it crosslinks collagen forming a scaffold for recruited bone marrow–derived CD11b$^+$ myeloid cells. This scaffold acts as a "premetastatic niche" and promote tumor outgrowth of disseminated metastatic tumor cells (22–25). Thus, VEGFR1$^+$ HPCs and CD11b$^+$ myeloid progenitor cells establish the premetastatic niche; VEGFR2$^+$ EPCs, among other cells, colonize and activate the angiogenic switch, leading to macrometastases formation (6, 22, 26, 27).

Tetrathiomolybdate is an oral copper chelator initially developed for the treatment of Wilson disease (28). It blocks key steps in angiogenesis by inactivating copper chaperones and decreasing copper-dependent enzymes (29). It was also suggested that tetrathiomolybdate could promote tumor dormancy (30, 31). Subsequent phase I and II studies in advanced malignancies showed that tetrathiomolybdate was safe and well-tolerated, but with limited efficacy in patients with advanced cancer (32–36).

We hypothesized that copper depletion using tetrathiomolybdate could prevent relapse by suppressing the EPC-mediated angiogenic switch and by disrupting copper-dependent LOX in the tumor microenvironment. We piloted this approach in patients with breast cancer who have no evidence of disease (NED) but who are at a very high risk of relapse after standard therapy. This includes N3 disease, stage II and higher triple-negative breast cancer (TNBC), and those with stage IV NED. To elucidate the mechanisms behind our clinical observations, we used mouse models of TNBC to understand whether tetrathiomolybdate affected the premetastatic niche so that it could better inform our choice of correlative studies on banked patient samples. Using a preclinical breast cancer mouse model, we also explored the relationship between copper depletion and its effects on the tumor microenvironment, specifically on LOX, and collagen crosslinking. We report here the clinical results of the first 75 patients who completed 24 months of tetrathiomolybdate therapy and the results of our concurrent laboratory experiments elucidating the effect of tetrathiomolybdate on the premetastatic niche in a mouse model of lung metastases. We have previously reported our clinical experience in 40 patients on tetrathiomolybdate for 12 months (37).

Materials and Methods

Tetrathiomolybdate

Tetrathiomolybdate is an oral copper chelator initially developed for the treatment of Wilson disease (28). Tetrathiomolybdate blocks key steps in angiogenesis by decreasing copper-dependent enzymes and can promote tumor dormancy (29–31). All treatment with tetrathiomolybdate was administered on an outpatient basis. Clinical grade tetrathiomolybdate (produced by GMP) was purchased in bulk from Sigma Aldrich Chemical Company under IND #71,380 held by L. Vahdat (Department of Medicine, Weill Cornell Medicine, New York, NY). Tetrathiomolybdate was stored under argon, and stability testing was routinely performed (38). Research pharmacists dispensed tetrathiomolybdate in gelatin capsules and maintained an inventory using the NCI Drug Accountability Record Form.

Treatment

Tetrathiomolybdate was administered for 2 years on the primary study. We administered tetrathiomolybdate in two phases, induction and maintenance: In the induction phase, patients were prescribed 180 mg of tetrathiomolybdate daily in four divided doses until Cp levels decreased to a target range of 5–17 mg/dL. Twenty-eight days of tetrathiomolybdate administration comprised one cycle. When Cp was within target, patients were switched to the maintenance phase. In the maintenance phase, patients took 100 mg of tetrathiomolybdate daily in divided doses. Doses were reduced in 20-mg increments to minimize toxicity and/or increased in 20-mg increments to maintain Cp target. Patients were taken off study for relapse or unacceptable toxicity. Patients completed medication logs for each cycle. The duration of the trial was 2 years. To evaluate compliance, patients were asked to document dose intake through daily dosage logs that were collected at the end of each cycle.

Extension studies. Patients with stage IIIC and stage IV NED were eligible to continue tetrathiomolybdate in 2-year increments on a series of extension studies, and those data will be reported separately.

Clinical study design

This phase II, open-label, single-arm study enrolled patients on an Institutional Review Board (IRB)-approved trial (NCT00195091, 0903-882, local IRB: 0309006307) at Weill Cornell Medical College Iris Cantor Breast Cancer Center (New York, NY). Written informed consent was obtained prior to undergoing any study-specific procedures in accordance with the Declaration of Helsinki. The schema is outlined in Supplementary Fig. S1. Patients were accrued between June 2007 and August 2014.

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Clinical study objectives

The primary endpoint was to assess the change in VEGFR2$^+$ EPCs in patients who were treated with oral tetrathiomolybdate for 2 years. Secondary endpoints were safety, event-free survival (EFS), effect of tetrathiomolybdate on VEGFR1$^+$ HPCs, and levels of plasma angiogenic factors and cytokines. Samples were banked for every cycle.

Patients. Patients were considered eligible for the study if they met the following criteria: at least 18 years of age; histologically confirmed stage II TNBC, stage III, or stage IV NED of all molecular subtypes; no radiographic, biochemical, or physical evidence of active breast cancer; more than 6 weeks from previous therapy including surgery, radiation, chemotherapy, biologic treatment; Eastern Cooperative Oncology Group (ECOG) performance status 0–1 and adequate organ function. Patients were stratified by molecular subtype according to the immunohistochemical marker profile described by Cheang and colleagues (39). For the purposes of this study, the TNBC and basal-like (ER and/or PR $\leq$ 10% and HER 2 neu–negative) were grouped together as TNBC. Protocol eligibility was expanded to include patients with stage II TNBC on March 25, 2009 due to new data suggesting that the risk of recurrence is comparable with a stage III non-TNBC (40). Concurrent hormonal therapy was permitted. HER2-positive patients were all required to have completed 1 year of standard trastuzumab therapy. Physical exam, laboratory studies (CBC, CMP, CEA, and CA15-3), imaging studies (either CT of chest, abdomen, and pelvis (CT C/A/P) and bone scan, or positron emission tomography (PET/CT) were required within 4 weeks prior to start of treatment.

Clinical and radiographic assessments

Patients were evaluated at baseline and every 4 weeks thereafter with physical examination and laboratory studies including complete blood count, complete metabolic panel, tumor markers, and research laboratory studies. Patients underwent imaging of investigator's choice, CT of chest, abdomen, and pelvis or PET/CT every 6 months and as needed to assess for relapse, using Response Evaluation Criteria in Solid Tumors (RECIST; 41).

Safety and tolerability

The National Cancer Institute Common Toxicity Criteria for Adverse Events (CTCAE) version 3.0 were used for toxicity and adverse event reporting (42). In the event of grade 3/4 toxicity, dosing was held until recovery. Treatment was resumed at the investigator's discretion at up to 90% of the previous dose. If recovery did not occur within 2 weeks, the patient was removed from study. In the event of grade 2 toxicity, the dose of tetrathiomolybdate was held until recovery and a new cycle could be initiated at 100%. If grade 2 toxicity recurred, dosing was held until recovery and the next cycle was resumed at 80% to 90% of prior dose. All patients were available to be evaluated for toxicity.

Measurement of hemangiogenic progenitor cells

Ten to 20 mL of venous blood was collected in EDTA-containing tubes and processed within 12 hours. Peripheral blood mononuclear cells were isolated by Ficoll density-gradient centrifugation. To quantitate circulating EPCs, cells were stained with CD133-PE (Miltenyi Biotec), VEGFR2-APC (R&D Systems), and CD45-PerCP (BD Biosciences). To quantitate HPCs, cells were stained with CD34-FITC (BD Biosciences), VEGFR1-APC (R&D Systems), and CD45-PerCP (BD Biosciences). Multicolor flow cytometry was performed as described previously (10).

Methods for quantification of LOXL2 levels in patient serum

Patient serum samples were collected in blood vacutainer tubes containing no anticoagulant. Blood was left at room temperature for at least 30 minutes to allow clotting. Blood samples were spun at 1,500 $\times$ g for 10 minutes at 4°C. Serum was removed and stored at -80°C until assayed.

Quantitation of blood serum LOXL2 levels

Patient LOXL2 serum levels were quantitated using an ELISA kit from US Biologicals following the manufacturer's protocol. One-hundred microliters of patient serum was added in duplicate to the wells of a microtiter plate coated with a biotin-conjugated antibody specific to LOXL2, along with concentration standards. After 2-hour incubation, avidin conjugated to horseradish peroxidase (HRP) was added to each well and incubated. TMB substrate solution was added and wells containing LOXL2 biotin–conjugated antibody and HRP enzyme–conjugated avidin exhibited a color change. The degree of color change was then measured with a spectrophotometric plate reader at 450 nm. Concentrations were calculated on the basis of the standard curve.

Statistical analysis

Descriptive statistics for demographic and biomarker variables were calculated for all patients enrolled in the trial. The intent-to-treat (ITT) population consisted of patients who had at least two doses of tetrathiomolybdate. The following outcomes were recorded: toxicity attributable to tetrathiomolybdate, time to progression of disease, overall survival, number of circulating hemangiogenic progenitor cells, and serum markers of interest. A sample size of 35 achieved a 90% power to detect a difference of 0.5 between EPC/mL at baseline and at last time point, with an estimated SD of 1.1 and two-sided α level of 0.05. After the first 35 patients were enrolled and a potential "signal" was observed in TNBC, we expanded the enrollment to 75 (accounting for $\sim$10% dropout) to further characterize the intervention in patients with TNBC. Each hypothesis testing procedure is as stated in the article. EFS and OS calculations were performed via Kaplan–Meier. Survival analyses for EFS, OS and time to first depletion event were computed using Fleming–Harrington tests with $\rho = 1$. For brevity, if the testing procedure was not stated then a mixed-model was used; details of all mixed models are available in Supplementary Statistical Methods.

All models involving "dose" reflect the actual dose taken as recorded in the patients' medication logs. For this reason, no patients were excluded from the analysis for compliance reasons.

The large number of inferential tests performed in this article necessitates a multiple comparisons adjustment. Details of the adjustment procedure are available in Supplementary Statistical Methods. The procedure implies that a significance level of alpha $= 0.0244$ should be used to assess significant hypotheses. Throughout the article, only hypotheses that meet this threshold are described as significant.

Analysis and graphics were computed using R: A Language and Environment for Statistical Computing, R Core Development Team (2013), and packages lme4, survival, and ordinal were used. All code was documented and adhered to the highest reproducibility standards.

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Preclinical study

The primary objective was to assess the effect of copper depletion on tumor burden in mice treated with tetrathiomolybdate or placebo (water). We used a breast cancer mouse model that closely resembled breast cancer progression and metastasis in patients with TNBC. In this model, highly metastatic human TNBC cells (MDA-LM2), stably expressing the luciferase and GFP transgene (1×10^6 cells) were implanted into the mammary fat pad of CB17-SCID mice ($n = 27$), and allowed to develop primary tumors (43). Upon tumor incidence (2 weeks postimplantation), one cohort ($n = 11$) was treated with tetrathiomolybdate (0.7 mg/day in drinking water for 3 weeks) and the other cohort ($n = 16$) with water as a control. For the metastasis studies, there were 10 controls and five tetrathiomolybdate–treated mice. For the premetastatic niche studies, there were six controls and six tetrathiomolybdate–treated mice.

Serum Cp level was used as a biomarker to determine copper status and monitor copper depletion 7 days post-tetrathiomolybdate treatment initiation (44, 45). Blood serum was collected and centrifuged at 3,000 × *g* at 4°C for 10 minutes. The serum was then pre-incubated in in sodium acetate buffer (pH5, 4°C) at 30°C for 5 minutes. *O*-dianisidine dihydrochloride (7.88 mmol/L, Sigma) reagent (preincubated at 30°C) was then added as a substrate to two replicate tubes of serum and incubated at 30°C for 30 minutes and 45 minutes, respectively. The reaction mixture was quenched with 9 mol/L sulfuric acid, and the absorbency was measured at 540 nm. The Cp oxidase activity was calculated as [(A45-A30) × 0.625 U]/mL. Once we achieved copper depletion (20%–30% baseline) as measured by Cp oxidase levels, we continued to monitor tumor progression by bioluminescence imaging (BLI) to further assess the effects of tetrathiomolybdate on the primary tumor (44, 45).

After 4 weeks, primary tumors were resected to prevent mortality due to primary tumor burden, and to develop metastases to the lungs. The mice were evaluated for lung metastases for an additional 2 weeks by BLI. Metastatic lungs harvested from both tetrathiomolybdate and water-treated mice were analyzed for collagen deposition by Picrosirius Red staining, according to the manufacturer's protocol (PolySciences, Inc.). Collagen fibers were visualized and images captured using both parallel and orthogonal light microscopy. Collagen fibers were quantified using ImageJ. Multiphoton second harmonic generation (SHG) imaging was used to characterize collagen quantity and collagen fiber length in rehydrated cross-sections of lungs using a custom-built multiphoton microscope as described previously (46).

We measured LOX antigen on these lungs as described previously (47). The assay was first optimized using a commercially available recombinant mouse LOX protein (rmLOX; R&D Systems, catalog no. 1564-LX). The assay reaction mixture consisted of 50 mmol/L sodium borate (pH 8.2), 1.2 mol/L urea, 50 μmol/L Amplex Red, 0.1 U/mL horseradish peroxidase, and 10 mmol/L 1,5-diaminopentane (cadaverine) substrate (3). The protein samples [hydrogen peroxide (H_2O_2), rmLOX, and lung lysates] were added to the reaction mix in the presence or absence of 500 μmol/L BAPN and were preincubated for 5 minutes at room temperature. The absorbance was measured at 590 nm using a microplate reader, at 1-minute intervals for a period of 10 minutes. Following optimization, we extracted protein from the metastatic lungs with a lysis buffer consisting of 4 mol/L urea, 0.02 mol/L

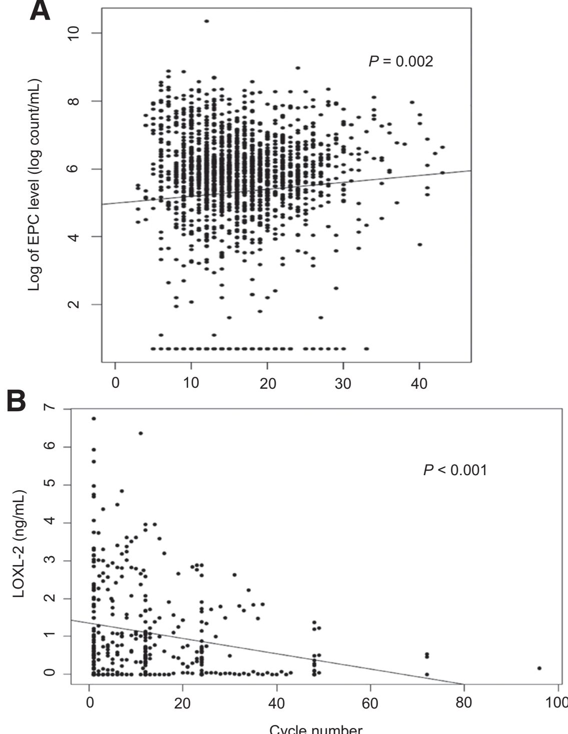

Figure 2.
Effect of tetrathiomolybdate on patients at a high risk for recurrence. **A,** Tetrathiomolybdate (TM)-associated copper depletion was strongly associated with a reduction in VEGFR2$^+$ EPCs using a mixed-effects model ($P = 0.002$). Each observation reflects a cycle (28 days); **B,** Lysyl oxidase 2 (LOXL-2) at cycle 12 and 24 were markedly reduced at both time points ($P < 0.001$). Both VEGFR2$^+$ EPCs and LOX condition the pre-metastatic niche and create a permissive environment for tumor metastases.

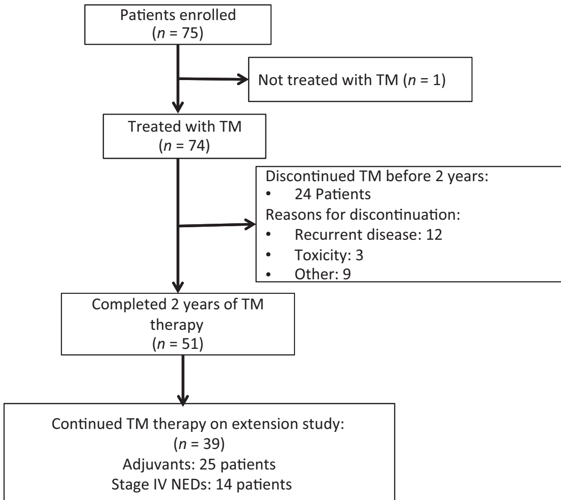

Figure 1.
Consort diagram of patients enrolled on the tetrathiomolybdate (TM) trial. This study reports on the first 75 patients who completed the primary 2-year study.

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borate (pH 7.8), phenylmethylsulfonylfluoride, and protease inhibitors (Roche). To maintain the activity of the LOX enzyme, we concentrated the protein extract using a 10 K micron filter (Millipore). We measured LOX activity of this concentrated protein as described previously. The fluorescent product was excited at 560 nm, and the emission was read at 590 nm every 5 minutes for 2 hours. LOX activity was measured as fluorescent units and normalized to untreated controls.

Mouse and human LOX levels in concentrated lung lysates were quantified by LOX ELISA following the manufacturer's instructions (USCN SEC580Hu and SEC580Mu).

Results

Patient characteristics

Between June 13, 2007 and August 1, 2014, 75 patients were enrolled as shown in Fig. 1. Supplementary Tables S1 and S2 describe patient baseline characteristics. The majority of patients were at high risk of relapse, including 55% of patients with stage III disease and 40% of patients with stage IV NED. The prior disease sites for stage IV NED patients include bone, chest wall, axilla, and visceral sites such as liver, brain, lung, and the peritoneum. Of note, 48% (36/75) of the patients had TNBC. The entire cohort received standard chemotherapy either in the adjuvant or metastatic setting prior to enrolling in this study.

Tetrathiomolybdate effectively depletes copper level as measured by Cp level

Median baseline Cp level at study entry was 28 mg/dL, which decreased to 14 mg/dL after one cycle (paired Wilcoxon: $P < 0.001$, $n = 73$). Median time to first depletion (Cp $\leq$ 17 mg/dL) was 4 weeks, with 85% of patients achieving at least one Cp of 17 or less within 8 weeks and 97% within 16 weeks.

The addition of a proton pump inhibitor (PPI) was significantly associated with improved tetrathiomolybdate absorption, which facilitated lowering the Cp level (paired Wilcoxon: $P = 0.001$, $n = 47$). As tetrathiomolybdate is a short-acting compound, the drug is administered between two and four times a day. Adherence was good with 55% of cycles having perfect adherence. Of the remainder 45%, of the cycles where adherence was not perfect, patients missed on average less than one pill (4 mg) a day. A mixed-effects model revealed no significant association between age ($P = 0.078$), body surface area ($P = 0.839$), or non-tamoxifen hormone therapies ($P = 0.482$) and Cp level. We observe no significant evidence of tachyphylaxis (effect of cycle number on Cp level) for patients who have continuous dosing of tetrathiomolybdate therapy (mixed model: $P = 0.485$).

Patients with TNBC are copper depleted significantly faster compared with other molecular subtypes

The amount of time to achieve first Cp $\leq$ 17 mg/dL was significantly reduced for TN compared with non-TN patients (survival model: $P < 0.001$, n1 = 39, n2 = 35; Supplementary Fig. S2). Median baseline Cp for patients with TNBC was 23 mg/dL compared with 31 mg/dL for other molecular subtypes (unpaired Wilcoxon: $P = 0.024$, n1 = 3, n2 = 27). Patients on tamoxifen achieved target levels of copper depletion significantly slower than their non-tamoxifen–prescribed counterparts (survival model: $P < 0.001$, n1 = 62, n2 = 13).

EPCs and LOXL2 are significantly reduced only in patients who are copper depleted

A mixed-effects model for EPC suggests that effective copper depletion drives down EPCs. Cp is highly significant in explaining EPC level and that a lower Cp correlates with lower EPC level ($P = 0.002$; Fig. 2A). Furthermore, TNBC subtype does affect EPC level ($P = 0.002$), even after accounting for the effect of Cp. In addition, a mixed-effects model for EPC level with tetrathiomolybdate dose suggests a significant negative correlation ($P = 0.005$). A difference in EPC levels between TNBC and non-TNBC patients at baseline was also observed (unpaired Wilcoxon: $P = 0.007$, n1 = 36, n2 = 38).

In the preclinical models, lysyl oxidase, a heavily copper-dependent enzyme critical for conditioning the premetastatic niche, was markedly reduced in the premetastatic niche of the lungs of the mice that were copper depleted; therefore, we sought to investigate whether circulating lysyl oxidase levels were affected in the patients with breast cancer who were copper depleted. We found that LOXL2 was significantly reduced at 12 months (paired Wilcoxon: $P < 0.001$, $n = 36$) and 24 months ($P < 0.001$, $n = 29$) in patients compared with baseline with a 55% decline at 12 months (Fig. 2B). In addition, Cp is correlated and highly significant in a mixed-effects model for LOXL2 ($P < 0.001$), as is tetrathiomolybdate dose ($P = 0.001$).

Tetrathiomolybdate is safe and well tolerated with a low (5.4 %) incidence of grade 3 or 4 toxicities

Tetrathiomolybdate was well tolerated in most patients, with only 5.4% of all cycles with grade 3 or 4 toxicities as shown in Table 1. The most common toxicities were sulfur eructation, neutropenia, and fatigue; however, most were grade 1 and 2. One patient who developed febrile neutropenia discontinued from the study. A patient with grade 3 anemia also discontinued from the study, but was later found to have vitamin B_{12} deficiency as the cause. If a grade 3 or 4 toxicity occurred, tetrathiomolybdate was held until resolution (average 5–10 days) and restarted at 20 mg a day lower dose.

Relationship between tetrathiomolybdate dose, Cp level, and toxicity

We explored the relationship between grade 3 or 4 toxicity, tetrathiomolybdate dose, and Cp level. We found the mean Cp for

Table 1. Adverse events of patients enrolled on study

Adverse event per cycle (total cycles = 1,396)	*N* (%) All grades	*N* (%) Grade 3/4
Hematologic		
Anemia	174 (12.5)	1 (0.07)
Neutropenia	292 (20.9)	47 (3.7)
Febrile neutropenia	1 (0.07)	1 (0.07)
Leukopenia	287 (20.6)	23 (1.6)
Thrombocytopenia	26 (1.9)	0 (0)
Gastrointestinal		
Sulfur burps	374 (26.8)	0 (0)
Nausea	14 (1.0)	0 (0)
Vomiting	4 (0.3)	0 (0)
Diarrhea	32 (2.3)	0 (0)
Constipation	6 (0.4)	0 (0)
Abdominal pain	1 (0.07)	0 (0)
General		
Fatigue	232 (16.6)	0 (0)
Neurologic		
Dizziness	10 (0.7)	0 (0)
Neuropathy	116 (8.3)	0 (0)

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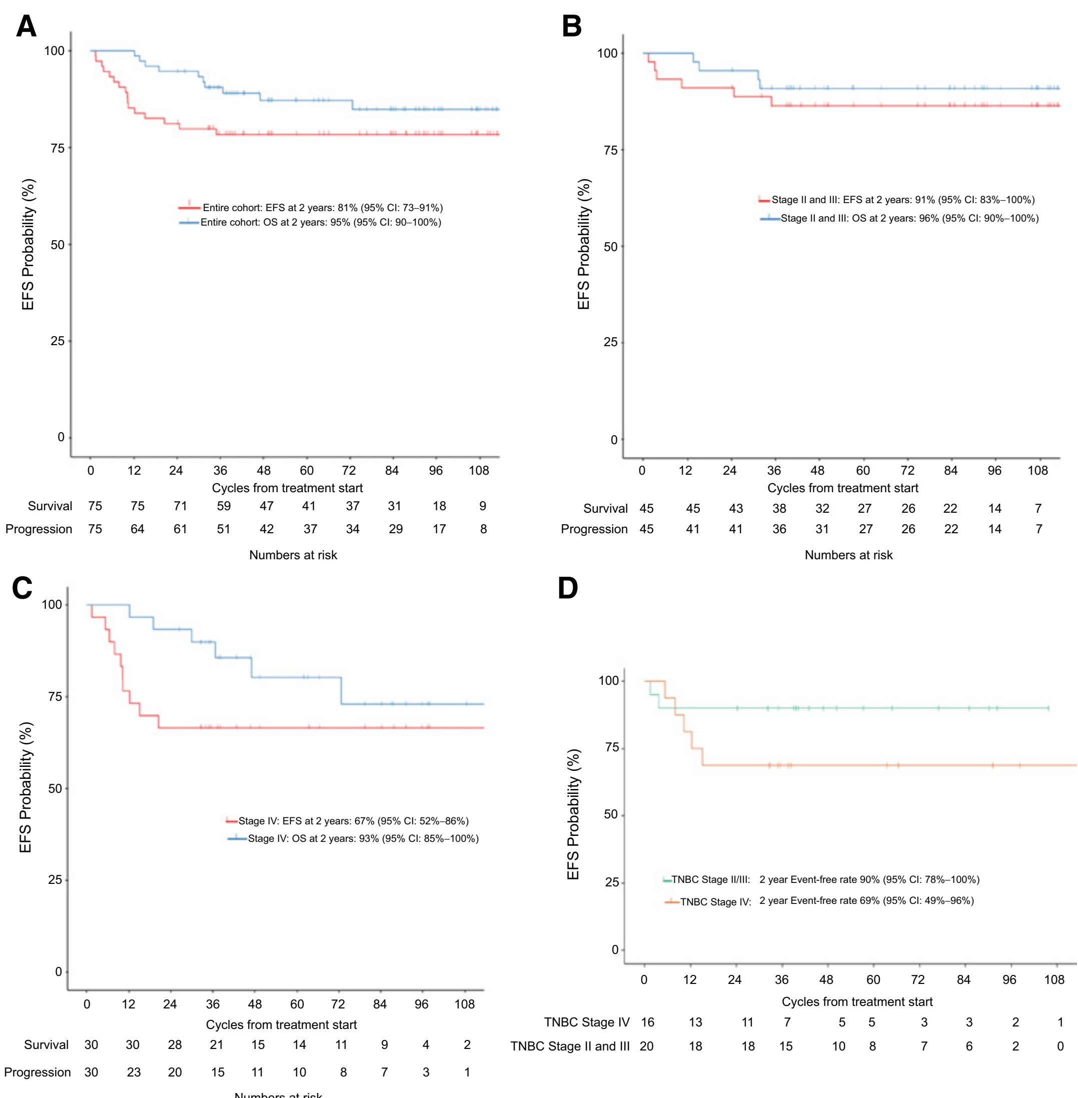

Figure 3.
Event-free (EFS) and overall survival (OS) 2-year (24 cycles) are shown in these Kaplan-Meier curves with associated confidence intervals. (**A**) EFS and OS for the entire cohort (stage II TNBC and stage III and IV NED) was 81% and 95%, respectively; (**B**) EFS and OS for the adjuvants was 91% and 96%, with no difference observed between TNBC and non-TNBC patients (data not shown) (**C**). For patients who were stage IV NED at study entry, 67% are event free, and OS is 93% (**D**) EFS for the TNBC patients that are adjuvants is 90%, and stage 4 NED is 69%.

cycles with these toxicities was 10.7 mg/dL (SD = 3.8 mg/dL). However, the mean tetrathiomolybdate dose prescribed was low at only 3.2 pills per day (SD = 1.6 mg/dL) or 64 mg a day. All grade 3 or 4 adverse events resulted from a dose of less than six pills per day (120 mg). This suggests that low Cp levels, rather than prescribed dose, are associated with toxicity. A mixed-effects ordinal regression model showed that Cp correlated negatively with adverse events toxicity ($P < 0.001$), that is, lower Cp levels increased the risk for high-toxicity grade-adverse events. Furthermore, dose was not significant in explaining adverse events toxicity ($P = 0.142$) after accounting for Cp levels. Adverse events toxicity risk decreased as patients underwent more treatment cycles ($P = 0.002$).

Outcome EFS and OS

Overall, with a median follow-up time of 6.3 years, the EFS and OS rates for the entire cohort were 72% and 84%, respectively. At 2 years, the EFS and OS for patients with stage II and III breast cancer were 91% and 96%, respectively. The EFS and OS for the patients with stage IV NED was 67% and 93%, respectively (Fig. 3A–C). The outcome did not differ significantly between TN and non-TN patients (survival model: $P = 0.814$ (EFS) and $P = 0.222$ (OS). The time to relapse for adjuvant TNBC patients was similar to non-TNBC patients ($P = 0.814$). The 2-year EFS for stage II, III TNBC is 90% and 69% for those who are stage IV NED (Fig. 3D). With prolonged follow-up, recurrences are rare after 2 years.

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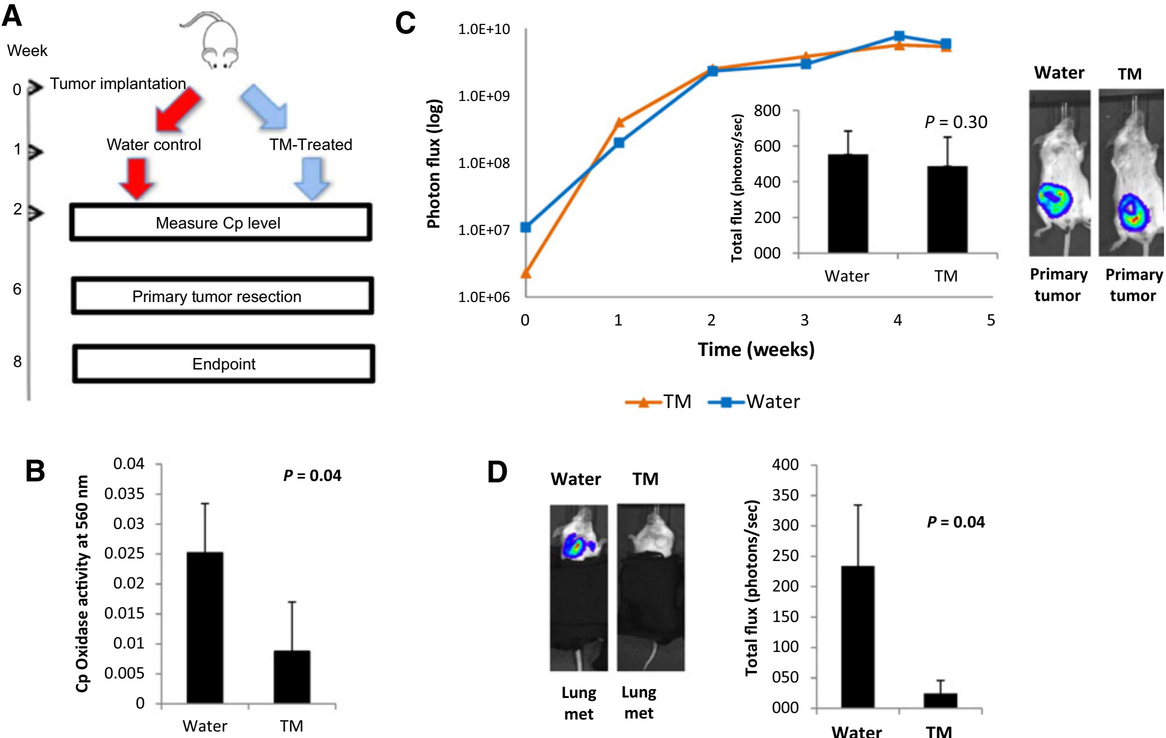

Figure 4.
Tetrathiomolybdate (TM) suppresses lung metastases *in vivo*. **A,** Schema of experimental timeline. **B,** Cp levels in water control and tetrathiomolybdate (TM)-treated (0.7 mg/day) mice 1-week post-tetrathiomolybdate administration, showing 64% copper depletion (CD) below baseline in the tetrathiomolybdate-treated group. **C,** Quantification of primary tumors at 6 weeks post-tumor implantation and 5 weeks post-tetrathiomolybdate administration. Top right, BLI of primary tumors derived from orthotopic injections into the mammary fat pad of 1×10^6 MDA- LM2-luciferase cells ($n = 10$ water control, $n = 5$ tetrathiomolybdate; TM). **D,** Quantification of lung metastases at 7 weeks post-tumor implantation, 6 weeks post-tetrathiomolybdate administration and 7 days postresection.

Sixteen patients relapsed on study (including four patients after 2 years). The median time to relapse for the patients who relapsed was 9.2 months from study enrollment with a range of 1.5 months to 32 months. Only four patients relapsed after more than 1 year on study. Of the 12 patients with recurrent disease within 1 year, six relapsed at ≤6 months, and the remainder relapsed between 6 months at ≤10 months). In this group, only 50% of the patients were copper depleted to target.

In preclinical models, tetrathiomolybdate suppresses lung metastases but has no effect on primary tumor. The investigational schema is shown in Fig. 4A. Tetrathiomolybdate therapy reduced Cp oxidase levels in the metastatic lungs of mice treated with tetrathiomolybdate versus water to acceptable levels (64% below baseline; Fig. 4B, $P = 0.04$). Tetrathiomolybdate-mediated copper depletion decreased secondary lung metastases as demonstrated by BLI, but did not have significant effects on the primary tumor (Fig. 4C, $P = 0.30$; Fig. 4D, $P = 0.04$).

LOX activity and levels are reduced in tetrathiomolybdate-treated premetastatic lungs. To determine whether LOX could be directly targeted by tetrathiomolybdate, we incubated recombinant LOX with tetrathiomolybdate. Strikingly, LOX activity as measured by Amplex Red Fluorescence Assay Kit (Molecular Probes), was dramatically reduced by tetrathiomolybdate comparable with BAPN (a known inhibitor of LOX activity). Notably, addition of copper rescued LOX activity in a dose-dependent fashion confirming that the loss of LOX activity was due to copper depletion by tetrathiomolybdate (Supplementary Fig. S3).

In vivo, LOX antigen was reduced in the premetastatic lungs of mice treated with tetrathiomolybdate and correspond to lower Cp levels than water-treated lungs (Fig. 5A, $P = 0.03$), Mouse LOX levels were unaffected.

Collagen deposition is decreased in tetrathiomolybdate-treated premetastatic lungs. Tetrathiomolybdate therapy diminished collagen deposition as revealed by Picrosirius staining and visualization for fibrillar collagen under polarized light in tetrathiomolybdate and water-treated premetastatic lungs and quantified by ImageJ software (Fig. 5B and C, $P = 0.01$). Accordingly, SHG imaging analysis confirmed that less collagen was deposited in lungs with tetrathiomolybdate treatment (Fig. 5D and E, $P = 0.04$) and that the collagen fibers formed with tetrathiomolybdate therapy were shorter relative to those formed in control animals (Fig. 5F, $P = 0.04$).

Discussion

Emerging evidence demonstrates that the interactions between tumor cells and the microenvironment play key roles in the

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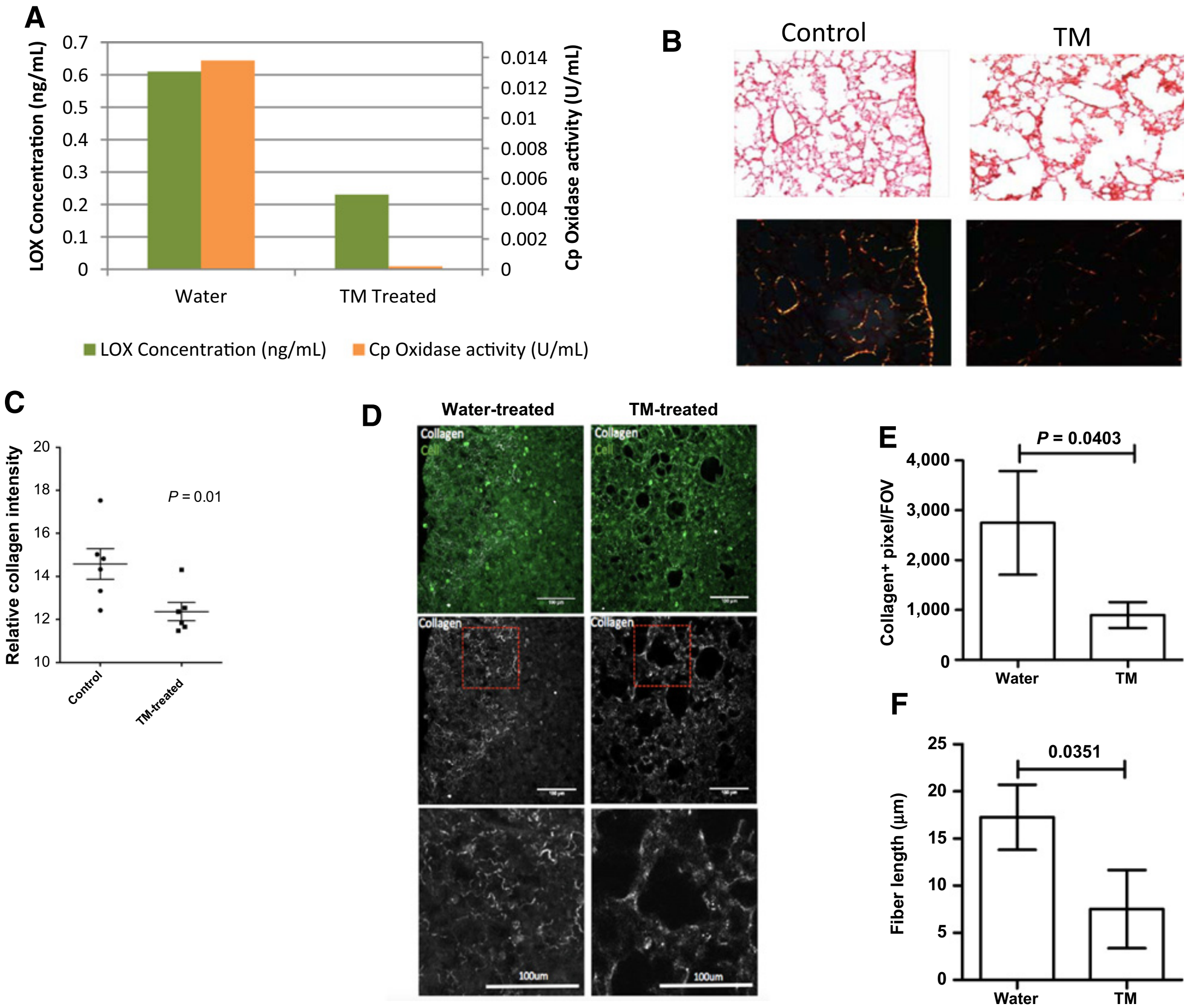

Figure 5.

Tetrathiomolybdate (TM) decreases human LOX levels and collagen deposition in premetastatic lungs *in vivo*. **A,** LOX was reduced in the TM-treated lungs ($P = .03$). Human LOX ELISA of one representative water ($N = 6$) and tetrathiomolybdate ($N = 6$) premetastatic lungs at 4 weeks after tumor implantation and 2 weeks post-tetrathiomolybdate administration. **B,** Picrosirius Red staining of premetastatic lungs measuring parallel (top) and orthogonal (bottom) polarized light intensity of fibrillar collagen. **C,** Average quantification of Picrosirius using ImageJ, including representative figures quantified within the boxed area (water, $n = 10$; tetrathiomolybdate $n = 5$; **D** and **E**). Accordingly, SHG imaging analysis confirmed that less collagen was deposited in lungs with tetrathiomolybdate treatment ($P = 0.04$) and that the collagen fibers formed with tetrathiomolybdate therapy were shorter relative to those formed in control animals $P = 0.04$; **F**.

metastatic process. To the best of our knowledge, this is the first study demonstrating that the tumor microenvironment can potentially be altered to remove critical components of the metastatic cascade that are necessary for tumor progression in patients with breast cancer at a high risk of relapse. We demonstrate that patients can be safely copper depleted for up to 2 years and that this is associated with a marked reduction of the VEGFR2+ EPCs (critical for the angiogenic switch) and LOXL2 two components of the tumor microenvironment that appear to be important for tumor progression. Our results suggest that if components of the tumor microenvironment, specifically bone marrow–derived VEGFR2$^+$ EPCs, are reduced (either through the inability to mobilize or quantitative reduction), then tumor progression might be less likely to occur. Taken a step further, we speculate that significant suppression of EPC surges may inhibit activation of the angiogenic switch and subsequent clinical relapse.

Several key themes emerge from this study, including that patients with TNBC are more effectively copper depleted than other molecular subtypes, and for patients who are copper depleted, serum LOX2 is also reduced. LOX is critical to conditioning the premetastatic niche in preclinical models of TNBC that metastasize to lung and was reduced with tetrathiomolybdate administration in our patients with breast cancer who were copper depleted.

As this is a single-arm study, the low number of EFS events while very encouraging, need to be confirmed in a larger randomized placebo-controlled study. We enrolled patients at very

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Published OnlineFirst October 21, 2016; DOI: 10.1158/1078-0432.CCR-16-1326

high risk of relapse in this study including 31% that were triple negative with involved axillary lymph nodes and 35% that were stage IV NED. In our trial, the EFS for patients with stage II/III TNBC was 90% and 50% for those with stage IV NED disease at a median follow-up time of 6.3 years. While selection bias can account for these results, we look forward to conducting additional studies that can control for those factors. We observed two patterns of relapses in this study. The first pattern was the development of overt metastases within 3 months of study entry and the second pattern was tumor recurrence around cycle 11 of tetrathiomolybdate therapy, less than a year after starting protocol therapy. We speculate that early relapses were occurring when enrolled on the tetrathiomolybdate trial (i.e., too late to make an effect) and the later relapses were predominantly (although not exclusively) in patients who were not adequately copper depleted. This is particularly encouraging because the median overall survival of stage IV TNBC is 13 months in multiple large datasets and the median overall survival in our study has not been reached. Similarly, the median overall survival for stage III TNBC is less than 40% at 4 years in most large datasets and in our cohort appears to be longer although a randomized trial is necessary to confirm this exploratory observation (40, 48).

This translational study also highlights the importance of a seamless laboratory to clinic and back again collaboration. After we observed what we considered a paucity of tumor recurrences (realizing that this is not a randomized trial) in our patients with stage IV NED TNBC, we sought to dissect what mechanisms might be responsible in a xenograft model of TNBC that metastasizes to the lungs. We found that the tumor microenvironment or premetastatic niche was significantly altered, specifically that LOX was significantly reduced and this in turn led to the inhibition of collagen crosslinking, which forms the scaffolding necessary for tumor progression. Going back to the banked human specimens, we found that LOX was significantly reduced in patients who were copper depleted with tetrathiomolybdate in our study similar to what was observed in the preclinical model optimizing our utilization of samples and resources.

Moving forward, we hypothesize that tetrathiomolybdate causes global changes in the tumor and host microenvironment, rendering it inhospitable for tumor progression. It is well known that the extracellular matrix (ECM) is critical for cancer–stromal cell interactions that allow and promote invasion and metastasis (49, 50). The ECM provides the structural and molecular framework for tumor progression facilitating focal adhesion, cell proliferation and motility, and availability of copper to be mobilized from existing pools is a critical component (51–55). Taken together, we believe that the effect of copper depletion on the VEGFR2$^+$ EPCs and LOXL2 are just two of a series of downstream effects that potentially render the microenvironment nonreceptive to tumor progression resulting in the promotion of tumor dormancy. Further studies to elucidate these steps in both the preclinical models of tumor progression and identify the group of patients who might benefit from this approach are warranted and underway.

Disclosure of Potential Conflicts of Interest

T. Cigler reports receiving speakers bureau honoraria from Novartis. C. Fischbach is a consultant/advisory board member for Leibniz Institute for Polymer Research Dresden. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Other (data entry): J. Guillaume-Abraham
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Acknowledgments

We would like to thank our donors who contributed to the study without whose support this project would not be possible. A special thanks to Divya Ramchandani, PhD who assisted with the preclinical studies. Finally, we would like to thank our patients enrolled on this study. They are equally committed to this study as we are and have voted with their feet in support of it.

Grant Support

This work was supported by the Anne Moore Breast Cancer Research Fund (all authors), Stephen and Madeline Anbinder Foundation (all authors), Susan G Komen for the Cure (to L. Vahdat), New York Community Trust (to L. Vahdat), Manhasset Women Against Breast Cancer (to L. Vahdat, V. Mittal), Breast Cancer Alliance of Greenwich (to L. Vahdat) Cancer Research and Treatment Fund (to L. Vahdat), and Berman Fund (to L. Vahdat), Keith Miller Foundation (to L. Vahdat), Anonymous (to L. Vahdat, V. Mittal), and Lefkofsky Foundation (to L. Vahdat, V. Mittal). Laboratory specimens were supported by The National Center for Advancing Translational Science of the NIH award number UL1TR000457.

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Received June 1, 2016; revised September 13, 2016; accepted September 26, 2016; published OnlineFirst October 21, 2016.

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Correction: Influencing the Tumor Microenvironment: A Phase II Study of Copper Depletion Using Tetrathiomolybdate in Patients with Breast Cancer at High Risk for Recurrence and in Preclinical Models of Lung Metastases

Nancy Chan, Amy Willis, Naomi Kornhauser, Maureen M. Ward, Sharrell B. Lee, Eleni Nackos, Bo Ri Seo, Ellen Chuang, Tessa Cigler, Anne Moore, Diana Donovan, Marta Vallee Cobham, Veronica Fitzpatrick, Sarah Schneider, Alysia Wiener, Jessica Guillaume-Abraham, Elnaz Aljom, Richard Zelkowitz, J. David Warren, Maureen E. Lane, Claudia Fischbach, Vivek Mittal, and Linda Vahdat

In the original version of this article (1), the abstract contained an incorrect value for event-free survival in stage IV NED patients with triple-negative breast cancer. The value (69%) was correct in the Results section and in Fig. 3D. The abstract has been corrected in the latest online HTML and PDF versions of the article. The authors regret the error.

Reference

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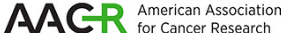
Published online September 15, 2020.
Clin Cancer Res 2020;26:5051
doi: 10.1158/1078-0432.CCR-20-3177
©2020 American Association for Cancer Research.

Published OnlineFirst October 21, 2016; DOI: 10.1158/1078-0432.CCR-16-1326



Clinical Cancer Research

Influencing the Tumor Microenvironment: A Phase II Study of Copper Depletion Using Tetrathiomolybdate in Patients with Breast Cancer at High Risk for Recurrence and in Preclinical Models of Lung Metastases

Nancy Chan, Amy Willis, Naomi Kornhauser, et al.

Clin Cancer Res 2017;23:666-676. Published OnlineFirst October 21, 2016.

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Annals of Oncology 24: 1491–1498, 2013
doi:10.1093/annonc/mds654
Published online 13 February 2013

Tetrathiomolybdate-associated copper depletion decreases circulating endothelial progenitor cells in women with breast cancer at high risk of relapse

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Received 19 July 2012; revised 21 November 2012; accepted 12 December 2012

Background: Bone marrow-derived endothelial progenitor cells (EPCs) are critical for metastatic progression. This study explores the effect of tetrathiomolybdate (TM), an anti-angiogenic copper chelator, on EPCs in patients at high risk for breast cancer recurrence.

Patients and methods: This phase 2 study enrolled breast cancer patients with stage 3 and stage 4 without evidence of disease (NED), and stage 2 if triple-negative. TM 100 mg orally was administered to maintain ceruloplasmin <17 mg/dl for 2 years or until relapse. The primary end point was change in EPCs.

Results: Forty patients (28 stage 2/3, 12 stage 4 NED) were enrolled. Seventy-five percent patients achieved the copper depletion target by 1 month. Ninety-one percent of triple-negative patients copper-depleted compared with 41% luminal subtypes. In copper-depleted patients only, there was a significant reduction in EPCs/ml by 27 ($P = 0.04$). Six patients relapsed while on study, of which only one patient had EPCs maintained below baseline. The 10-month relapse-free survival was 85.0% (95% CI 74.6%–96.8%). Only grade 3/4 toxicity was hematologic: neutropenia (3.1% of cycles), febrile neutropenia (0.2%), and anemia (0.2%).

Conclusions: TM is safe and appears to maintain EPCs below baseline in copper-depleted patients. TM may promote tumor dormancy and ultimately prevent relapse.

Key words: breast cancer, endothelial progenitor cells, tetrathiomolybdate

introduction

Despite improvements in adjuvant therapy of breast cancer over the past two decades, there is significant risk of relapse in a high-risk subset of patients. Although the definition of high risk has been evolving, it still includes patients with stage 3 breast cancer and those with stage 4 with no evidence of disease (NED). The risk of relapse in stage 3 patients is 50%–75% over 5 years, and patients with stage 4 breast cancer will inevitably recur even when temporarily rendered disease-free by surgery, radiation, or chemotherapy [1]. Reflecting the increasing importance of biology over stage, now included in the definition is the triple-negative subset [lack of expression of estrogen receptor (ER)/progesterone receptor (PR), nor overexpression of human epidermal growth factor 2 (HER2)]. These patients have a poor prognosis in earlier stages and represent a disproportionately increased percentage with metastatic disease [2–4].

The tumor microenvironment and its components, including stromal fibroblasts and endothelial and inflammatory cells, play a major role in the establishment, progression, and metastatic dissemination of cancer [5–11]. Using preclinical models of breast cancer that metastasize to the lungs, the premetastatic niche is comprised of recruited bone marrow-derived cells, including endothelial progenitor cells (EPCs; $CD45^{dim}$, $CD133^{+}$, $VEGFR2^{+}$), that modulate the angiogenic switch for the progression of avascularized micrometastases to vascularized macrometastases. $VEGFR1^{+}$ hematopoietic progenitor cells (HPCs) and $CD11b^{+}$ myeloid progenitor cells establish the premetastatic niche and recruit EPCs, among other cells, to activate this angiogenic switch [12, 13]. EPC deficiency results in impaired macrometastatic formation as a

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result of severe angiogenesis inhibition [9]. We extended these analyses to breast cancer patients, in which significant increases in EPCs and HPCs were observed immediately before overt relapse, suggesting that these cells comprise a critical component for the propagation of macrometastases [14].

While there are many important components of angiogenesis, copper is emerging as essential through experiments that demonstrate decreased endothelial cell proliferation, blood vessel formation, and tumor growth with copper depletion [15–18]. Copper is a required cofactor in the expression, activation, and secretion of key activators of angiogenesis through multiple mechanisms including NF-κB and HIF-1 alpha. Copper is a key component of enzymes, including superoxide dismutase-1, vascular adhesion protein-1, and lysyl oxidase, implicated in the priming of the tumor microenvironment [19–22]. Copper may also play a role in migration and invasion as perinuclear copper is translocated to the leading edge of endothelial cell projections during angiogenesis and is transported across the cell membrane [23].

Tetrathiomolybdate (TM), an oral copper chelator developed for the treatment of Wilson's disease, blocks angiogenesis through the inactivation of copper chaperones and decreased incorporation of copper into copper-containing enzymes [24]. Copper levels needed for physiologic functions are lower than those favored by tumor angiogenesis; therefore, copper must be depleted to a therapeutic window. This is achieved by measuring serum ceruloplasmin (Cp), a major extracellular copper transporter, used as a surrogate marker of copper availability [25]. In non-human primates, copper depletion has been shown to decrease peripheral circulation of EPCs [26]. In an HER2/neu breast cancer mouse model, TM was studied as a chemo-preventive agent and it delayed tumor development by >200 days, suggesting that TM maintained these micrometastatic tumors in a dormant-like state [20, 27]. Phase I/II studies of TM in advanced malignancies demonstrated safety and promising efficacy, particularly in patients with minimal residual disease [28–30].

Encouraged by these data, we investigated TM as a drug to promote tumor dormancy in breast cancer patients with NED but at high risk of relapse. We hypothesized that targeting the tumor microenvironment via copper depletion prevents relapse by disrupting the EPC-mediated angiogenic switch required for the progression of micro- to macrometastasis. We further hypothesized that TM might promote tumor dormancy, as reflected by a decrease in circulating EPCs. We report here the results for the first 12 months of copper depletion.

methods

study design

This phase II, open-label, single-arm study enrolled patients on an Institutional Review Board approved trial (NCT00195091, 0903-882, 0309006307) at Weill Cornell Medical College–Iris Cantor Breast Cancer Center. Written informed consent was obtained before undergoing any study-specific procedures in accordance with the Declaration of Helsinki.

study objectives

The primary objective was to assess the change in the number of EPCs in patients treated with TM. Secondary objectives were to evaluate safety, relapse-free survival (RFS), number of HPCs, and levels of plasma angiogenic factors and cytokines.

patients

Female patients were eligible for inclusion in the study if they met the following criteria: at least 18 years of age; histologically confirmed stage 3, stage 4 with NED, or stage 2 triple-negative breast cancer; lack of radiographic, biochemical, or physical evidence of recurrent breast cancer; >6 weeks from previous chemotherapy, biologic therapy, surgery, or radiation; ECOG performance status 0–1; and adequate organ function.

Patients were stratified by molecular subtype according to immunohistochemical marker profile. Stage 2 triple-negative breast cancer patients were included because their estimated risk of relapse is equivalent to stage 3 hormone-receptor-positive patients [31]. Only concurrent hormonal therapy was permitted. All HER2-positive patients were required to have completed 1 year of standard trastuzumab therapy. Physical examination, laboratory studies, and imaging studies [computerized tomography of chest, abdomen, and pelvis (CT c/a/p) and bone scan or positron emission tomography (PET)/CT scan] were required <4 weeks before enrollment.

treatment

TM was administered to outpatient in two phases, induction and maintenance.

induction

TM 180 mg daily in four divided doses until Cp levels decreased to a target range of 5–16 mg/dl. Twenty-eight days of TM comprised one cycle. Cp levels were tested every 2 weeks for the first 4 weeks, then weekly until target Cp was reached. When Cp was within target, patients were switched to maintenance.

maintenance

TM 100 mg was taken daily in divided doses. Doses were reduced in 20 mg increments to minimize toxicity and/or increased in 20 mg increments every 2 weeks to maintain Cp target. Patients were taken off study due to relapse or unacceptable toxicity. Patients brought completed medication logs. The duration of the trial was 2 years.

Clinical grade TM was purchased in bulk from Sigma-Aldrich Chemical Company (Milwaukee, WI) under IND 71380. TM was stored under argon and stability testing was routinely carried out [32]. Research pharmacists dispensed TM in gelatin capsules and maintained an inventory, using the NCI Drug Accountability Record Form.

clinical and radiographic assessments

Patients were seen monthly for physical examination and laboratory studies including complete blood count, complete metabolic panel, tumor markers, and research studies. Imaging of investigator's choice, CT c/a/p or PET/CT, was done every 6 months, using RECIST.

safety

The National Cancer Institute – Common Toxicity Criteria for Adverse Events (CTCAE), version 3.0, were used for adverse event reporting. In the event of grade 3/4 toxicity, dosing was held until recovery. Treatment was resumed at investigator's discretion at 50% of the previous dose. If recovery did not occur within 2 weeks, the patient was removed from study. In the event of grade 2 toxicity, the dose of TM was held until recovery and a new cycle could be initiated at 100%. If grade 2 toxicity recurred, dosing was held until recovery and the next cycle was resumed at 50%.

hemangiogenic progenitor cells and angiogenic factors

Ten to 20 ml of venous blood was collected in EDTA-containing tubes, and peripheral blood mononuclear cells were isolated by Ficoll density-gradient centrifugation within 12 h. EPCs were defined as $CD45^{dim}$, $CD133^{+}$, $VEGFR2^{+}$, and HPCs as $CD34^{+}$, $CD45^{+}$, $VEGFR1^{+}$. Flow cytometry was carried out as previously described [14]. Plasma SDF-1 was detected by enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, MN) and c-kit, VEGFR2, and matrix metalloproteinases (MMP)-1, -3, and -9 by multiplex assays (Meso Scale Discovery, Gaithersburg, MD) as per manufacturer's protocol.

statistical analysis

Descriptive statistics for demographic and angiogenic variables were calculated for all patients. Incidence of adverse events and their associated 95% confidence intervals were estimated using standard methods for proportions. RFS was evaluated using survival analysis techniques. Baseline Cp and EPC values were compared with subsequent time points by the Wilcoxon signed-rank or Mann–Whitney, as appropriate. To assess the association between Cp and EPC over time, three independent mixed effects linear models with subject as a random effect were used to account for the correlation between observations on the same subject. A sample size of 35 achieves a 90% power to detect a difference of 0.5 between EPC/ml at baseline and at last time point, with an estimated standard deviation of 1.1 and two-sided alpha level of 0.05. All analyses were carried out in R: A Language and Environment for Statistical Computing, R Development Core Team, Vienna, Austria, 2011.

results

patient characteristics

Between 1 June 2007 and 30 June 2010, 40 patients were enrolled (Figure 1). One patient withdrew consent after enrollment and did not ingest TM. A total of 426 cycles were administered to 39 patients in their first 12 months of therapy (mean 10.7 cycles/patient). Twenty-seven patients remain currently on study. Reasons for discontinuation include relapse (six), toxicity (three), patient preference (two), and loss to follow up (one). Baseline characteristics are shown in Table 1. The median age was 50 years (range 29–66). The majority of patients had a very high risk of relapse (i.e. exceeding 60% relapse risk at 10 years), including any subtype stage 4 NED (30.0%), stage 3 triple-negative (12.5%), and stage 3 HER2-positive (17.5%). Twenty-six (65.0%) patients were receiving endocrine therapy while on trial. The median time between completing last treatment and initiating TM was 4.6 months.

effect of TM on copper levels

TM effectively decreased copper levels in a majority of patients

The ITT population consisted of 40 patients. The mean baseline Cp level was 29.7 mg/dl (range 20–47), which decreased to a mean Cp level of 14.2 mg/dl (7–26) while on treatment, $P < 0.0001$ (Figure 2A). Seventy-five percent of patients copper-depleted by month 1, with the target for copper depletion defined as an average Cp <17 mg/dl. Patients receiving TM for at least 4 weeks decreased their Cp level by 41.9% (range −0.10 to 75.0) from baseline. The mean baseline

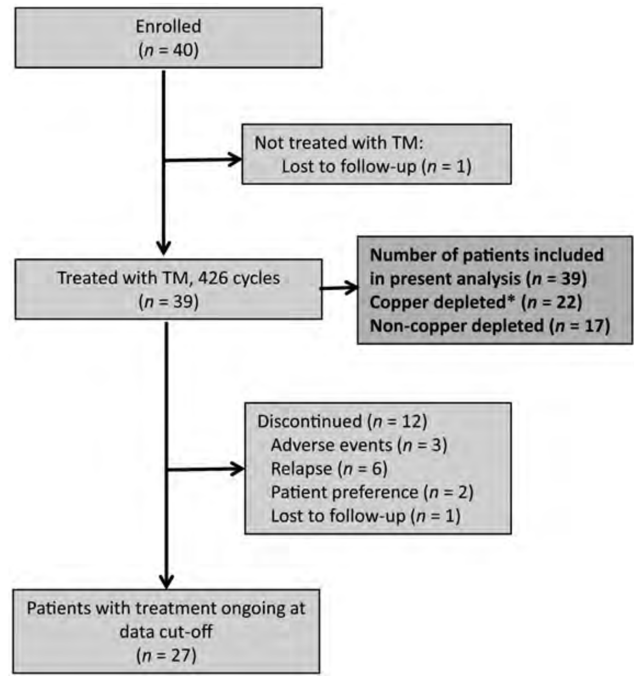


Figure 1. Schema of breast cancer patients on trial. The asterick indicates that copper depletion is defined by a ceruloplasmin < 17 mg/dl.

Cp of copper-depleted patients and non-copper-depleted patients was not significantly different (28.1 versus 31.4 mg/dl; $P = 0.19$). The mean Cp on treatment of copper-depleted patients and non-copper-depleted patients was 14.5 and 22.1 mg/dl, respectively, $P < 0.0001$ (Figure 2B). Copper-depleted patients spent a mean of 78% of time (range 55–100%) within target Cp levels during the study. Those on tamoxifen ($N = 10$) had higher baseline Cp levels of 35.9 mg/dl (range 29–47) compared with 27.8 (22–36) on aromatase inhibitors ($N = 13$). Mixed effects linear models of Cp over time showed a significant association with type of hormone therapy ($P = 0.01$), concomitant proton pump inhibitor (PPI) ($P = 0.01$), and dosage of TM ($P < 0.05$). Cp levels did not significantly correlate with age, body mass index, or stage.

triple-negative breast cancer patients copper-deplete most effectively

Patients with triple-negative disease had a lower Cp at baseline (mean 25.9 mg/dl) compared with patients with luminal subtypes (31.7). Ninety-one percent of triple-negative patients copper-depleted compared with luminal (36–45%) and HER2-positive subtypes (50%–67%), though the test for interaction was not significant (Figure 2C).

effect of TM on circulating bone marrow-derived progenitor cells

reduction of EPCs observed only in copper-depleted patients

The mean baseline number of EPCs in the ITT population was 39.8 cells/ml (range 0–207), which decreased over time to 36.3 (0–179) at 1 year of TM therapy ($P = 0.52$). In copper-depleted patients, mean EPCs/ml decreased from baseline to last dose

Table 1. Baseline patient demographics and clinical characteristics in the intent-to-treat population

Characteristic	Results ($N = 40$)
Median age, years (range)	50 (29–66)
Race/ethnicity (%)	
White	32 (80)
Black	0
Hispanic	5 (12.5)
Asian/Pacific	2 (5)
Other	1 (2.5)
ECOG performance status (%)	
0	36 (90)
1	4 (10)
AJCC stage	
Stage 2, *N* (%)	2 (5)
Stage 3, *N* (%)	26 (65)
Stage 4 NED, *N* (%)	12 (30)
Primary tumor characteristics	
Median tumor size, cm (range)	3.5 (1.2–7)
Median number of positive lymph nodes, *N* (range)	9 (0–42)
Prior metastatic sites in stage 4 NED patients, *N*	
Chest wall	7
Liver	3
Brain	1
Bone only	1
Axilla	1
Peritoneum	1
Molecular subtype, *N* (%)	
Luminal A (ER+/HER2−/Ki67 < 14%)	11 (27.5)
Luminal B (ER+/HER2−/Ki67 ≥ 14%)	11 (27.5)
Luminal-HER2 (ER+ and/or PR+/HER2+)	4 (10.0)
HER2-enriched (ER+/PR+/HER2+)	3 (7.5)
Triple-negative (ER−/PR−/HER2−)	11 (27.5)
Triple-negative patients by stage ($N = 11$)	
Stage 2, *N* (%)	2 (18.1)
Stage 3, *N* (%)	5 (45.4)
Stage 4 NED, *N* (%)	4 (36.3)
Prior antitumor therapy for primary breast cancer, *N* (%)	
Anthracycline and/or taxane-based	26 (15.0)
Trastuzumab	7 (17.5)
High-dose chemo with stem cell support	2 (5.0)
Prior antitumor therapy for metastasis, *N* (%)	
Local treatment (surgery and/or radiation)	7 (17.5)
Hormone therapy	4 (10)
Anthracycline	1 (2.5)
Taxane	1 (2.5)
Capecitabine	2 (5.0)

ECOG, Eastern Cooperative Oncology Group; NED, no evidence of disease; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor 2; AJCC, American Joint Committee on Cancer.

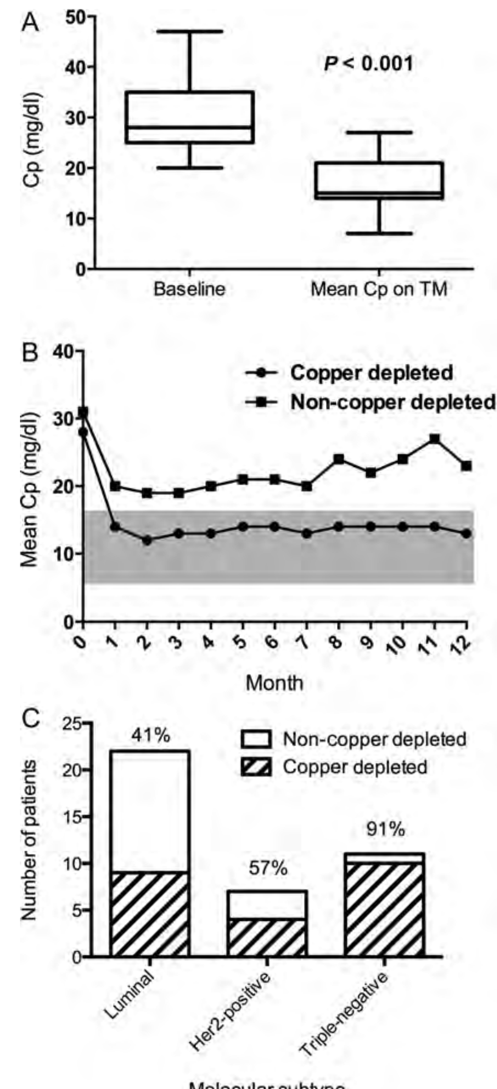
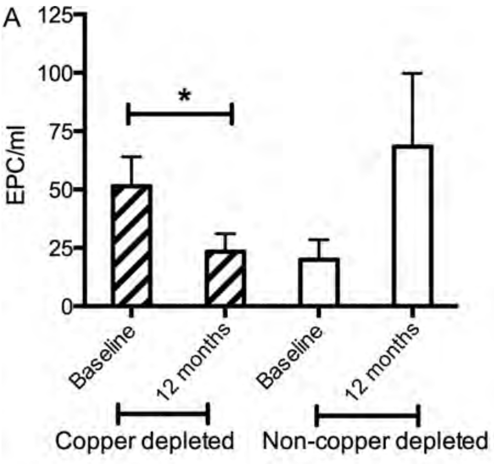


Figure 2. (A) The mean ceruloplasmin (Cp) level significantly decreases in all patients from baseline of 29.7–14.2 mg/dl while on treatment with tetrathiomolybdate (TM). The horizontal line indicates mean; box, standard deviation; whiskers, minimum to maximum values. (B) Mean Cp remains suppressed over time only in copper-depleted patients. The shaded area indicates target for copper depletion. (C) More triple-negative breast cancer patients achieve copper depletion than other molecular subtypes. The percentage of patients copper-depleted is noted above the bar.

by 27 ($P = 0.04$, Figure 3A). In patients who did not achieve the copper depletion target, mean EPCs/ml increased by 61 ($P = 0.95$). There was no significant difference between the baseline values of copper-depleted and non-copper-depleted subgroups. High-risk subtypes (triple-negative, HER2-positive, and stage 4 NED) had higher mean EPC/ml levels at baseline (45.6, range 0–207) compared with the luminal stage 3 patients (27.7, range 0–114). A decrease in Cp from baseline corresponded to a decrease in EPCs from baseline in triple-negative patients more so than the other molecular subtypes (Figure 3B). Use of non-tamoxifen hormone therapy ($P = 0.01$), PPI ($P = 0.001$), Cp levels ($P = 0.04$), and dosage of TM ($P = 0.01$) were statistically significant in explaining the decrease of EPCs over time when a multivariable model was used. Additionally, there was a significant interaction between the PPI group and time ($P = 0.007$), reflecting a decreasing monthly average trend of EPCs in the patients taking PPI. The interaction between Cp and dosage of TM is significantly greater than zero ($P = 0.03$), suggesting that mean EPCs increased in patients with rising Cp levels. EPC levels did not



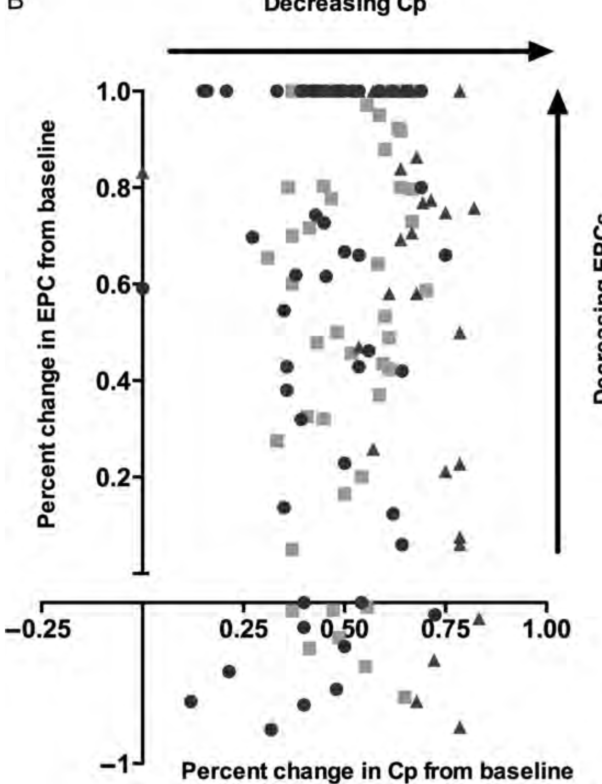


Figure 3. (A) Endothelial progenitor cells (EPCs) significantly decrease from baseline only in copper-depleted patients. The bar indicates mean with standard error of measurement; *$P < 0.05$. (B) Decrease in ceruloplasmin (Cp) corresponds to a decrease in EPCs in majority of copper-depleted patients and an exploratory analysis by molecular subtype. Square indicates luminal; circle, triple-negative; triangle, human epidermal growth factor 2 (HER2)-positive.

significantly correlate with age, body mass index, stage, or molecular subtype.

lack of effect of copper depletion on HPCs

The mean baseline HPC/ml for all patients was 2590 (range 66.7–19 300); it was 2170 (range 195–10 700; non-significant) after 12 months of therapy. HPCs did not significantly correlate with copper depletion status.

Table 2. Summary of incidence of drug-related adverse events in the intent-to-treat population, comprising 426 cycles. *N* indicates the number of cycles affected by the adverse event.

Adverse event	Grade 1, *N* (%)	Grade 2, *N* (%)	Grade 3, *N* (%)	Grade 4, *N* (%)
Hematologic				
Anemia	43 (10.1)	7 (1.6)	1 (0.2)	0
Neutropenia	39 (9.2)	28 (6.6)	6 (1.4)	7 (1.6)
Febrile neutropenia			1 (0.2)	0
Leukopenia	36 (8.5)	29 (6.8)	5 (1.2)	0
Thrombocytopenia	7 (1.6)	0	0	0
Gastrointestinal				
Sulfur burps	79 (18.5)	0	0	0
Nausea	3 (0.7)	0	0	0
Vomiting	2 (0.5)	0	0	0
Diarrhea	5 (1.2)	1 (0.2)	0	0
Constipation	2 (0.5)	0	0	0
Abdominal pain	5 (1.2)	0	0	0
General				
Fatigue	29 (6.8)	0	0	0
Neurologic				
Dizziness	0	1 (0.2)	0	0
Neuropathy	6 (1.4)	0	0	0

circulating markers of angiogenesis

There were no significant effects on circulating angiogenic markers, including VEGFR2, ckit, SDF1, MMP-1, -3, or -9. The mean SDF1 decreased only in copper-depleted patients at 12 months, and increased in patients before relapse (supplementary Figure S1, available at *Annals of Oncology* online). Similarly, mean MMP-1 and -3 increased before relapse (supplementary Figure S2, available at *Annals of Oncology* online).

toxicity

Overall TM was well tolerated (Table 2).

hematologic toxicity

Sixty-seven (15.7%) cycles were complicated by grade 1/2 neutropenia in 23 (59.0%) patients and 13 (3.1%) cycles by grade 3/4 neutropenia in 9 (23.1%) patients. One patient required hospital admission for neutropenic fever and was taken off study. Fifty (11.7%) cycles were complicated by grade 1/2 anemia in 14 (35.9%) patients. Only one (0.2%) cycle was affected by grade 3 anemia in a patient later diagnosed with B12 deficiency. No patients required growth factor support.

non-hematologic toxicity

There was no grade 3 or 4 non-hematologic toxicity. One patient with grade 2 diarrhea, likely due to lactose used as a filler in the TM pills, left the study. Sulfurous eructations resolved with initiation of PPI therapy in >90% of patients.

clinical outcomes

Twenty-seven patients remain relapse-free on study. Six patients (15%) recurred during their first 12 months on TM; three with stage 3 disease (one triple-negative) relapsed after 2, 3, and 10

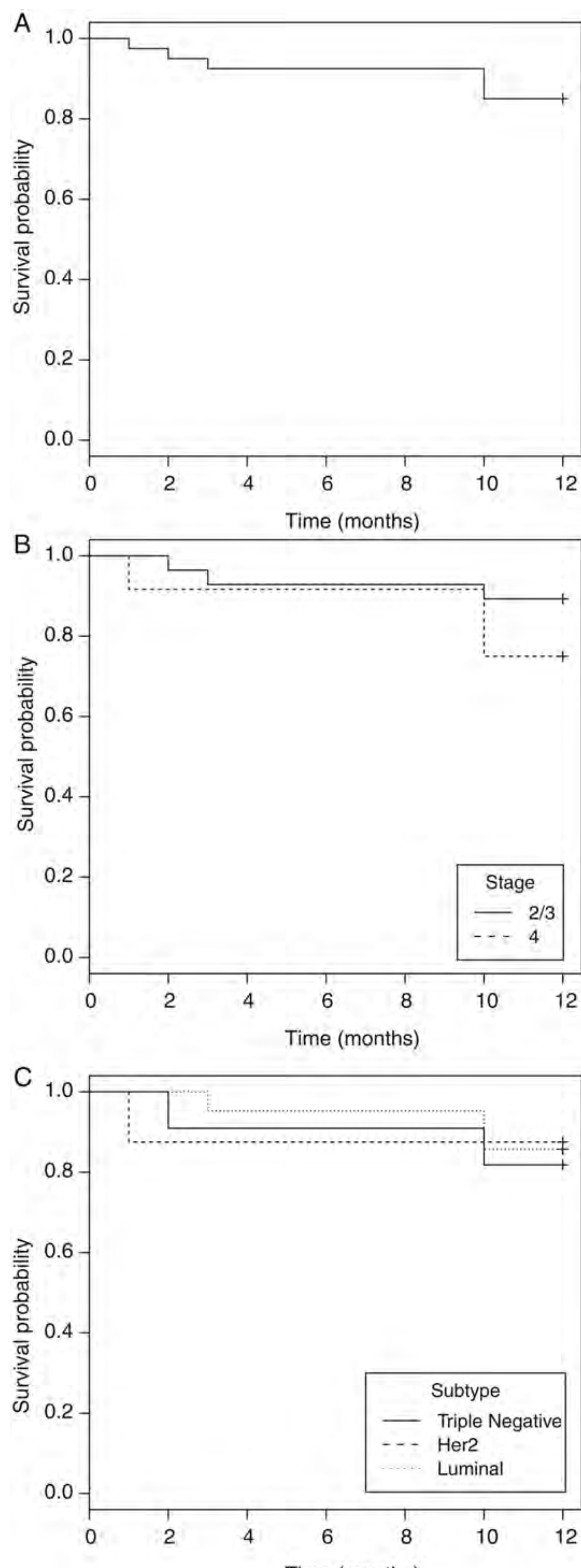


Figure 4. Relapse-free survival (RFS) in (A) all patients, (B) by stage [stage 2/3 versus stage 4 without evidence of disease (NED)], and (C) by molecular subtype (luminal subtypes versus human epidermal growth factor 2 (HER2)-positive versus triple-negative). The 10-month RFS in all patients was 85.0% (95% CI 74.6%–96.8%). The 10-month RFS was lower in stage 4 NED patients compared with stage 2 and 3 patients (75.0% versus 89.3%), and in triple-negative patients compared with luminal patients (81.8% versus 85.7%).

months and three with stage 4 NED (one triple-negative, one HER2-positive, one luminal) relapsed after 1, 10, and 10 months of therapy. Of these relapsed patients, Cp decreased to target in four patients but EPCs were maintained below baseline in only one patient. RFS curves are shown in Figure 4.

discussion

Understanding the process of breast cancer metastases is critical to eradicating tumor progression. We have conducted a series of translational studies demonstrating that bone marrow-derived hematopoietic progenitors are a critical component of this process. This is the first human trial utilizing a copper depletion strategy to modulate EPCs, an essential component of the microenvironment, in breast cancer patients with an extraordinarily high-risk of relapse from occult residual disease. Since tumor progression may be dependent on critical copper-dependent processes, we hypothesized that copper depletion could prevent an overt relapse by the inhibition of the EPC-mediated angiogenic switch.

In this study, we demonstrated that TM effectively and rapidly depletes copper levels in the majority of patients without untoward effects. In fact, we have safely copper-depleted a proportion of patients for >65 months on an extension study. Concomitant administration of a PPI significantly correlated with copper depletion and may be considered an adjunct to TM treatment. We observed a significant, sustained reduction in EPCs with copper depletion. EPCs did not significantly change in patients unable to achieve or maintain the Cp target. Taken together, copper depletion may inhibit the production, release, or mobilization of EPCs from the bone marrow, leading to a suppressed angiogenic switch and maintained tumor dormancy. Moreover, these findings suggest that TM may have direct effects on the tumor microenvironment at the level of the bone marrow niche, the sanctuary site for EPCs. Though HPCs did not significantly change in patients receiving TM, our previous studies showed that HPCs predicted relapse and progression of disease, suggesting that these cells may also be important in the metastatic cascade, but upstream of the processes was affected by copper depletion [14].

As per subgroup analysis, triple-negative patients copper-depleted more effectively compared with luminal patients. Though the test for interaction was not significant, this study was not powered to show these differential effects. Angiogenesis-related genes are frequently overexpressed in triple-negative tumors [33], and antiangiogenic agents may be more effective in this subset. Intriguingly, several studies evaluating bevacizumab, a monoclonal antibody targeting VEGF, suggested greater efficacy in triple-negative patients [34, 35].

To gain a better understanding of the targets of TM, we measured circulating markers of angiogenesis. SDF1 and its

receptor CXCR4, involved in angiogenesis and metastatic progression, may have a role in EPC recruitment [36, 37]. In breast cancer patients, expression of SDF1 has been inversely correlated with survival [38]. In our study, SDF1 decreased with copper depletion but increased before relapse. Likewise, MMPs increased in patients before relapse. MMPs regulate tumor growth and invasion [36] and provide a permissive bone marrow niche to facilitate mobilization of progenitor cells [39]. Our observations suggest that SDF1 and MMPs may have important roles in metastatic progression.

Though a randomized clinical trial is necessary to assess survival, efficacy measures were promising. Of 11 triple-negative patients on study, only 2 relapsed. One patient did not adequately copper-deplete despite incremental dose increases. The other patient progressed within 2 months of TM therapy, suggesting that active neoangiogenesis was occurring at the time of enrollment, which could not be halted due to delayed effects of copper depletion. We are cautiously optimistic about the low incidence of relapse and have extended the study to 6 years in selected patients. Two stage-4 NED triple-negative patients remain disease-free at 65 and 49 months on TM therapy, which is encouraging given the dismal median survival of 9 months in metastatic triple-negative patients [2].

In summary, we have conducted the first phase-II study employing copper chelation in high-risk breast cancer patients with minimal residual disease. As the evidence accumulates in favor of copper depletion to prevent relapse, a large, randomized, multicenter trial enriched with triple-negative and stage 4 NED patients may be of utility.

acknowledgements

We would like to thank Dr Sofia Merajver and Dr George Brewer, whose guidance was critical in many of the practical issues with initiating this clinical trial, and the Weill Cornell Translational Core Laboratory for processing the samples.

funding

We greatly appreciate the generous support from the following: Anne Moore Breast Cancer Research Fund, Stephen and Madeline Anbinder Foundation, Rozaliya Kosmandel Research Fund, Susan G. Komen for the Cure, New York Community Trust, Cancer Research and Treatment Fund, and Berman Fund. SMHR was partially supported by the grant from the Clinical Translational Science Center (CTSC) (UL1-RR024996) [no other grant numbers].

disclosure

The authors have declared no conflicts of interest.

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Annals of Oncology 24: 1498–1505, 2013
doi:10.1093/annonc/mdt011
Published online 13 February 2013

Promoter methylation of *BRCA1* in triple-negative breast cancer predicts sensitivity to adjuvant chemotherapy

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Received 24 March 2012; revised 10 November 2012; accepted 31 December 2012

Background: *BRCA1* function is inactivated through *BRCA1* promoter methylation in a substantial number of triple-negative breast cancers. We investigated the impact of *BRCA1*-methylation status on the efficacy of adjuvant chemotherapy in patients with triple-negative breast cancer or with non-triple-negative breast cancer.
Methods: *BRCA1* promoter methylation was assessed in 1163 unselected breast cancer patients. Methylation was evaluated using a methylation-specific PCR (MSP) assay.
Results: In the subgroup of 167 triple-negative breast cancer patients who received adjuvant chemotherapy, patients with *BRCA1*-methylated tumors had a superior 10-year disease-free survival (DFS)(78% versus 55%, $P = 0.009$) and 10-year disease-specific survival (DSS) (85% versus 69%, $P = 0.024$) than those with *BRCA1*-unmethylated tumors, and *BRCA1* methylation was an independent favorable predictor of DFS and DSS in a multivariate analysis in this subgroup [DFS: hazard ratio (HR) = 0.45; 95% confidence interval (CI) 0.24–0.84; $P = 0.019$; DSS: HR = 0.43; 95% CI = 0.19–0.95; $P = 0.044$]. In contrast, in 675 non-triple-negative breast cancer patients who received adjuvant chemotherapy, *BRCA1* methylation was an unfavorable predictor of DFS and DSS in univariate analysis (DFS: HR = 1.56; 95% CI 1.16–2.12; $P = 0.003$; DSS: HR = 1.53; 95% CI = 1.05–2.21; $P = 0.026$).
Conclusions: Triple-negative breast cancer patients with *BRCA1*-methylated tumors are sensitive to adjuvant chemotherapy and have a favorable survival compared with patients with *BRCA1*-unmethylated triple-negative tumors.
Key words: *BRCA1* methylation, chemotherapy, triple-negative breast cancer

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Treatment of Wilson Disease With Ammonium Tetrathiomolybdate

III. Initial Therapy in a Total of 55 Neurologically Affected Patients and Follow-up With Zinc Therapy

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Background: It is unclear what anticopper drug to use for patients with Wilson disease who present with neurologic manifestations because penicillamine often makes them neurologically worse and zinc is slow acting.

Objective: To evaluate the frequency of neurologic worsening and drug adverse effects with ammonium tetrathiomolybdate.

Design: Open-label study of 55 untreated patients (22 of them new) presenting with neurologic Wilson disease treated with tetrathiomolybdate varying from 120 to 410 mg/d for 8 weeks and then followed up for 3 years. Neurologic function was assessed with scored neurologic and speech tests.

Setting: A university hospital referral setting.

Patients: All untreated, newly diagnosed patients with neurologic Wilson disease.

Intervention: Treatment with tetrathiomolybdate.

Main Outcome Measures: Neurologic function was evaluated by neurologic and speech examinations. Drug adverse effects were evaluated by complete blood cell counts and biochemical measures.

Results: Only 2 (4%) of 55 patients treated with tetrathiomolybdate showed neurologic deterioration, compared with an estimated 50% of penicillamine-treated patients. Five of the 22 new patients exhibited bone marrow suppression and 3 had aminotransferase elevations. These numbers are higher than in the original 33 patients and appear to be due primarily to a more rapid dose escalation.

Conclusions: Tetrathiomolybdate shows excellent efficacy in patients with Wilson disease who present with neurologic manifestations. With rapid escalation of dose, adverse effects from bone marrow suppression or aminotransferase elevations can occur.

Arch Neurol. 2003;60:379-385

From the Departments of Human Genetics (Dr Brewer, Mr Dick, and Ms Sitterly), Internal Medicine (Drs Brewer and Askari), and Neurology (Drs Hedera and Fink and Ms Kluin); Division of Speech-Language Pathology, Department of Physical Medicine and Rehabilitation (Ms Kluin); and Department of Pediatrics (Dr Carlson), University of Michigan, Ann Arbor. The University of Michigan has recently licensed the antiangiogenic uses of tetrathiomolybdate to Attenuon LLC, San Diego, Calif, and both Dr Brewer and Mr Dick have equity in Attenuon LLC. The authors have no relevant financial interest in this manuscript.

THE INITIAL TREATMENT of patients with Wilson disease who present with neurologic symptoms is problematic. Of the 3 commercially available anticopper agents, penicillamine often makes about half of these patients irreversibly neurologically worse,[1] trientine hydrochloride is untested but its actions are much like those of penicillamine, and zinc is too slow acting. To fill this therapeutic void, we have been developing ammonium tetrathiomolybdate.[2-4]

Tetrathiomolybdate acts differently than previous anticopper drugs. It forms a tripartite complex with copper and protein that is very stable.[5-8] Given with food, tetrathiomolybdate complexes food copper with food protein, rendering that copper, along with endogenously secreted copper in saliva, gastric juice, and intestinal secretions, unabsorbable. This puts the patient in an immediate negative copper balance. Given away from food, tetrathiomolybdate is absorbed into the blood and there complexes freely available and potentially toxic copper with blood albumin. This complexed copper cannot be taken up by cells and is therefore nontoxic.[8] The lightly bound and potentially toxic copper of organs is in equilibrium with that in the blood, and further copper toxicity in various organs in Wilson disease is quickly stopped, generally in 1 to 2 weeks, with tetrathiomolybdate therapy.

We have previously published 3 articles in this journal about the development of tetrathiomolybdate for this therapeutic purpose.[2-4] The most recent, in 1996,[4] presented data on 33 patients with neurologic presentations who were treated with tetrathiomolybdate up until that time. In this article, we present 22 additional pa-

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Table 1. Initial Data on the New Sample of 22 Patients With Wilson Disease

Patient No./ Sex/Age, y	Hepatic Copper, µg/g	Urine Copper, µg/d	Treatment History (No. of Weeks)	Major Signs and Symptoms
169/F/25	NA	127	Penicillamine (1½)	Dysarthria, dystonia, severe chorea, nonambulatory
172/F/20	873	111	Zinc acetate (2)	Dysarthria, incoordination*
173/F/20	240	NA	Penicillamine (2)	Mild dysarthria and chorea
175/F/21	NA	154	Penicillamine (2)	Anarthria, severe dystonia and incoordination, nonambulatory
181/M/38	654	381	None	Severe dysarthria, dystonia, tremor, incoordination, nonambulatory
183/M/30	414	607	None	Tremor, incoordination
194/M/11	403	122	None	Mild dystonia and incoordination
200/M/28	809	626	None	Mild dysarthria, incoordination, tremor
203/M/30	689	220	Zinc acetate (3)	Mild dysarthria, incoordination, tremor
205/M/28	397	319	None	Mild dysarthria, dystonia, incoordination
206/F/27	1077	115	Zinc acetate (2)	Severe dysarthria, mild dystonia, incoordination
208/M/21	441	NA	Penicillamine (3)	Anarthria, severe dystonia, incoordination, nonambulatory
209/F/27	285	NA	Zinc acetate (4)	Severe dysarthria, dystonia, tremor, incoordination, nonambulatory
210/M/23	326	310	None	Dysarthria, mild dystonia, incoordination
211/F/28	989	260	None	Mild dysarthria and dystonia
212/M/36	609	234	None	Mild dysarthria, dystonia, tremor, incoordination, psychiatric problems
214/M/31	670	213	Penicillamine (3)	Mild dysarthria, dystonia, tremor
216/F/35	511	251	None	Severe dysarthria, dystonia, tremor, incoordination
218/M/25	856	302	None	Mild dysarthria and tremor
222/F/43	NA	175	None	Dysarthria, dystonia, tremor, incoordination
223/M/24	319	340	Penicillamine (1)	Mild dysarthria, dystonia, severe tremor, incoordination
227/F/37	766	128	None	Dysarthria, dystonia, tremor, incoordination

Abbreviation: NA, not available.
*Incoordination in all patients was related to bradykinesia.

tients, for a total of 55. In addition, we summarize the efficacy and toxicity data in the entire cohort of 55 patients.

METHODS

The patients were diagnosed as having Wilson disease by means of standard criteria previously extensively published.[9-14] Some of the presenting symptoms and diagnostic data gathered at the time of first admission in the 22 new patients described herein are shown in **Table 1**. In addition to the underlying diagnosis of Wilson disease, all patients were diagnosed as having symptoms of a movement disorder attributable to Wilson disease. The institutional review board of the University of Michigan Medical School, Ann Arbor, reviewed and approved the project.

Each patient was admitted for up to 8 weeks in the General Clinical Research Center of the University of Michigan Hospital, Ann Arbor. After initial studies to confirm the diagnosis, obtain informed consent, and establish baseline neurologic and speech function, therapy with tetrathiomolybdate was initiated. In many patients the drug was started at 120 mg/d, with 20 mg between meals 3 times daily and 20 mg with meals 3 times daily. If the patient strongly desired a bedtime snack, a fourth 20-mg dose was given with the snack, for a total initial dose of 140 mg/d. In most patients the between-meals doses were then rapidly escalated during a several-day period, usually to a total dose of about 200 to 260 mg/d. In some patients, the dose was not escalated. The tetrathiomolybdate treatment used in the 22 new patients is given in **Table 2**. In addition to initial dose, Table 2 gives maximum and average dose data. Patients also started zinc therapy early in their 8-week stay, usually 50 mg 3 times per day. Patients did not receive additional tetrathiomolybdate after the initial 8 weeks of therapy.

Two types of toxic effects from tetrathiomolybdate have been encountered. One is an overtreatment anemia, often accompanied by leukopenia, and occasionally by thrombocytopenia. The other is a mild further elevation of aminotransferase enzymes, due to unknown mechanisms. When either was encountered, the patient's tetrathiomolybdate dose was decreased and often the patient was given a drug holiday.

During the 8-week admission, a quantitative neurologic test and a quantitative speech examination were carried out at roughly weekly intervals, by previously published methods,[2-4] standardized for, and extensively evaluated in, Wilson disease. The neurologist (P.H., M.C., and J.K.F.) and speech (K.J.K.) evaluators were not blinded during this open-label study. The main purpose of these weekly tests was to detect neurologic deterioration during initial treatment. An increase of 5 points (scale, 0-38) on the quantitative neurologic examination, or an increase of 3 points (scale, 0-7) on the speech examination, is taken as evidence of significant neurologic deterioration. The patients were discharged on a regimen of zinc maintenance therapy, then returned for an annual visit. The neurologic and speech tests were repeated on an annual basis. The main purpose of these annual examinations was to evaluate the extent of neurologic recovery, if any.

During the 8-week period, assays of "safety variables" were carried out to detect adverse effects of tetrathiomolybdate therapy. These include complete blood cell counts, liver function tests, and amylase, lipase, creatinine, serum urea nitrogen, uric acid, urine protein, and iron variables, all carried out by standard technique in use at the University of Michigan Health System hematologic and biochemistry laboratories.

RESULTS

Table 3 shows the results of the weekly quantitative neurologic testing. Patient 211 showed a 6-point deterioration in week 2, so we scored her as showing deterioration, although her scores varied quite widely during the next 3 weeks. None of the other patients showed a 5-point deterioration (increase) in score. The data demonstrated the minor fluctuations in symptoms from one time to another that are attributable to the level of stress, anxi-

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Table 2. Data on Tetrathiomolybdate Dosage, Complications, and Dosage Interventions*

Patient No.	Dosage, mg/d Starting	Dosage, mg/d Maximum	Dosage, mg/d Average	Complications	Interventions
169	140	140	120	...	...
172	120	320	300	...	...
173	120	140	120	Mild anemia, leukopenia	None
175	120	200	180	Anemia, leukopenia, thrombocytopenia, and aminotransferase elevations	Drug holiday
181	120	260	180	...	...
183	140	260	240	...	...
194	120	200	140	...	...
200	140	260	260	...	...
203	140	200	200	...	...
205	200	200	180	Mild anemia, leukopenia	Drug holiday, then dosage reduction to 60 mg/d
206	140	200	140	Aminotransferase elevations	Drug holiday, then dosage reduction to 60 mg/d
208	120	200	100	Aminotransferase elevations	Dosage reduction to 80 mg/d
209	120	200	120	Mild anemia, leukopenia, thrombocytopenia	Drug holiday, then dosage reduction to 80 mg/d
210	140	140	140	...	...
211	140	140	140	...	...
212	140	200	200	...	...
214	140	200	200	...	...
216	140	200	120	Mild anemia, leukopenia	Drug holiday, then dosage reduction to 80 mg/d
218	120	120	120	...	...
222	120	120	120	...	...
223	120	120	120	...	...
227	120	140	120	...	...

*Ellipses indicate none.

ety, fatigue, etc, that can impact on the expression of these signs and symptoms.

Table 4 shows similar data for the speech quantitative testing. No patient showed a 3-point deterioration (increase) in score. Again, the data showed the minor fluctuations in dysarthria from one time to another that are attributable to the psychological and physical status of the patient on the day of testing, as discussed in the preceding paragraph.

Table 5 shows the results of repeat quantitative neurologic testing on annual return visits of the 19 patients who returned, compared with the baseline, which is the first recorded score during the initial 8-week admission. One patient died of variceal bleeding before return, and 2 dropped out. Only 1 patient (patient 210) showed significant (≥5 points) deterioration, in this case between baseline and year 1, and this was clearly related to noncompliance with zinc therapy. Almost all of the patients showed some improvement in scores, generally with most of the improvement between baseline and year 1. This is perhaps best demonstrated by the improvement in mean scores at the bottom of Table 5, which shows that average improvement was predominant in that period and was statistically significant.

Table 6 shows the results of repeat quantitative speech testing on annual return visits of the 19 patients who returned, compared with the baseline, which is the first recorded score during the initial 8-week admission. No patient showed significant (≥3 points) deterioration in score. Many patients showed some improvement in their score over baseline, with most of that improvement occurring between baseline and year 1. This is demonstrated by the mean scores at the bottom of Table 6, which show statistically significant improvement between baseline and year 1. However, unlike the neurologic data, the means for each year in Table 6 suggest that, in some patients, improvement in speech may continue as long as year 3, and indeed, the means of years 2 and 3 are very close to being significantly different.

We saw 2 adverse effects from tetrathiomolybdate therapy in this study (Table 2). Five patients (patients 173, 175, 205, 209, and 216) exhibited bone marrow suppression (**Table 7**), which is attributable to overtreatment and bone marrow depletion of copper. Bone marrow suppression began between weeks 3 and 6 in the 5 patients. Three patients (patients 175, 206, and 208) exhibited elevations of serum aminotransferase enzymes (**Table 8**), due to unknown mechanisms. Enzyme elevations began at the beginning of week 4 in the 3 patients. Mild alkaline phosphatase elevations are expected, because of the initiation of zinc therapy. This is a harmless result of increased induction of the enzyme in the liver by zinc.[15] Both the bone marrow suppression and the aminotransferase elevations were responsive to a drug holiday and/or a reduction in tetrathiomolybdate dose (Table 2).

No patient showed abnormalities of serum urea nitrogen, creatinine, uric acid, and urine protein levels during the 8 weeks of tetrathiomolybdate therapy (data not shown).

COMMENT

In terms of efficacy, the major issue is to avoid the initial neurologic deterioration that occurs about 50% of the time with penicillamine therapy and results in about 25% of patients having permanent, additional, drug-induced

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Table 3. Weekly Quantitative Neurologic Scores During the 8 Weeks of Initial Tetrathiomolybdate Therapy*

Patient No.	Weeks of Therapy								
	0	1	2	3	4	5	6	7	8
169	. . .	18.5	. . .	. . .	16	16	. . .	. . .	14.5
172	. . .	5	4.5	. . .	. . .	2	. . .	. . .	3
173	1.5	. . .	. . .	1.5	. . .	. . .	. . .	1	1.5
175	28	. . .	. . .	25	23	24	. . .	26	. . .
181	22	14.5	15.5	20	20	20	18	. . .	. . .
183	. . .	. . .	3.5	3.5	4	. . .	. . .	. . .	2.5
194	6	6.5	7.5	6.5	. . .	5	4.5	4	. . .
200	4	6.5	5.5	4	2.5	. . .	5	6	. . .
203	8.5	8	10	8.5	8.5	. . .	7.5	. . .	. . .
205	4.5	3	. . .	. . .	. . .	2.5	2	2	. . .
206	8.5	9	7	. . .	8	5.5	6	. . .	. . .
208	23	23.5	23	22.5	23	26	25	26	. . .
209	22	19.5	19	23	21.5	22.5	. . .	. . .	. . .
210	3.5	3.5	. . .	3.5	3.5	4	4	. . .	. . .
211	5.5	6	11.5	5.5	. . .	10	. . .	. . .	. . .
212	. . .	. . .	6	4	4	2	. . .	. . .	. . .
214	3	2.5	2	2	. . .	. . .	. . .	. . .	. . .
216	19	19	. . .	21.5	. . .	. . .	. . .	23	21.5
218	7	. . .	4.5	1.5	. . .	1.5	2.5	. . .	3.5
222	9	8.5	7.5	6	7.5	6	5.5	. . .	. . .
223	9.5	5	5	. . .	10	8	8	9.5	. . .
227	8.5	8.5	11	. . .	11	10	6.5	. . .	. . .
Mean	10.7	9.8	8.9	9.2	11.7	10.3	7.9	11.1	7.8
SD	7.9	6.4	5.7	8.3	7.6	8.3	6.5	10.2	7.5
No. of patients	18	17	16	15	16	16	12	9	6

*Score range is 0 (normal) to 38. Ellipses indicate not measured.

Table 4. Weekly Quantitative Speech Scores During the 8 Weeks of Initial Tetrathiomolybdate Therapy*

Patient No.	Weeks of Therapy								
	0	1	2	3	4	5	6	7	8
169	4.5	. . .	4.5	4.5	4.5	. . .	6	4.5	4.5
172	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
173	3	2	2	2	2	2	2	2	. . .
175	6	5.5	5.5	. . .	5.5	5.5	. . .	6	6
181	4.5	4.5	5.5	5.5	7	5.5	7	7	. . .
183	2	1	1	1	1	1	1	. . .	. . .
194	3.5	3.5	3.5	3.5	3.5	. . .	3.5	3.5	3.5
200	3.5	2.5	3.5	2	2	2	2	1.5	. . .
203	3	3	3	3	3	3	. . .	. . .	. . .
205	3	3	. . .	. . .	3	3	3	. . .	. . .
206	5	5	5	5	5	6	5	5	. . .
208	5.5	5	5	5	5	5	5	5	5
209	5	5	6	5.5	5	5.5	5.5	. . .	. . .
210	4.5	4.5	4.5	4.5	4.5	4.5	. . .	. . .	. . .
211	4.5	4.5	4.5	5	4.5	4.5	5	. . .	. . .
212	2	2	2	2	2	1.5	. . .	. . .	. . .
214	3	2	2	2	2	2	. . .	. . .	. . .
216	6	7	7	7	7	7	7	. . .	. . .
218	1	1	1	1	1	1	. . .	1	1
222	3	3	3	3	3	3	3	3	. . .
223	2.5	2.5	2.5	2.5	2.5	. . .	2.5	2.5	. . .
227	3	3	. . .	3	3	3	3	3	. . .
Mean	3.7	3.5	3.7	3.5	3.6	3.6	4.0	3.7	3.9
SD	1.3	1.5	1.6	1.6	1.7	1.8	1.8	1.7	1.6
No. of patients	22	21	20	20	22	19	16	13	6

*Score range is 0 (normal) to 7. Ellipses indicate not measured.

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Table 5. Yearly Quantitative Neurologic Scores After Initial Tetrathiomolybdate and Maintenance Zinc Acetate Therapy

Patient No.	Baseline	Year* 1	2	3
169	18.5	19.0	18.5	15.5
172	5.0	2.5	2.0	0.5
173	1.5	0.0	1.0	4.0
175	Dropped out			
181	22.0	5.0	7.0	9.5
183	3.5	5.0	2.0	1.0
194	6.0	0.0	3.0	1.0
200	4.0	1.0	1.0	. . .
203	8.5	4.0	2.0	4.0
205	Deceased			
206	8.5	5.5	11.0	9.5
208	23.0	21.0	17.0	16.5
209	Dropped out			
210	3.5	12.0	6.0	3.5
211	5.5	1.0	1.5	2.0
212	6.0	2.0	2.5	0.5
214	3.0	1.0	0.0	0.0
216	19.0	6.0	4.0	3.5
218	7.0	3.0	. . .	1.5
222	9.0	1.5	1.0	0.5
223	9.5	1.0	. . .	. . .
227	8.5	2.0	. . .	. . .
Mean	9.0†	4.9†	5.0	4.6
SD	6.4	5.9	5.5	5.2
No. of patients	19	19	16	16

*Ellipses indicate not measured.
†A paired *t* test comparing baseline vs year 1 is significantly different at $P<.002$.

Table 6. Yearly Quantitative Speech Scores After Initial Tetrathiomolybdate and Maintenance Zinc Acetate Therapy

Patient No.	Baseline	Year* 1	2	3
169	4.5	5.0	4.0	4.0
172	3.5	2.5	1.5	1.0
173	3	1.5	2.0	3.0
175	Dropped out			
181	4.5	3.0	2.0	1.0
183	2.0	1.0	0.0	0.0
194	3.5	3.5	3.0	2.0
200	3.5	1.0	0.5	. . .
203	3.0	. . .	2.5	2.5
205	Deceased			
206	5.0	5.0	5.5	5.5
208	5.0	5.0	5.0	5.0
209	Dropped out			
210	4.5	5.5	4.5	. . .
211	4.5	3.0	2.0	1.5
212	2.0	1.0	0.5	0.0
214	3.0	1.0	. . .	0.5
216	6.0	4.0	4.0	3.0
218	1.0	. . .	1.0	1.0
222	3.0	2.5	. . .	1.5
223	2.5	1	. . .	0.5
227	3.0	1.5	. . .	. . .
Mean	3.5†	2.8†	2.5‡	2.0‡
SD	1.2	1.6	1.7	1.6
No. of patients	19	17	15	16

*Ellipses indicate not measured.
†A paired *t* test comparing baseline vs year 1 is significantly different at $P<.001$.
‡A paired *t* test comparing year 2 vs year 3, on the 11 patients in whom both samples were obtained, gives $P = .055$.

damage.[1] In these 22 patients, only 1 was classified as neurologically worsening during the initial 8 weeks of therapy (Table 3), and none was classified as having deterioration in speech (Table 4). Putting these data together with data from the earlier 33 patients described,[4] of whom 1 deteriorated, we have seen a total of 2 neurologic deteriorations in 55 patients treated, for a rate of 3.6%. We speculate that an occasional patient will exhibit continued neurologic deterioration irrespective of the anticopper drug used, whereas penicillamine catalyzes a "drug-induced" deterioration. Our working hypothesis on why the latter happens is that penicillamine aggressively mobilizes copper from the liver, increasing blood copper levels and, in the process, further elevating brain copper levels for a time.

The low rate of neurologic deterioration when tetrathiomolybdate is used for initial therapy is a very positive result, because it allows subsequent recovery starting at a much higher baseline than if the patient deteriorates. During the ensuing period of maintenance therapy, for which we use zinc, substantial improvement occurs (Tables 5 and 6), probably through recovery of neurons that were damaged but not killed by the copper-induced inflammatory process. Most of the improvement occurs during the first year, although with speech, improvement may occur during a longer period (Table 6).

The neurologic and speech recovery data over time in the present study are compared with the original study[4] in **Table 9**. These data show the consistency between the 2 studies in terms of mean baseline values, occurrence of most of the improvement during year 1, and the degree of average improvement.

Alternatives to tetrathiomolybdate for initial therapy, besides penicillamine, include zinc, which appears to be favored by Hoogenraad et al.[16] However, we view zinc as rather slow acting for acutely ill patients, taking perhaps 4 to 6 months to control copper toxicity, during which time the disease may progress. Another alternative is trientine. Although its mechanism is similar to that of penicillamine, it is a more gently acting drug and may not share penicillamine's propensity to make the disease worse initially. We are currently in the midst of a double-blind clinical trial comparing tetrathiomolybdate and trientine for initial use in patients with a neurologic presentation.

A formal toxicity study of tetrathiomolybdate had not been done before these studies, although one is now under way. Approval by the US Food and Drug Administration for this clinical trial was based on extensive animal studies of tetrathiomolybdate during several decades, in which the only toxic effects found were due to copper deficiency.

Adverse effects from tetrathiomolybdate in these studies have been limited to mild bone marrow suppression producing anemia, leukopenia, and occasionally thrombocytopenia, and to mild elevations of aminotrans-

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Table 7. Blood Count Studies During the 8 Weeks of Initial Tetrathiomolybdate Therapy

	Weeks of Therapy								
	0	1	2	3	4	5	6	7	8
Mean Blood Counts in the 17 Patients Who Were Stable									
HGB, g/dL	13.2	13.1	13.0	13.2	13.3	13.2	12.9	12.4	13.0
WBCs/µL	4300	4800	4200	6200	4200	4000	4400	4100	3900
Platelets, $\times 10^3$/µL	121	117	117	133	119	124	135	132	134
No. of patients	17	17	17	17	16	16	12	11	5
Mean Blood Counts in the 5 Patients Who Showed Bone Marrow Suppression									
HGB, g/dL	13.8	12.2	12.0	11.5	11.0	10.5	9.8	10.8	10.3
WBCs/µL	5800	4200	4500	4000	3700	5800	3500	4400	4600
Platelets, $\times 10^3$/µL	112	90	102	104	98	90	86	122	130
No. of patients	5	5	5	5	5	4	4	3	2

Abbreviations: HGB, hemoglobin; WBCs, white blood cells.

Table 8. Liver Function Studies During the 8 Weeks of Initial Tetrathiomolybdate Therapy

	Weeks of Therapy								
	0	1	2	3	4	5	6	7	8
Mean Liver Function Values in the 19 Patients Who Were Stable									
Bilirubin, mg/dL	0.9	0.6	0.6	0.6	0.7	0.7	0.7	0.6	0.6
Albumin, g/dL	3.6	3.4	3.4	3.5	3.5	3.5	3.5	3.4	3.5
ALT, IU/L	36	36	37	37	34	56	63	54	74
AST, IU/L	38	34	32	31	32	32	41	36	44
LDH, IU/L	168	170	163	161	161	194	204	166	157
Alk phos, U/L	93	94	99	102	103	118	116	111	122
No. of patients	19	18	18	19	17	17	13	11	6
Mean Liver Function Values in the 3 Patients Who Showed Aminotransferase and Alk Phos Elevations									
Bilirubin, mg/dL	0.9	0.9	0.6	0.6	0.8	0.8	0.9	0.4	0.4
Albumin, g/dL	3.7	3.5	3.4	3.3	3.2	3.1	3.4	3.3	3.2
ALT, IU/L	81	84	82	288	378	413	354	174	89
AST, IU/L	67	84	53	125	148	139	148	50	36
LDH, IU/L	251	301	221	210	198	188	251	190	164
Alk phos, U/L	137	133	137	170	212	225	271	469	489
No. of patients	3	3	3	3	3	3	2	1	1

Abbreviations: Alk phos, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.
SI conversion factor: To convert bilirubin to micromoles per liter, multiply by 17.1.

ferase enzymes. The bone marrow effects appear to be due to depletion of copper and are responsive to a drug holiday or dose reduction. The frequency of anemia or leukopenia was much higher in the current study (5 of 22 patients) than in the original study (1 of 33 patients). One difference is that the number of patients receiving a daily dose of 200 mg or more was only 15 of 33 in the original study and was 14 of 22 in the present study. A second difference is that the dose escalation was considerably more rapid in the current study, usually taking less than a week, while it occurred during 2 to 3 weeks in the original study. Probably the most important difference is that in the original study, escalation was based on the presence of free copper in the blood. This was copper unaccounted for by ceruloplasmin or tetrathiomolybdate binding. In the current study, escalation was more arbitrary, aimed at quelling copper toxicity quickly, since we had seen so little problem with higher doses in the original study.

The other adverse effect, aminotransferase elevations in 3 of 22 patients, was not detected at all in our original study of 33 patients. We are not sure what causes aminotransferase elevations, but we speculate that tetrathiomolybdate is removing copper from various hepatic pools, including metallothionein, and that this causes some additional hepatitis. Since we have not seen this adverse effect from tetrathiomolybdate use in a variety of other clinical uses such as for cancer[17] and macular degeneration, where it is used for antiangiogenic purposes, nor in a variety of animal studies, we suspect it occurs only in the face of high hepatic copper loading. Again, we suspect that the reason we saw aminotransferase elevations here but not in the original 33 patients relates to the more rapid and arbitrary tetrathiomolybdate dose escalation.

Both of these adverse effects are quickly responsive to a drug holiday and/or dose reduction. Both clearly are related to dose. For example, in the present study, 7 of the 8 adverse effects occurred in the 14 patients receiving 200 mg or more of tetrathiomolybdate per day (Table 2), whereas only 1 occurred in the 8 patients who received 140 or 120 mg. Since our data indicate no effi-

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Table 9. Neurology and Speech Scores Over Time in the 2 Studies

	Baseline	Year 1	2	3
Neurology Scores				
Original study[4]				
Mean	8.1	4.8	2.5	3.5
SD	6.1	6.2	4.8	3.9
No. of patients	26	17	10	11
Present study				
Mean	9.0	4.9	5.0	4.6
SD	6.4	5.9	5.5	5.2
No. of patients	19	19	16	16
Speech Scores				
Original study				
Mean	3.3	2.1	1.8	1.8
SD	1.7	1.6	1.3	1.7
No. of patients	24	22	15	15
Present study				
Mean	3.5	2.8	2.5	2.0
SD	1.2	1.6	1.7	1.6
No. of patients	19	17	15	16

cacy advantage of higher doses, we now recommend daily doses no higher than 120 mg/d for the initial treatment of Wilson disease, to minimize adverse effects.

In summary, tetrathiomolybdate shows excellent efficacy for the initial treatment of patients presenting with the movement disorder symptoms of Wilson disease. Only 2 (4%) of 55 patients worsened during the 8 weeks of tetrathiomolybdate therapy, compared with an estimated 50% who are treated initially with penicillamine. This stabilization of the clinical state during the initial period while copper toxicity is controlled then allows very good recovery of much neurologic function during the succeeding year or two.

Two adverse effects predominate. One is overtreatment bone marrow suppression. Since the bone marrow requires copper for cellular proliferation, higher doses of tetrathiomolybdate causing bone marrow depletion of copper result in bone marrow cellular suppression. The other adverse effect is elevation of serum aminotransferase enzymes, possibly due to hepatic mobilization of copper in livers already loaded with copper. Both adverse effects are dose related and occur much less frequently if the daily dose of tetrathiomolybdate does not exceed 120 mg. Since there does not appear to be an efficacy advantage of higher tetrathiomolybdate doses, we recommend 120 mg/d for initial therapy in Wilson disease, to minimize adverse effects. Both adverse effects, if they do occur, are quickly responsive to drug holiday and/or dose reduction.

Accepted for publication November 5, 2002.

Author contributions: *Study concept and design* (Dr Brewer); *acquisition of data* (Drs Brewer, Hedera, Carlson, Askari, and Fink, Mss Kluin and Sitterly, and Mr Dick); *analysis and interpretation of data* (Drs Brewer, Hedera, and Askari, Ms Kluin, and Mr Dick); *drafting of the manuscript* (Dr Brewer and Ms Sitterly); *critical revision of the manuscript for important intellectual content* (Drs Brewer, Hedera, Carlson, and Fink, Ms Kluin, and Mr Dick); *statistical expertise* (Mr Dick); *obtained funding* (Dr Brewer); *administrative, technical, or material support* (Drs Brewer, Hedera, and Askari, Mr Dick, and Ms Sitterly); *study supervision* (Drs Brewer, Carlson, and Fink).

This study was supported by grant FD-U-000505 from the US Food and Drug Administration's Orphan Products Office, Rockville, Md, and by the General Clinical Research Center of the University of Michigan Hospitals, supported by grant MO1-RR00042 from the National Institutes of Health, Bethesda, Md. Dr Brewer is supported in part by a grant from Attenuon LLC, San Diego, Calif.

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Treatment of Wilson Disease With Ammonium Tetrathiomolybdate

IV. Comparison of Tetrathiomolybdate and Trientine in a Double-blind Study of Treatment of the Neurologic Presentation of Wilson Disease

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Objective: To compare tetrathiomolybdate and trientine in treating patients with the neurologic presentation of Wilson disease for the frequency of neurologic worsening, adverse effects, and degree of neurologic recovery.

Design: A randomized, double-blind, controlled, 2-arm study of 48 patients with the neurologic presentation of Wilson disease. Patients either received 500 mg of trientine hydrochloride 2 times per day or 20 mg of tetrathiomolybdate 3 times per day with meals and 20 mg 3 times per day between meals for 8 weeks. All patients received 50 mg of zinc 2 times per day. Patients were hospitalized for 8 weeks, with neurologic and speech function assessed weekly; discharged taking 50 mg of zinc 3 times per day, and returned annually for follow-up.

Setting: A university hospital referral setting.

Patients: Primarily newly diagnosed patients with Wilson disease presenting with neurologic symptoms who had not been treated longer than 4 weeks with an anticopper drug.

Intervention: Treatment with either trientine plus zinc or tetrathiomolybdate plus zinc.

Main Outcome Measures: Neurologic function was assessed by semiquantitative neurologic and speech examinations. Drug adverse events were evaluated by blood cell counts and biochemical measures.

Results: Six of 23 patients in the trientine arm and 1 of 25 patients in the tetrathiomolybdate arm underwent neurologic deterioration ($P<.05$). Three patients receiving tetrathiomolybdate had adverse effects of anemia and/or leukopenia, and 4 had further transaminase elevations. One patient receiving trientine had an adverse effect of anemia. Four patients receiving trientine died during follow-up, 3 having shown initial neurologic deterioration. Neurologic and speech recovery during a 3-year follow-up period were quite good.

Conclusion: Tetrathiomolybdate is a better choice than trientine for preserving neurologic function in patients who present with neurologic disease.

ClinicalTrials.gov Identifier: NCT00004339

Arch Neurol. 2006;63:521-527

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WILSON DISEASE IS AN autosomal recessive disease of a toxic reaction to copper, primarily affecting the brain and liver.[1-5] The disease is due to mutations in the *ATP7B* gene,[6-8] which produces a protein required for biliary excretion of the body's excess copper.

Three anticopper drugs are currently approved for Wilson disease. Penicillamine, a copper chelator that causes excretion of copper in the urine,[9] is effective in Wilson disease but has a long list of adverse effects.[10] Trientine hydrochloride is also a copper chelator that enhances urinary excretion of copper, is better tolerated than penicillamine,[11] and has not been evaluated in patients presenting with neurologic symptoms. Zinc, approved for maintenance therapy, induces intestinal cell metallothionein, which binds copper from food and endogenous secretions, preventing its transfer to blood,[12-14] thus producing a block of intestinal absorption of copper.

Treatment of patients initially seen with neurologic symptoms from Wilson disease has been problematic. Using a retrospective survey, we found that 50% of pa-

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Table 1. Initial Data on Participating Patients

Patient/Sex/Age	Pretreatment History	Hepatic Copper Level, μg/g	Urine Copper Level, μg/d	Ceruloplasmin Level, mg/dL
Patients Who Received TM				
230/F/26		294	258	3.5
234/M/27				2.1
236/M/34	20 d of penicillamine	915	133	6.7
237/M/23		253	444	2.9
239/F/44		410	152	13.3
240/F/32	Recrudescence after stopped taking penicillamine			2.0
242/M/29				2.4
245/M/36		734	222	6.6
247/M/45	11 d of penicillamine	724	153	5.2
249/M/49		814	185	14.6
251/M/13		726	275	10.2
253/M/27	Recrudescence after stopped taking penicillamine		218	9.2
256/M/23	14 d of penicillamine	566	217	3.2
257/F/29	Recrudescence after stopped taking penicillamine		226	4.6
261/M/41	Recrudescence after stopped taking penicillamine		219	2.0
265/M/41		786	404	16.5
266/M/35	Recrudescence after stopped taking penicillamine		167	3.9
268/F/22	21 d of trientine	1033		10.7
271/F/39			187	11.3
273/M/32			168	12.9
274/M/23			207	1.0
275/M/42		378	360	2.0
281/M/28		508	405	8.4
282/M/31		783	234	6.0
285/M/27		727	203	2.0
Mean (SD)		643 (231)	240 (89)	6.5 (4.6)
Sample size*		15	21	25
Patients Who Received Trientine Hydrochloride				
76/M/28		697		11.3
232/M/37			624	6.8
233/M/17				14.6
238/M/29		841	132	9.3
241/F/17		1104	155	16.1
243/F/18	21 d of penicillamine and trientine	934		2.0
248/F/25	7 d of penicillamine	953		12.5
250/F/24	7 d of trientine	1070		4.2
252/M/16		262	373	8.4
254/F/13		893		3.9
258/F/21	6 d of penicillamine		115	4.0
259/F/17			147	4.0
260/M/18				7.2
262/F/32			403	7.2
267/M/26	21 d of penicillamine			13.8
269/F/24	9 d of penicillamine			7.8
270/M/43		615	123	9.1
272/F/19		572	840	18.8
278/F/26		1112	192	2.0
279/M/34	28 d of penicillamine	857	163	2.0
280/M/37			156	3.6
284/F/13		190	166	2.0
287/M/25	7 d of penicillamine	510	187	8.7
Mean (SD)		758 (296)	270 (218)	7.8 (4.9)
Sample size†		14	14	23
Normal range		20-50	20-50	18-35

Abbreviation: TM, tetrathiomolybdate.
*Nineteen men, 6 women.
†Eleven men, 12 women.

tients presenting with neurologic symptoms treated with penicillamine had neurologic deterioration, and 77% of these were in the first weeks of therapy.[15] The likely mechanism is that during mobilization of large stores of copper in the liver, blood copper levels are elevated, causing a further elevation of copper levels in the brain. The outcome for patients who deteriorated was often very bad in that half of them never recovered to their prepenicil-

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Table 2. Patients' Neurologic Scores

	Baseline	Week 1	2	3	4	5	6	7	8	38
24 Patients in the TM Arm Who Did Not Show Neurologic Deterioration										
Mean (SD)	7.7 (5.1)	7.4 (5.3)	7.7 (5.7)	7.5 (5.5)	7.6 (5.7)	8 (5.3)	7.2 (5.2)	5.8 (3.8)	5.1 (3.2)	
Sample size	24	16	17	21	21	21	17	14	12	
1 Patient in the TM Arm Who Showed Neurologic Deterioration										
Patient 251	7.5		7.5	9.5	13		11.5			
17 Patients in the Trientine Hydrochloride Arm Who Did Not Show Neurologic Deterioration										
Mean (SD)	8.9 (7.2)	8 (5)	8.6 (7.4)	8.5 (7.6)	9.1 (6.4)	8.1 (6.5)	8.2 (6.9)	10.0 (8.7)	9.3 (11.1)	
Sample size	17	11	12	12	15	15	15	7	5	
6 Patients in the Trientine Arm Who Showed Neurologic Deterioration										
Patient										
76	7.0	8.0	10.0	15.0	17.5	18.0	18.0	20.0		
233	10.5	12.0			18.5		19.5	20.5	22.5	
238	3.5		2.0	7.5	10.5	10.0		9.5		
243	15.0	15.0	14.0	14.0	14.0	14.5	20.5	17.0		
260	11.5	11.5	12.0	11.5	12.0	10.5	10.5		10.0	17.5
287	11.0	9.8	10.8	14.8	15.0	14.8	15.0	17.3	17.3	

Abbreviation: TM, tetrathiomolybdate.

lamine baseline and many were seriously disabled. Thus, we believe that penicillamine is contraindicated for the initial treatment of the patient with neurologic symptoms from Wilson disease. We admit that this view is not universally accepted, and some writers question the data of Brewer et al[15] and still recommend penicillamine for treating these patients. We simply point out that no one has formally and prospectively studied the risk from penicillamine-induced neurologic deterioration, and until they do, the best risk estimate is the data in Brewer et al.[15]

Zinc therapy is not the answer because it takes 4 to 6 months to control the toxic effects of copper. During this prolonged period of ongoing copper toxicity, the disease may progress on its own. Indeed, this occurred in 1 of 3 patients presenting with neurologic disease who we treated with zinc as the sole therapy.

To fill this need, we have developed a new drug, tetrathiomolybdate (TM),[16-19] which acts by forming a tripartite complex with copper and protein. Given with food, TM binds food copper and endogenously secreted copper with food proteins and prevents absorption of the complexed copper. Given without food, TM is absorbed into the blood and there complexes available copper with albumin, making the copper unavailable for cellular uptake. In a 55-patient, open-label trial of TM therapy in patients presenting with neurologic symptoms, only 2, or 3.6%, showed neurologic deterioration reaching our criteria.[19]

To evaluate the safety and efficacy of new treatments for Wilson disease in patients presenting with neurologic symptoms, we carried out a double-blind trial comparing TM and trientine, and the results are reported herein.

METHODS

The patients were diagnosed as having Wilson disease by means of standard criteria previously published. Selected diagnostic data are presented in **Table 1**. In addition to the underlying diagnosis of Wilson disease, all patients were diagnosed as having symptoms of a movement disorder attributable to Wilson disease. If patients had received treatment for longer than 28 days with penicillamine or trientine, they were excluded. Most patients were newly diagnosed, but a few were accepted who had been receiving long-term treatment with penicillamine, stopped their therapy more than 1 year prior to consideration, and then developed new neurologic symptoms. Pretreatment history is given in Table 1. The institutional review board of the University of Michigan Medical School, Ann Arbor, reviewed and approved the project.

Each patient was admitted for 8 weeks in the General Clinical Research Center of the University of Michigan Hospital, Ann Arbor. After initial studies to confirm the diagnosis, obtain informed consent, and establish baseline neurologic and speech function, patients were randomized to 1 of 2 treatment arms using a table of random numbers. In arm 1, patients received TM in doses of 20 mg 3 times daily with meals and 20 mg 3 times daily between meals. In arm 2, patients received 500 mg of trientine hydrochloride 2 times daily between meals. Tetrathiomolybdate and trientine were placed in identical-appearing capsules. Matching placebo capsules were used so that all patients received the same number of doses at the same time. All patients received 50 mg of zinc 2 times daily.

Criteria for adverse effects included anemia (a replicable hemoglobin value < 80% of baseline), leukopenia (a replicable white blood cell count < 80% of baseline), and transaminase elevations consisting of a replicable quadrupling of baseline values of either aspartate aminotransferase or alanine aminotransferase. In the event of anemia or leukopenia, a drug holiday was given until recovery, then the drug treatment was restarted at half levels. A subsequent drop of 20% of the blood value involved resulted in discontinuation of the drug treatment. A quadrupling of transaminase values resulted in discontinuation of the drug regimen.

During the 8-week hospital admission, a quantitative neurologic test and a quantitative speech test were carried out at weekly intervals. These methods have been previously published and are standardized for and previously evaluated in Wilson disease.[19] The neurologists and speech pathologist were

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Table 3. Patients' Speech Scores

	Baseline	Week 1	2	3	4	5	6	7	8	38
24 Patients in the TM Arm Who Did Not Show Neurologic Deterioration										
Mean (SD)	3.1 (1.6)	3.3 (1.6)	3.3 (1.6)	3.3 (1.8)	3.0 (1.7)	3.2 (1.6)	2.8 (1.6)	3.3 (1.5)	3.0 (2.0)	
Sample size	24	18	20	16	20	22	14	16	15	
1 Patient in the TM Arm Who Showed Neurologic Deterioration										
Patient 251	5.0	5.0	5.0	5.5	6.0	6.0				
17 Patients in the Trientine Hydrochloride Arm Who Did Not Show Neurologic Deterioration										
Mean (SD)	3.6 (1.6)	3.6 (1.6)	3.5 (1.4)	3.9 (1.3)	3.7 (1.8)	3.8 (1.8)	3.6 (1.7)	3.3 (1.7)	3.6 (2.4)	
Sample size	17	15	13	15	13	12	15	13	4	
6 Patients in the Trientine Arm Who Showed Neurologic Deterioration										
Patient										
76	5.0	6.0	5.0	5.5	5.0			4.5	4.5	
233	4.5		5.0	6.0		5.5	5.5	5.5	5.5	
238	4.0	3.0	3.0	4.0	3.0	4.5		4.0		
243	5.0	5.0	4.5	4.5	4.5	5.5	5.5	6.0	4.5	
260	4.0	3.5	3.5	4.0	4.0	4.0	4.0		4.0	6.0
287	5.0	4.5	6.0	5.5		7.0	6.0	5.0		

Abbreviation: TM, tetrathiomolybdate.

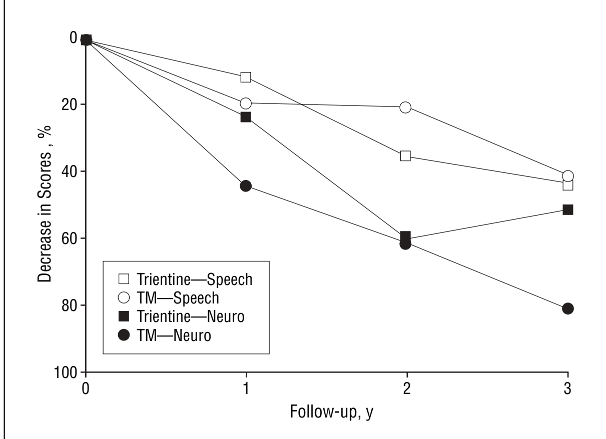

Figure. The improvements in neurologic scores (neuro) and speech scores (speech) during years of follow-up in patients treated with tetrathiomolybdate (TM) and trientine.

blinded. A replicable increase of 5 points (scale, 0-38) on the quantitative neurologic examination or a replicable increase of 3 points (scale, 0-7) on the speech examination was taken as evidence of significant neurologic deterioration. The patients were discharged from the hospital taking a regimen of zinc maintenance therapy and then returned for annual visits for 3 years, with repeat studies of the neurologic and speech examinations.

During the 8-week admission, assays of safety variables were carried out. These included complete blood cell counts; liver function tests; blood levels of amylase, lipase, creatinine, urea nitrogen, uric acid, and iron variables; and urine protein levels, all carried out by standard techniques in use at the University of Michigan Health System hematologic and biochemistry laboratories. Blood ceruloplasmin was also assayed in these laboratories.

Urine was collected for copper measurements in acid-washed, trace element–free containers, and urine and serum copper levels were measured by atomic absorption. Nonceruloplasmin plasma copper (sometimes called "free" copper) levels were determined by subtracting 3 μg for every 1 mg/dL of ceruloplasmin from the serum copper, expressed as microgram per deciliter.

RESULTS

The average neurologic scores for the patients who did not deteriorate in the TM arm during the 8-week admission and the individual weekly scores for the single patient who reached criteria for neurologic deterioration are presented in **Table 2**. The average neurologic scores for the patients who did not deteriorate in the trientine arm during the 8-week admission are also presented in Table 2. Five patients reached criteria for neurologic deterioration during the 8 weeks, and a sixth (patient 260) was reported by his family to have deteriorated significantly shortly after discharge to his home in Venezuela. On readmission 9.5 months after his initial hospital admission, he was found to have reached criteria for worsening in spite of evidence of good compliance with maintenance therapy. The individual weekly scores for these 6 patients are presented at the bottom of Table 2. A careful review of plasma copper, urine copper, and nonceruloplasmin plasma copper levels found very similar results in the patients who deteriorated compared with those who did not deteriorate (data not shown).

Neurologic deterioration in 6 of 23 patients in the trientine arm compared with 1 of the 25 patients in the TM arm was statistically significant ($P<.05$). The baseline neurologic scores of all 48 patients averaged about 8.4 and was not significantly different between the 2 arms. The mean baseline neurologic scores of the patients who worsened was 9.5, not significantly different than the whole sample.

The average speech scores for the patients who did not deteriorate neurologically in the TM arm during the 8-week admission are presented in **Table 3**, along with individual weekly scores for the 1 patient who deteriorated neurologically. The average speech scores for the patients who did not deteriorate neurologically in the trientine arm during the 8-week admission are also pre-

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Table 4. Adverse Effects From Anticopper Drugs in the 48 Patients

Treatment Arm	Patient	Hemoglobin Level, g/dL		White Blood Cell Count, ×10³/μL	
		Baseline	At Criteria	Baseline	At Criteria
		Anemia/Leukopenia			
Trientine hydrochloride	252	13.1	11.1	3.9	2.8*
TM	230	12.8	8.9*	3.0	1.5*
	265	13.7	11.2	3.9	1.9*
	274	13.4	10.8*	2.2	2.1

Treatment Arm	Patient	AST Level, U/L		ALT Level, U/L	
		Baseline	At Criteria	Baseline	At Criteria
		Transaminase Elevations†			
TM	237	24	72	28	320*
	240	23	96*	35	333*
	242	55	168	46	504*
	281	23	72	27	240*

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; TM, tetrathiomolybdate.
*Indicates criterion being reached.
†No patients were taking trientine.

sented in Table 3, along with individual weekly scores for the 6 patients who deteriorated neurologically. No patient in either arm reached criteria for speech deterioration, although 5 of the 7 patients who deteriorated neurologically showed some worsening in speech score.

Baseline speech scores of the patients who did not deteriorate neurologically averaged 3.32, and the baseline speech scores of the 7 patients who deteriorated neurologically averaged 4.64 and were significantly higher ($P<.04$). This suggests that a high baseline speech score is predictive of neurologic deterioration during treatment. In fact, none of 25 patients with a baseline speech score of 3.5 or less deteriorated, while 7 of 24 patients with baseline scores of 4.0 or higher deteriorated.

Long-term neurologic recovery was quite good in those patients who returned for follow-up. During a 3-year period, patients initially treated with TM recovered an average of 81% of their neurologic function and patients treated with trientine, 51% (**Figure**). The lower value for the trientine arm at 3 years is probably a sampling artifact from the particular patients who returned at 3 years because at 2 years, TM- and trientine-treated patients were equivalent at about 60%. Recovery of speech function also occurred (Figure) but not to the same degree as neurologic recovery on a percentage basis.

During the 8 weeks of drug therapy, 3 patients in the TM arm and 1 patient in the trientine arm reached criteria for anemia and/or leukopenia, while 4 patients in the TM arm and zero patients in the trientine arm reached criteria for transaminase elevations (**Table 4**). A careful review of copper and molybdenum data on the patients receiving TM found very similar results for the plasma copper, urine copper, nonceruloplasmin plasma copper, and urine molybdenum levels in the patients showing adverse effects compared with those who did not. However, the plasma molybdenum level was significantly higher during the first 2 weeks of therapy in those who showed adverse effects. This difference is unexplained but may be related to the TM-produced adverse effects. There were no negative effects on other safety variables with either drug.

Table 5. Deaths in the 48 Patients

Treatment Arm	Patient	Months Until Death	Cause of Death
Trientine hydrochloride	233	22	Severe neurologic impairment, general inanition, infection
	260	11.5	Neurologic deterioration beginning soon after hospital discharge, general inanition
	280	12	Severe neurologic impairment initially, no improvement, general inanition
	287	6	Severe neurologic impairment, pulmonary congestion
TM	249	14	Severe neurologic impairment initially, no improvement, late neurologic worsening, general inanition
	251	17.5	Leukemia

Abbreviation: TM, tetrathiomolybdate.

Two patients in the TM arm died during follow-up (**Table 5**). One of these was the patient who had neurologic deterioration (patient 251). However, he died of leukemia presumably unrelated to Wilson disease or its therapy. Four patients in the trientine arm died during follow-up (Table 5). Three of these (patients 233, 260, and 287) were patients who deteriorated neurologically while receiving trientine therapy.

Values for 24-hour urine copper and nonceruloplasmin plasma copper initially, at 7 to 8 weeks, and at 1 year are given in **Table 6**. The 7- to 8-week value for urine copper for trientine-treated patients reflects the effect of the drug on urinary copper excretion. Urine copper values at 1 year show that the urine copper has come under good

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Table 6. 24-Hour Urine Copper and Nonceruloplasmin Plasma Copper Values*

	TM			Trientine Hydrochloride		
	Initial	7-8 wk	1 y	Initial	7-8 wk	1 y
24-h urine copper level, µg	240 (20)	213 (23)	89 (10)	270 (60)	1102 (50)	116 (30)
Nonceruloplasmin plasma copper level, µg/dL	17.2 (2.3)	11.8 (4.3)	7.4 (1.7)	10.7 (2.2)	11.8 (3.6)	7.3 (1.5)

Abbreviation: TM, tetrathiomolybdate.
*Values are expressed as mean (SE).

Table 7. Liver Function Test Values*

	TM			Trientine Hydrochloride		
	Initial	7-8 wk	1 y	Initial	7-8 wk	1 y
Albumin level, g/dL	4.0 (0.1)	3.4 (0.1)	3.7 (0.14)	3.0 (0.1)	3.2 (0.1)	3.8 (0.12)
AST level, U/L	48.1 (12.4)	62.5 (20)	34.0 (2.45)	42.9 (5.1)	41.2 (5.1)	34.1 (3.2)
ALT level, U/L	55.5 (13.7)	64.1 (8.8)	45.2 (4.25)	44.2 (3.9)	62.4 (19.9)	50.4 (5.4)
Bilirubin level, mg/dL	1.17 (0.19)	0.74 (0.09)	0.7 (0.09)	1.03 (0.11)	0.64 (0.06)	0.7 (0.12)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; TM, tetrathiomolybdate.
SI conversion factor: To convert bilirubin to micromoles per liter, multiply by 17.1.
*Values are expressed as mean (SE).

control (while the normal value is ≤50 µg, values lower than 125 µg are viewed as under good control).[13] The normal value for nonceruloplasmin plasma copper is about 10 ug/dL, so the values at 1 year show good control.

Results of liver function tests initially, at 7 to 8 weeks, and at 1 year are presented in **Table 7**. The serum albumin value was lower than normal in 10 of 25 patients in the TM arm initially, and this had declined to 6 of 18 at 1 year. For the trientine arm, the serum albumin value was lower than normal in 9 of 23 patients initially and declined to 3 of 14 at 1 year. Mean aspartate aminotransferase and alanine aminotransferase levels tended to decline over 1 year. At 1 year, aspartate aminotransferase values were higher than normal (35 U/L is the upper limit of normal) in 5 of 18 patients in the TM arm and in 6 of 14 patients in the trientine arm. At 1 year, alanine aminotransferase values were higher than normal (45 U/L is the upper limit of normal) in 10 of 18 patients in the TM arm and in 10 of 14 patients in the trientine arm. Continued mild elevations of these enzymes are quite common in patients with Wilson disease receiving maintenance therapy. Regarding total bilirubin level, 9 of 25 patients in the TM arm showed values higher than normal (1.1 mg/dL), initially, and this was reduced to 3 of 22 at 1 year. In the trientine arm, 7 of 23 patients showed values higher than normal initially, and this was reduced to none of 19 at 1 year.

COMMENT

In this study, trientine, used as initial therapy for patients with neurologic symptoms of Wilson disease, showed a 26% risk (6 of 23 patients) of being associated with neurologic deterioration. Tetrathiomolybdate showed a 4.0% risk (1 of 25 patients) of being associated with neurologic deterioration, almost exactly the same risk seen in our open study of TM (3.6% [2/55]). The difference in risk between trientine and TM in the current study is statistically significant ($P<.05$).

Long-term neurologic recovery overall was very good (Figure). Speech recovery was fair and did not differ between the 2 arms. Trientine was well tolerated. Only 1 patient developed anemia/leukopenia. Tetrathiomolybdate showed a frequency of about 12% of anemia and/or leukopenia (3 of 25 patients) and about 16% of transaminase elevations (4 of 25 patients). These problems were easily handled by dose reduction and/or drug holiday. The frequency of these problems with the 120-mg dose of TM given for 8 weeks has led to a new trial of 120 mg of TM for 2 weeks followed by 60 mg for 14 weeks, which is currently ongoing.

This study suggests that neurologic deterioration during initial treatment with trientine is a grave prognostic sign. Of the 6 patients treated with trientine who deteriorated, 3 died. Of the other 17 patients who did not deteriorate, only 1 died. Two of the 3 patients treated with trientine who deteriorated but did not die ended up with severe, permanent neurologic impairment. One of these ended up much worse than his baseline, and one ended up about the same as baseline. One patient treated with trientine who had neurologic deterioration ended up doing well, but this patient is the only 1 of 6 patients treated with trientine who deteriorated initially and did well in the end.

These data indicate that TM, given together with zinc, is the preferred treatment over penicillamine and trientine for the neurologic presentation of Wilson disease. A direct comparison of TM and zinc has not been done and could be considered. It has been our view that zinc is too slow acting and the disease may progress during the first 6 months of zinc therapy, and indeed, this happened in 1 of 3 patients we treated with zinc

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prior to TM availability. With the present dosage regimen of TM, patients should be followed up weekly, particularly beginning at week 3, for anemia and/or leukopenia or transaminase elevations. If one of these occurs, the drug should be temporarily stopped and after a few days, resumed at half dose. Tetrathiomolybdate is still an experimental drug. It should become available commercially in the next year.

Accepted for Publication: December 2, 2005.
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Funding/Support: This work was supported by grant FD-U-000505 from the US Food and Drug Administration's Orphan Products Office and by the General Clinical Research Center of the University of Michigan Hospitals, which is supported by grant MO1-RR00042 from the National Institutes of Health.
Financial Disclosure: The University of Michigan has recently licensed the antifibrotic and anti-inflammatory usesof tetrathiomolybdate to Pipex, Inc, Miami, Fla. Dr Brewer has equity in and is a consultant to Pipex, Inc.

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Announcement

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Ammonium tetrathiomolybdate enhances the antitumor effect of cisplatin via the suppression of ATPase copper transporting beta in head and neck squamous cell carcinoma

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Received May 17, 2019; Accepted September 26, 2019

DOI: 10.3892/or.2019.7367

Abstract. Platinum-based antitumor agents have been widely used to treat head and neck squamous cell carcinoma (HNSCC) and numerous other malignancies. Cisplatin is the most frequently used platinum-based antitumor agent, however drug resistance and numerous undesirable side effects limit its clinical efficacy for cancer patients. Cancer cells discharge cisplatin into the extracellular space via copper transporters such as ATPase copper transporting beta (ATP7B) in order to escape from cisplatin-induced cell death. In the present study, it was demonstrated for the first time that the copper chelator ammonium tetrathiomolybdate (TM) has several promising effects on cisplatin and HNSCC. First, TM suppressed the ATP7B expression in HNSCC cell lines *in vitro*, thereby enhancing the accumulation and apoptotic effect of cisplatin in the cancer cells. Next, it was revealed that TM enhanced the antitumor effect of cisplatin in HNSCC cell tumor progression in a mouse model of bone invasion, which is important since HNSCC cells frequently invade to facial bone. Finally, it was demonstrated that TM was able to overcome the cisplatin resistance of a human cancer cell line, A431, via ATP7B depression *in vitro*.

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Key words: copper transporter, head and neck squamous cell carcinoma, ATP7B, cisplatin

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a common malignant tumor (1,2). Despite advances in the early detection, diagnosis, and management of HNSCC, the long-term survival rate of HNSCC patients has improved only marginally over the past decades, and more effective strategies for the treatment of HNSCC are thus required (3). Although immunotherapy with anti-programmed cell death protein 1 (anti-PD-1) and therapy with anti-epidermal growth factor receptor (anti-EGFR) were recently approved in many countries for the treatment of HNSCC patients, cisplatin is still the most widely used antitumor drug for HNSCC (4-6).

First synthesized in 1844 (7), cisplatin, or *cis*-diammin-edichloroplatinum (II), is a metallic (platinum) coordination compound with a square planar molecular geometry. Cisplatin is activated via the replacement of a chloride ligand with H_2O in the cancer cell cytoplasm. Activated cisplatin binds to the N7 atom of guanines in DNA, which in turn generates intra-strand DNA crosslinks (ICLs) (8). ICLs are cytotoxic lesions with a covalent linkage between opposite strands of double-stranded DNA. ICLs lead to defects of vital DNA metabolic processes such as transcription and DNA replication in cancer cells, resulting in cancer cell death (9). HNSCC patients usually exhibit a good response to cisplatin chemotherapy however later relapse, since the development of cisplatin resistance markedly reduces the clinical effectiveness of this agent (10). Some research indicates that cisplatin resistance can result from variations of genetic and protein expression at the cellular level (11,12). Another mechanism of cisplatin resistance consists of reducing the cellular accumulation of cisplatin by increasing its efflux and suppressing its influx (13). It was revealed that the copper transporter ATP7B (ATPase copper transporting beta) is linked to cisplatin efflux from cancer cells (14). Copper transporter expression

is closely linked to the serum copper concentration (15). The copper chelator ammonium tetrathiomolybdate (TM) may be effective for the treatment of copper metabolism disorder and Wilson's disease (16). We previously demonstrated that copper chelators have a bone-protective effect against bone-invasive HNSCC cells (17), however, the potential influence of TM on the accumulation of cisplatin in cancer cells in HNSCC is not clear. The present findings provide the first evidence that the copper chelator TM enhances the antitumor effect of cisplatin via ATP7B suppression in an HNSCC mouse model.

Materials and methods

Reagents. SLC31A1/CTR1 antibody (anti-rabbit, polyclonal; cat. no. GTX48534) was purchased from GeneTex. ATP7A (anti-rabbit, polyclonal; ID product code ab125137) was purchased from Abcam. ATP7B (anti-rabbit, polyclonal; cat. no. NB100-360) was purchased from Novus Biologicals. Cleaved caspase-3 (Asp175) (5A1E) (anti-rabbit, monoclonal; product no. 9664), Ki-67 (D2H10) (anti-rabbit, monoclonal; product no. 9027), and horseradish peroxidase (HRP)-conjugated IgG antibody (goat anti-rabbit, monoclonal; product no. 7074) were purchased from Cell Signaling Technology, Inc.

Cell lines and culture conditions. The human oral squamous cell carcinoma lines HSC-2 (#JCRB0622), HSC-3 (#JCRB0623), and HSC-4 (#JCRB0624) and the human skin squamous cell carcinoma line A431 (#JCRB004) were obtained from the Human Science Research Resources Bank (Osaka, Japan). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. All cell lines were cultured in an atmosphere of 10% CO_2 at 37˚C. The *cis*-dichloro-diamine-platinum (CDDP)-resistant subline A431/CDDP-R was derived from the previously established human epidermoid carcinoma cell line A431 (18). The subline A431/CDDP-R, which was established by mutagenic induction, was revealed to have 2.7 times more resistance to CDDP than the parent cell line A431 based on the half maximal inhibitory concentration (IC_{50}) (19).

Tissue microarray analysis. The expression of ATP7B was analyzed in head and neck cancer tissue and in a normal tissue microarray (#OR601c; US Biomax). The antigen was activated by cooking in a citric acid solution. For the immunohistochemical analysis, the specimens were incubated with anti-ATP7B antibody (1:250) overnight at 4˚C. The slides were then treated with a streptavidin-biotin complex (EnVision System Labeled Polymer, HRP; Dako; Agilent Technologies, Inc.) for 60 min at a dilution of 1:100. The immunoreaction was visualized with the use of a DAB substrate-chromogen solution (Dako Cytomation Liquid DAB Substrate Chromogen System; Dako; Agilent Technologies, Inc.). The cells were counted using a light microscope and evaluated.

Western blot analysis. Protein determination performed by Bradford assay. A total of 15 μg protein were mixed with 4X Laemmli sample buffer (Bio-Rad Laboratories, Inc.) and boiled at 95˚C for 5 min. The samples were electrophoresed in 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc.) and blocking with 5% skim milk for 1 h. The membranes were incubated with primary (4˚C, for 24 h) and secondary (room temperature, for 1 h) antibodies according to the ECL chemiluminescence protocol (product no. RPN2109; Amersham Biosciences; GE Healthcare Life Sciences) to detect secondary antibody binding. Antibodies against ATP7A (1:1,000), ATP7B (1:1,000), CTR1 (1:1,000), caspase-3 (1:1,000), cleaved caspase-3 (1:1,000), β-actin (1:10,000) and GAPDH (2,000) were used as primary antibodies. HRP-conjugated anti-rabbit antibody (1:2,000) was used as the secondary antibody. A ChemiDoc MP system (Bio-Rad Laboratories, Inc.) was used for the analysis of western blots.

Flow cytometric analysis. HSC-3 cells were treated with cisplatin with or without TM for 24 h. Cells were washed and fixed, then incubated with Annexin V-FITC and PE (cat. no. 88-8005-72, cat. no. 00-6990; eBioscience; Thermo Fisher Scientific, Inc.). After staining, the cells were washed, suspended in the FACS staining buffer, and analyzed on a FACS Aria III flow cytometer using FlowJo. Ver.10 (both from BD Biosciences).

Real-time PCR analysis. Total RNA from cells was extracted using RNA easy Mini kit (Qiagen Sciences, Inc.). cDNA synthesis was performed with 1 μg of total RNA using PrimeScript (Takara Bio, Inc.). Real time PCR analysis was carried out with iQ SYBR Green Mix using the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). Detection was carried under following cycle conditions. Initial denaturation at 95˚C for 30 sec, followed by 41 cycles of 95˚C for 10 sec and 60˚C for 35 sec.

The primer sequence for ATP7B was forward, 5-GCC AGCATTGCAGAAGGAAAG-3 and reverse, 5-TGATAA GTGATGACGGCCTCT-3; and for β-actin, forward, 5-GAA AATCTGGCACCACACCTT-3 and reverse, 5-TTGAAGGTA GTTTCGTGGAT-3.

The relative fold change values were evaluated by normalization to β-actin expression via the $2^{-\Delta\Delta Cq}$ method (20).

Cell proliferation assay. The HSC-2 and HSC-3 cells were each plated in six-well plates at a density of $1x10^5$ cells/well. Two 6-well plates, one for each cell line, were used for each of the four groups: A control, cisplatin-treated, TM-treated, and cisplatin+TM-treated group. After 72 h, the cells were counted using a TC20 automated cell counter (Bio-Rad Laboratories, Inc.).

Immunocytochemical analysis. HSC-3 cells were plated on culture slides (BD Falcon; BD Biosciences) at a density of $1x10^3$ cells/well. After cisplatin or TM treatment, the number of apoptotic cells was assessed by the DeadEnd™ Fluorometric TUNEL System (Promega, Corporation).

Cisplatin concentration assay. The cisplatin concentrations in the cell suspensions were measured by a cisplatin assay kit (MicroMolar Cisplatin Assay Kit; ProFoldin). The samples, buffer, and chelate color solution were mixed and incubated for

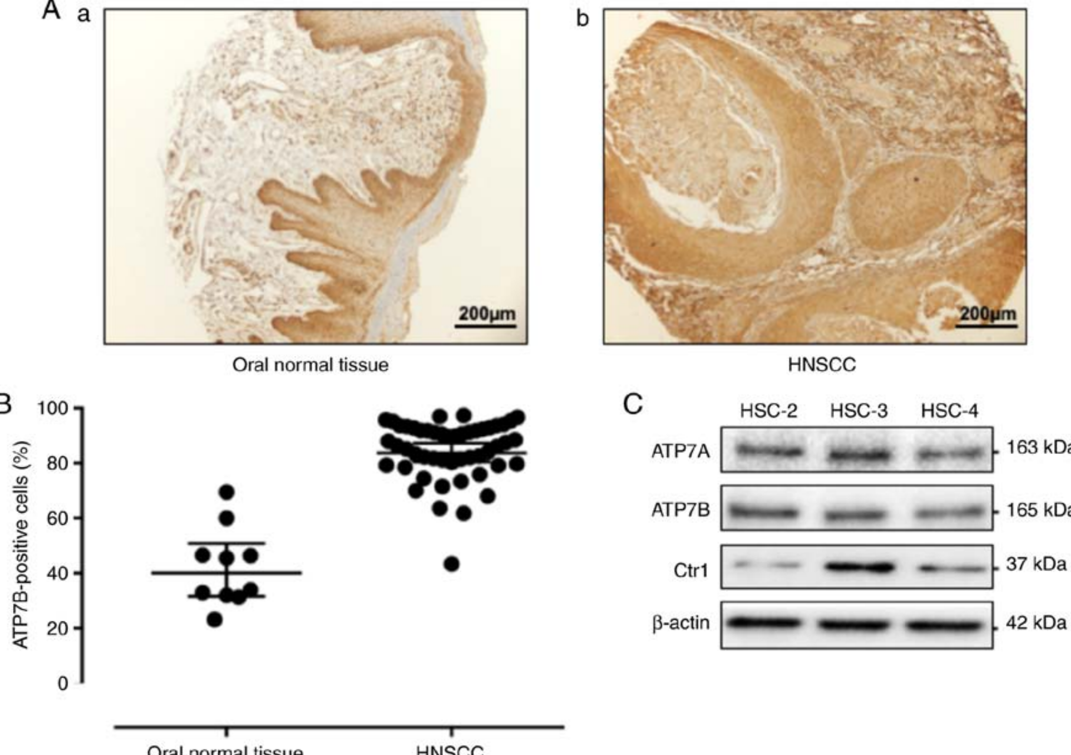

Figure 1. Expression of ATP7B in head and neck normal tissues, head and neck cancers, and human oral squamous cell lines. (A) Immunohistochemistry results of ATP7B expression in (a) head and neck normal tissue and (b) HNC. (B) Scatterplot of the ATP7B-positive areas in the head and neck normal tissues (n=11) and HNSCC (n=70). Error bars: Mean ± SD. There was a significantly increased expression of ATP7B in the HNSCC samples (P<0.0001). (C) The expression of ATP7B in the human oral squamous cell lines (HSC-2, -3, -4) analyzed by western blotting. HNC, head and neck cancer; HNSCC, head and neck squamous cell carcinoma; ATP7B, ATPase copper transporting beta.

60 min at 65˚C. The absorbance was then read at a wavelength of 535 nm using a microplate reader (SH-1000; Hitachi).

Animal experiments. A mouse model of bone invasion by human oral squamous cell carcinoma was established in 5-week-old male BALB/c nude mice (n=6 per group; n=24 total; mean body weight, 19.5 g; Charles River Laboratories) by i.p. inoculation of 1×10^5 HSC-3 cells into the bone marrow space of the right tibial metaphysis under general anesthesia with 0.4 mg/kg of medetomidine, 4.0 mg/kg of midazolam and 5.0 mg/kg of butorphanol Mice were maintained in SPF cages. Body condition scoring was applied and body weight was monitored daily. At 7 days after the tumor cell inoculation, the mice were divided into four groups (control, cisplatin-treated, TM-treated, and cisplatin+TM-treated). The cisplatin group was treated with a single intraperitoneal injection of 100 μl of cisplatin (5 mg/kg). The TM group was orally administered 200 μl of a solution containing TM (1 mg) in phosphate-buffered saline (PBS) 5x/week for 2 weeks. The cisplatin+TM-treated group was treated with both agents at the doses used in the cisplatin group and TM group. At the end of the experimental period (day 35), the mice were sacrificed with cervical dislocation by formal trained researcher under anesthesia with 0.4 mg/kg of medetomidine, 4.0 mg/kg of midazolam and 5.0 mg/kg of butorphanol (i.p) and the right tibias of the nude mice that had been injected with the cancer cells were excised and then fixed in 4% paraformaldehyde phosphate buffer solution. There were no differences in body weight in the control (24.9 g), cisplatin (24.1 g), TM (24.5 g) and dual-treated group (23.9 g) at the end of the experiment. The criteria of humane endpoints for euthanasia was loss of >20 percent of body weight compared to the age-matched controls. Death of the animal was verified by cessation of cardiovascular and respiratory movements. All of the animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the Okayama University Graduate School of Medicine and Dentistry (approval no. OKU-2018663).

In vivo radiography and assessment of osteolytic lesion areas. Osteolytic bone destruction in the mice was assessed on radiographs. The bones were placed against films (22x27 cm; Fuji Industrial Film FR; Fuji Photo Film) and exposed to soft X-rays at 35 kV for 15 sec with the use of a Sofron apparatus (Sofron). The radiolucent bone lesions were observed microscopically (IX81; Olympus Corporation), and the areas were quantified with Lumina Vision/OL image software (Mitani Corporation). A micro-CT image was obtained with a SKYSCAN scanner (Bruker Japan).

Immunohistochemical analysis. Each tibial bone was fixed in 10% formalin at room temperature for 48 h, decalcified, and then embedded in paraffin. Serial sections were then prepared (5 μm-thick). The specimens were incubated with ATP7B (1:250), Ki-67 (1:250) or IL-6 (1:100) antibodies overnight at 4˚C, followed by Alexa Fluor 488 anti-rabbit IgG

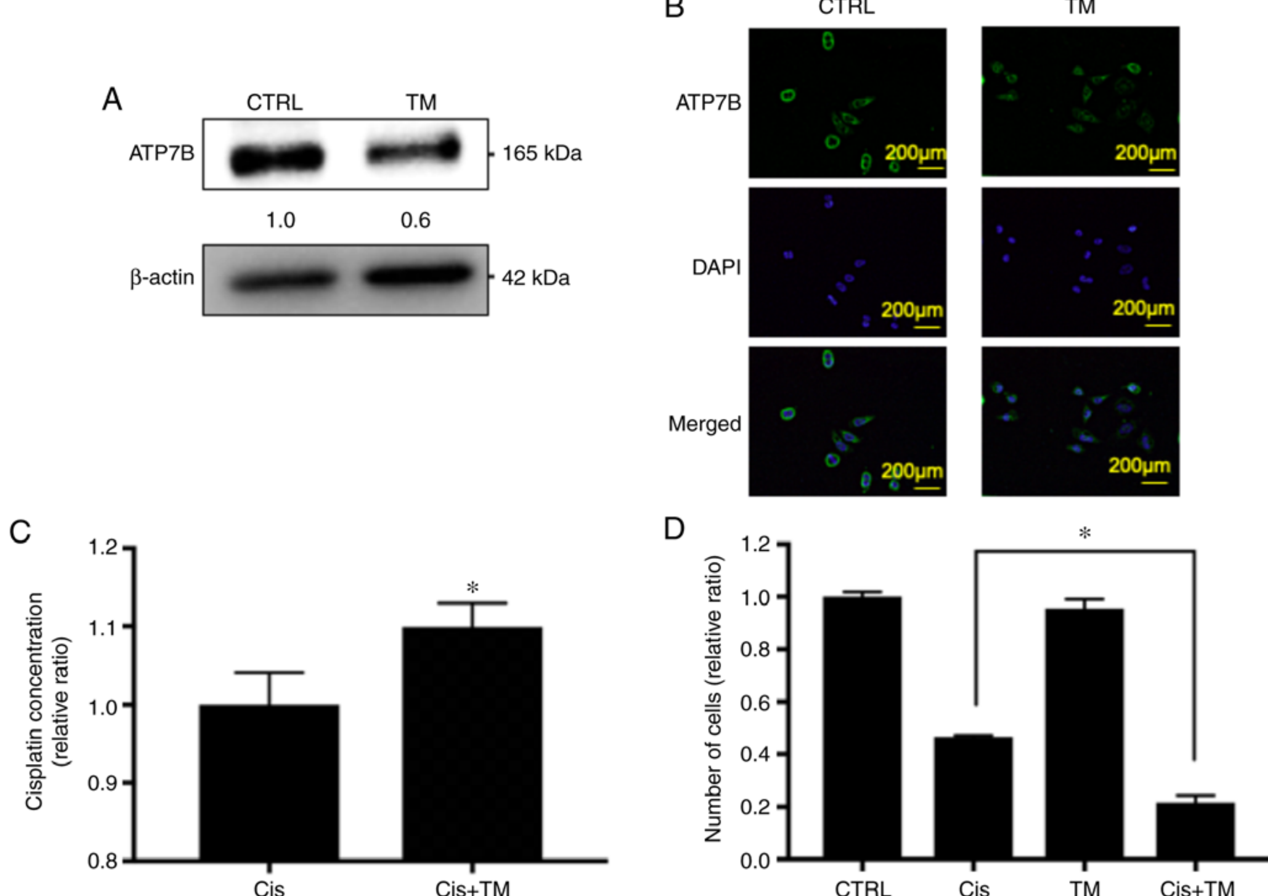

Figure 2. Effect of TM on ATP7B expression and the synergistic antitumor effect of cisplatin in HNSCC cell proliferation *in vitro* via cisplatin accumulation. HSC3 cells were plated in triplicate and treated with 5 μM TM for 24 h. (A) The expression of ATP7B in control HSC-3 cells and TM-treated HSC-3 cells was analyzed based on the image blot density revealed by western blotting. (B) Immunofluorescence analysis of ATP7B and DAPI in the cells. Upper images, ATP7B (green); middle images, DAPI (blue); lower images, merged. Sections were incubated with rabbit anti-ATP7B (1:100), then with Alexa Fluor 488 anti-rabbit IgG (1:1,000) and encapsulated with DAPI. (C) The cisplatin concentration in HSC-3 cells was evaluated after 6-h cisplatin treatment with or without 24-h TM pre-incubation. (D) Antitumor and synergistic antitumor effect of TM against HSC-3 HNSCC cells. HSC-3 cells were treated with cisplatin with or without TM for 24 h. Live cells were counted with trypan blue reagent. Data represent the relative ratio (the control is indicated as 1.0). *P<0.05 cisplatin + TM group vs. the cisplatin treated group. TM, tetrathiomolybdate; ATP7B, ATPase copper transporting beta; HNSCC, head and neck squamous cell carcinoma.

(1:1,000) as a secondary antibody. Nuclei were counterstained with Fluoroshield mounting medium with DAPI (product no. ab104139; Abcam).

Statistical analysis. The data were analyzed using an unpaired Student's t-test for comparisons of two groups and by performing a one-way analysis of variance (ANOVA) and a post hoc Bonferroni or Dunnett's test for multiple group comparisons. Graph Pad Prism, ver. 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). was used for all analyses. The results are expressed as the mean ± standard deviation (SD). Probability (P)-values <0.05 were considered to indicate a statistically significant difference.

Results

ATP7B expression in the human HNSCC tissue. Fig. 1A provides a representative histological pattern of normal oral tissue and HNSCC tissue. ATP7B was expressed significantly higher in the HNSCC samples compared to the normal epithelium samples (P<0.0001) (Fig. 1B). To determine whether HNSCC cells expressed ATP7B *in vitro*, western blot analysis was performed in HSC-2, HSC-3 and HSC-4 cells. As revealed in Fig. 1C, the results of the western blot analysis revealed a high expression of ATP7B in the HNSCC cells. CTR1 is a cisplatin influx transporter. The human head and neck carcinoma cell line HSC-3 markedly expressed CTR1. Thus, HSC-3 cells were used for the subsequent experiment.

TM enhances the antitumor effect of cisplatin on oral squamous cell carcinoma cells. The effect of TM on the expression of the cisplatin efflux transporter ATP7B was then assessed in HNSCC cells by western blotting, immunocytochemical analysis and Real-time PCR. First, TM was added to an HSC-3 culture medium for 24 h, and as revealed in Figs. 2A and S1, the protein expression of ATP7B in the TM-treated HSC-3 cells was decreased by 40%, while the mRNA expression of ATP7B in the TM-treated HSC-3 cells was not altered. Next, immunocytochemical analysis was performed, and the results indicated that ATP7B expression in the cell membrane was decreased by TM treatment, confirming the results of the western blotting (Fig. 2B).

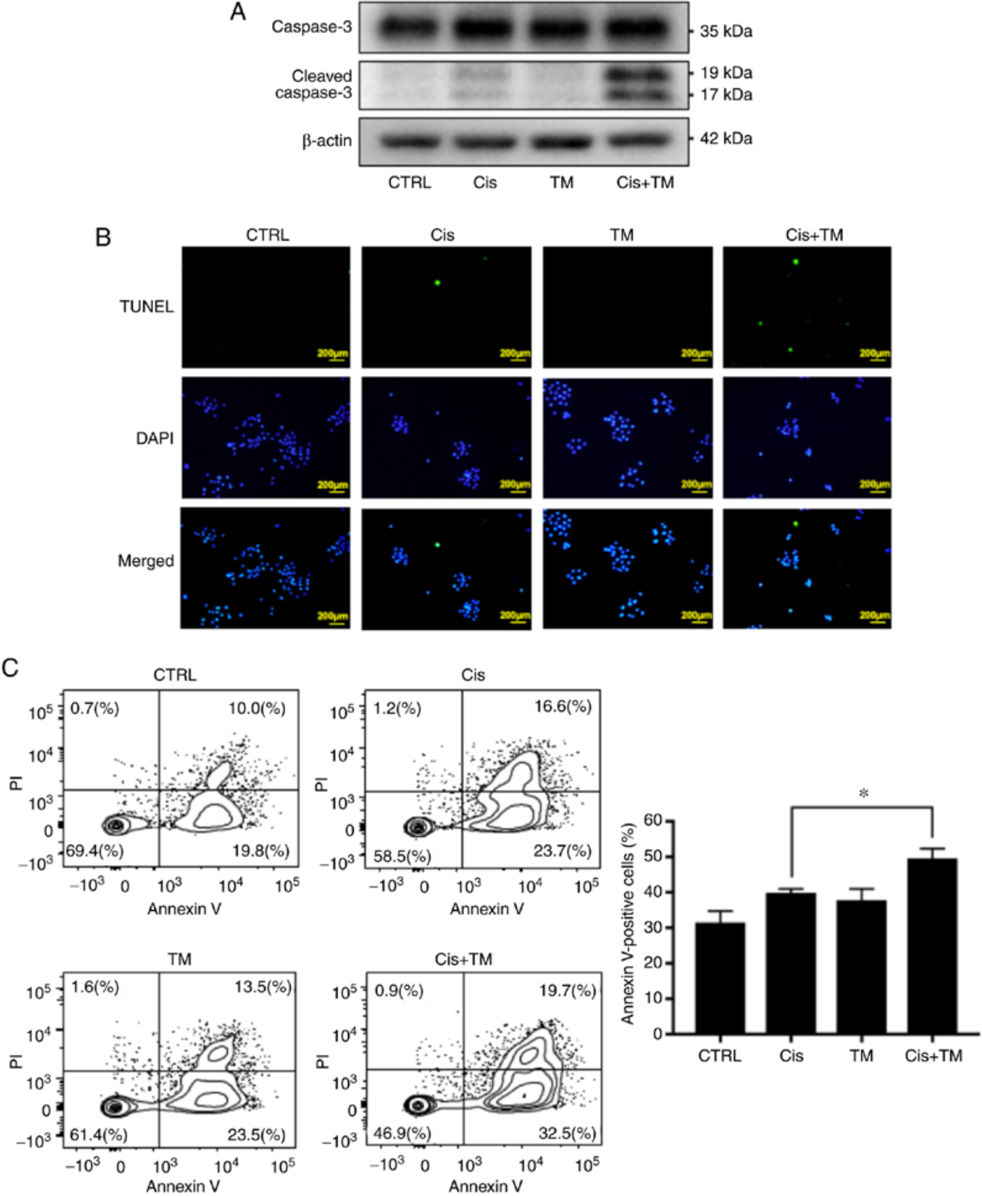

Figure 3. Apoptosis effects of TM and cisplatin on HNSCC cells grown *in vitro*. (A) The expression of total and cleaved caspase-3 in HSC-3 cells treated with or without cisplatin and TM. Total and cleaved caspase-3 in HSC-3 cells was evaluated based on the image blot density revealed by western blotting. (B) Immunofluorescence analysis of Tunnel and DAPI in the HSC-3 cells. Upper images, Tunnel (green); middle images, DAPI (blue), lower images, merged. (C) Flow cytometric analysis: Propidium iodide (PI) and Annexin V staining of HSC-3 cells treated with cisplatin or with cisplatin+TM for 24 h. Apoptopic cells were evaluated with FACS Aria III. TM, tetrathiomolybdate; HNSCC, head and neck squamous cell carcinoma. Data are expressed as the mean ± SD. *P<0.05. Cisplatin+ TM-treated vs. cisplatin-treated cells.

Based on these data, it was hypothesized that TM may enhance the antitumor effect of cisplatin in HNSCC cells via an accumulation of cisplatin. It was therefore evaluated whether TM increased the cisplatin accumulation in the HNSCC cell lines, by performing a platinum assay. As revealed in Fig. 2C, the cisplatin concentration in the HSC-3 cells was increased by pretreatment with TM. Next, to analyze the additive antitumor effect of TM and cisplatin against HNSCC cells *in vitro*, a trypan blue staining assay was performed. As revealed in Fig. 2D, TM did not affect the number of viable HSC-3 cells up to 48 h after treatment compared with the control. Cisplatin decreased the proliferation of HSC-3 cells compared with the control group. Notably, the TM+cisplatin dual treatment significant decreased the cell proliferation compared to the single treatment of cisplatin. The antitumor effect of cisplatin consists of inducing apoptosis by DNA cross-linking in cancer cells. The cleavage of caspase-3 is well known as an indicator of apoptosis. The effect of TM on cisplatin-induced cleavage of caspase-3 was therefore assessed by performing a western blot analysis. As revealed in Fig. 3A, TM and cisplatin did not affect the expression of total caspase-3. Furthermore, TM did not directly induce the cleavage of caspase-3. However, TM enhanced the cisplatin-induced cleavage of caspase-3. Moreover, TM enhanced the cispl-atin-induced DNA fragmentation as evaluated by fluorescence

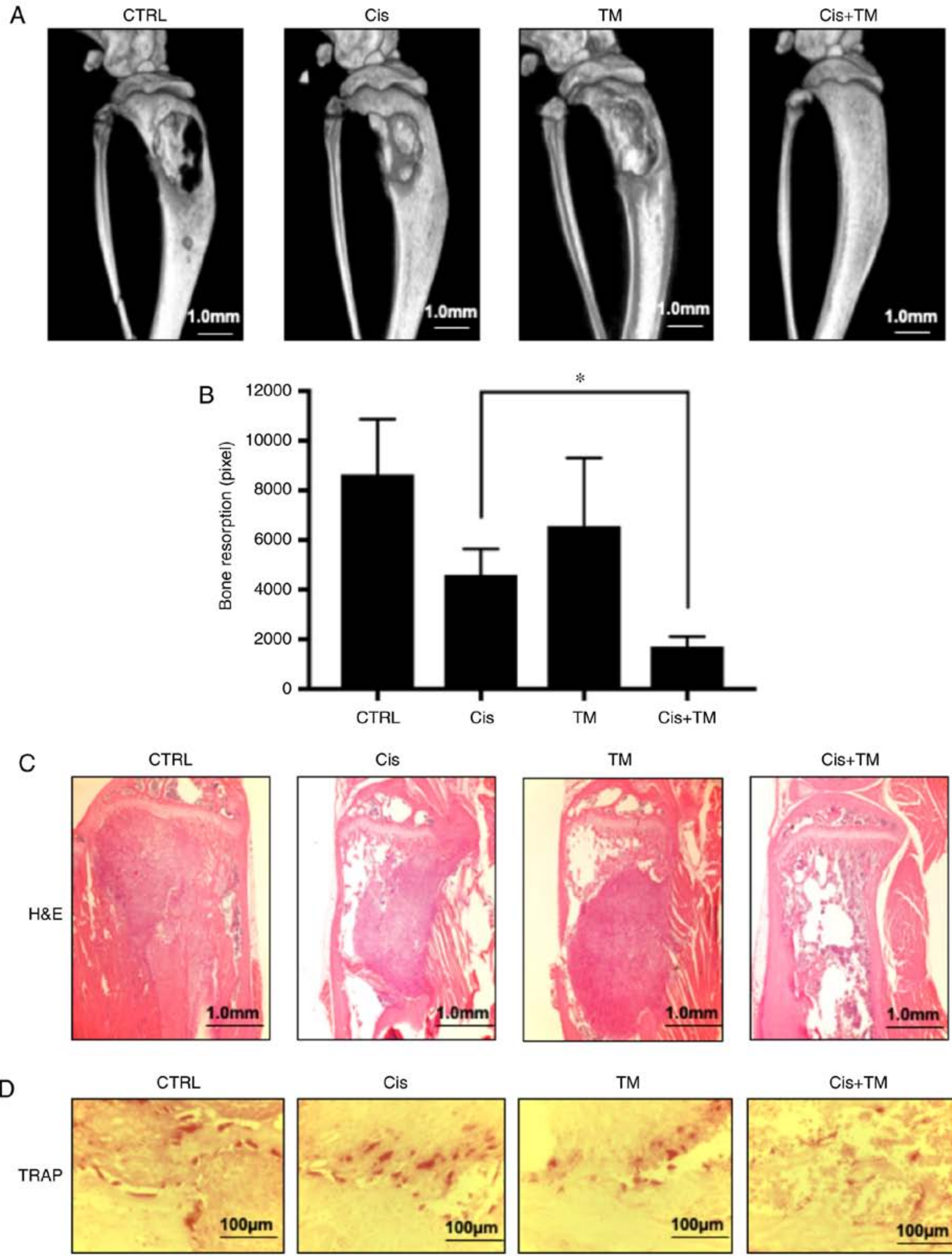

Figure 4. Effects of tetrathiomolybdate (TM) on the mouse model of cancer-associated bone destruction. (A) Representative radiographs and micro-CT of a tibia from a mouse with HSC-3 injected bone invasion after a single treatment of TM or low-dose cisplatin or a combination treatment of TM+low-dose cisplatin. (B) Bone resorption area with a soft X-ray photograph image, assessed with the LuminaVision imaging software program (Mitani Corporation). Data are expressed as the mean ± SD. $^{*}P<0.05$. Cisplatin+ TM-treated vs. cisplatin-treated mice. (C) Histological and histomorphometric analysis of the bone marrow in the bone invasion mouse model. The sections of mouse tibial bone marrow were stained with hematoxylin and eosin. (D) Osteoclast visualized with TRAP stain. TM, tetrathiomolybdate; H&E, hematoxylin and eosin; TRAP, tartrate-resistant acid phosphatase.

tunnel staining assay (Fig. 3B). Flow cytometric analysis was also performed to evaluate the effect of TM on cisplatin-induced HNSCC apoptosis. The plots of Annexin V/FITC-A vs. propidium iodide-A from the gated cells revealed the populations corresponding to viable and non-apoptotic (Annexin V^-PI^-) and (Annexin V^+PI^+, Annexin V^+PI^-) apoptotic cells. TM did not directly induce apoptosis of HSC-3 cells (control 29.8%, TM 37.0%). However, TM significantly enhanced the effect of

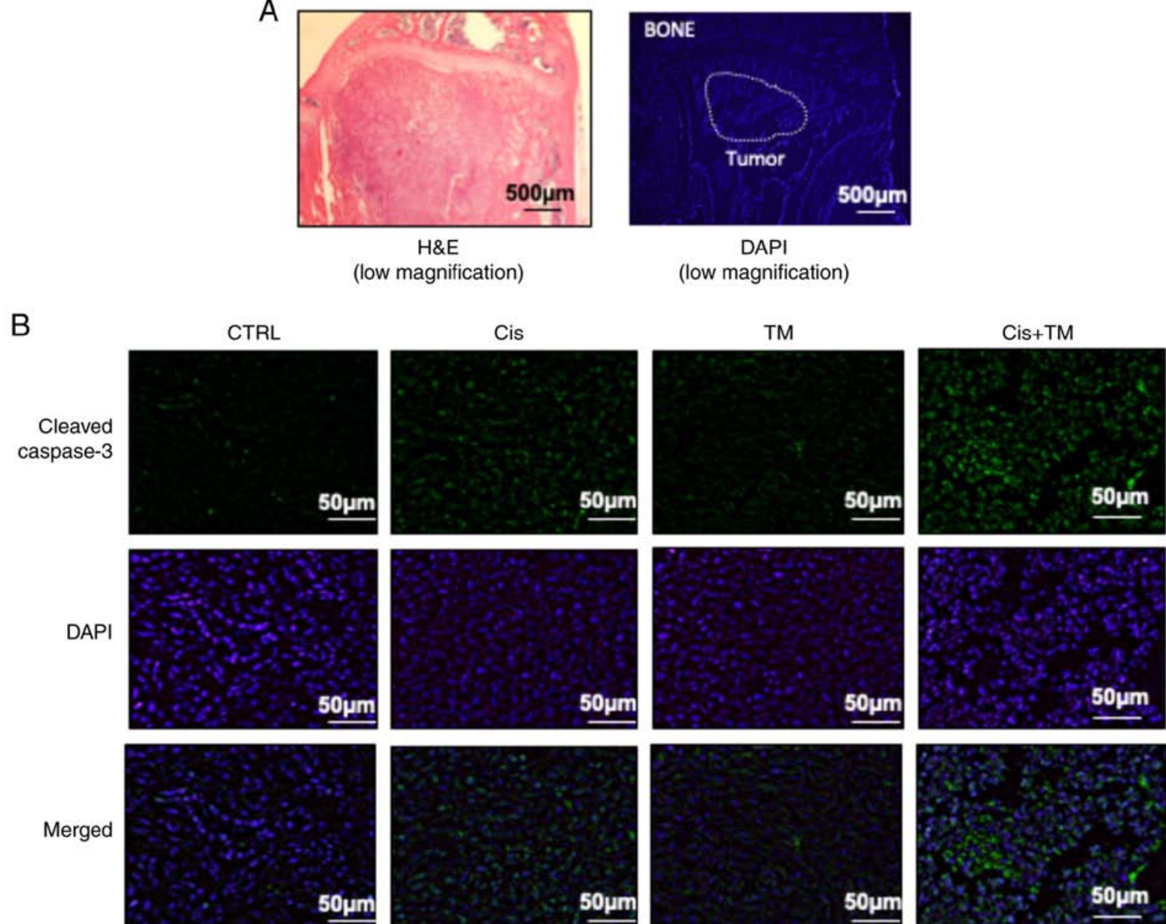

Figure 5. Immunofluorescence analysis of the tumor in the bone marrow in the mouse model of bone invasion. Representative sections of HSC-3 injected mouse tibial bone marrow stained with (A, low magnification) H&E and DAPI; (B, green: High magnification) cleaved caspase-3 and DAPI stained with blue (n=6/group). H&E, hematoxylin and eosin.

cisplatin-induced apoptosis (cisplatin 40.3%, cisplatin with TM 52.2%) (Fig. 3C).

TM enhances the anticancer effect of cisplatin and bone resorption in vivo. To analyze the dual treatment effect of TM+cisplatin *in vivo*, an HNSCC bone invasion mouse model was established using HSC-3 cells. The mice were administered TM (1 mg/kg) and/or a low dose cisplatin (5 mg/kg) 5x/week beginning 7 days after the tumor inoculation, and the tumor volume was measured on day 35. The dual treatment effect of TM+low-dose cisplatin on osteolytic bone destruction induced by oral squamous carcinoma was determined by conducting soft X-ray and micro-CT examinations. As revealed in Fig. 4A and B, the osteolytic lesions were clearly visible in the tibiae of the mice with bone invasion induced by HSC-3 cells treated with the vehicle (control) only. The use of TM or low-dose cisplatin alone tended to suppress the osteolytic bone destruction and tumor burden in the bone marrow. Notably, few destructive lesions were detected in the tibiae of the mice treated with TM + low-dose cisplatin. The total area of radiographic osteolytic lesions from all tibiae was significantly suppressed by the TM+cisplatin treatment compared to treatment with cisplatin treatment ($P<0.05$). Hematoxylin and eosin (H&E) staining revealed tumor growth in bone marrow. The dual treatment with TM+cisplatin markedly decreased the tumor burden (Fig. 4C). Morisawa *et al* demonstrated that TM has an anti-bone resorption effect by suppressing osteoclastogenesis via suppression of RANKL in osteoblasts (17). As revealed in Fig. 4D, treatment with TM decreased the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts in the bone marrow tumor invasion front. The present findings are consistent with those of Morisawa *et al* Imunohistochemical analysis of tumor-inoculated mouse tibia bone marrow was performed. Fig. 5A reveals a representative low magnification image of an HSC-3-inoculated mouse tibial bone marrow section stained with H&E and DAPI. The immunohistochemical analysis revealed a marked increase in the number of cleaved caspase-3-positive tumor cells in HSC-3 tumor sections from the cisplatin-treated mice. Whereas TM alone did not increase the expression of cleaved caspase-3, the dual treatment of TM+cisplatin enhanced the expression of cleaved caspase-3 compared to the treatment with either TM or cisplatin alone (Fig. 5B). The expression of the cell growth indicator Ki-67 was also evaluated in low-dose cisplatin-treated mice. Although TM did not affect Ki-67 expression, the TM+cisplatin dual treatment significantly decreased the expression of Ki-67 (Fig. 6A). TM treatment did not affect the expression of IL-6 which is an important

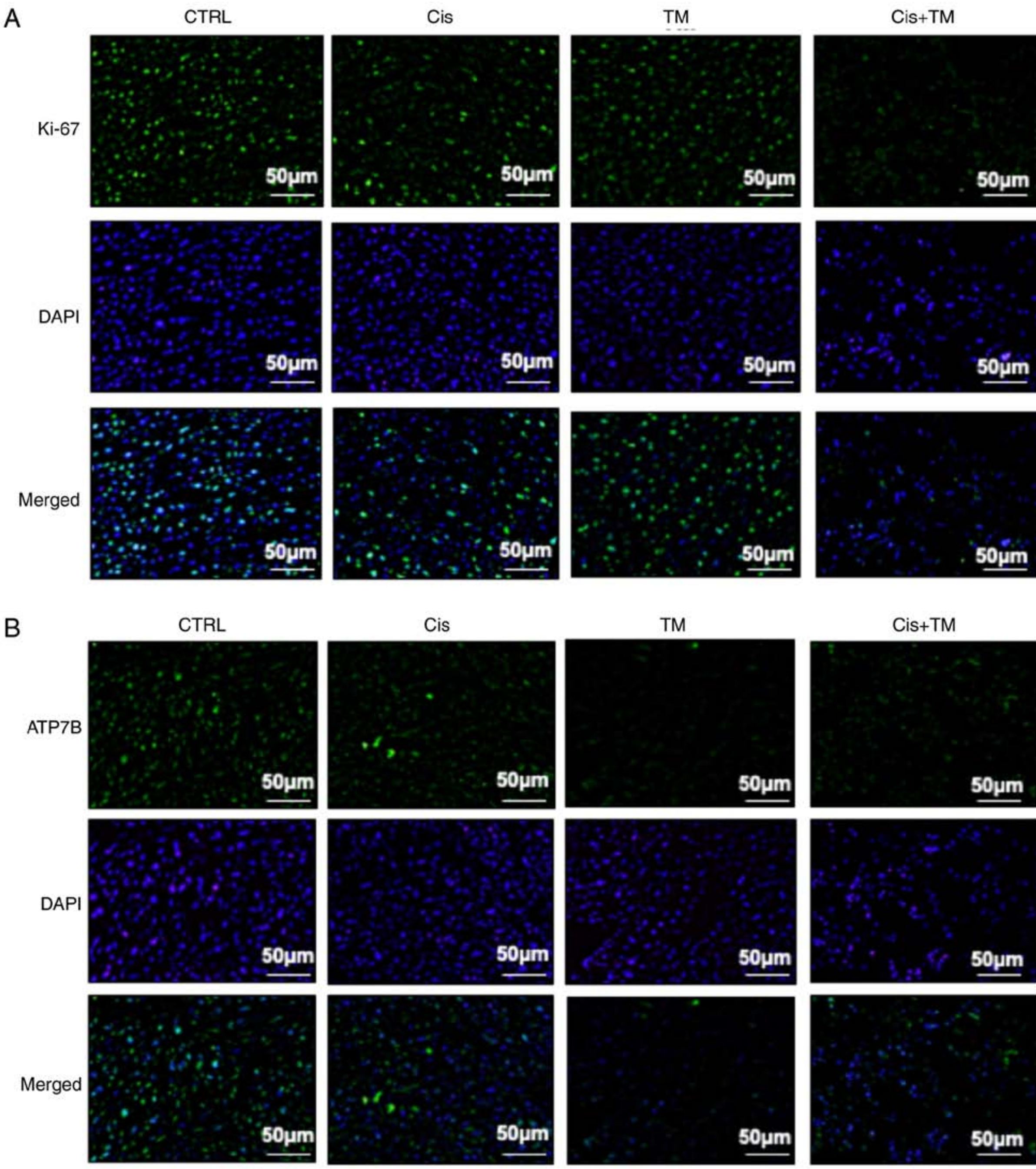

Figure 6. Immunofluorescence analysis of the tumor in the bone marrow in the mouse model of bone invasion. (A, green: High magnification) KI-67, (B, green: High magnification) ATP7B and DAPI stained with blue (n=6/group). ATP7B, ATPase copper transporting beta.

bone remodeling factor (Fig. S2). Collectively these results indicated that TM enhanced the antitumor effect of cisplatin. Finally, immunohistochemical evaluation was conducted to determine the effect of TM on the *in vivo* expression of the copper transporter ATP7B. Notably, TM decreased the expression of ATP7B compared with the control group. These data indicated that TM enhanced the antitumor effect of cisplatin via an accumulation of cisplatin in the cancer cells (Fig. 6B).

TM overcomes cisplatin resistance. The accumulation of cisplatin is decreased in most of the available cisplatin-resistant cell lines, and an active efflux system for cisplatin exists in some of these cell lines (4-12). The results of the present investigations indicated that TM treatment may overcome cisplatin resistance. Mese *et al* established the cisplatin-resistant human skin squamous cell carcinoma cell line A431 (18). This cell line (A431-CDDP-R) was used to evaluate the ability of TM treatment to overcome cisplatin resistance. Notably, the parental A431 cells only slightly expressed ATP7B. In contrast, the A431/CDDP-R cells strongly expressed ATP7B (Fig. 7A). To assess the ability of TM treatment to overcome cisplatin resistance in A431/CDDP-R cells, the cells were pretreated with TM for 24 h. The cells were then treated with cisplatin for 24 h. A western blot analysis revealed that the TM treatment

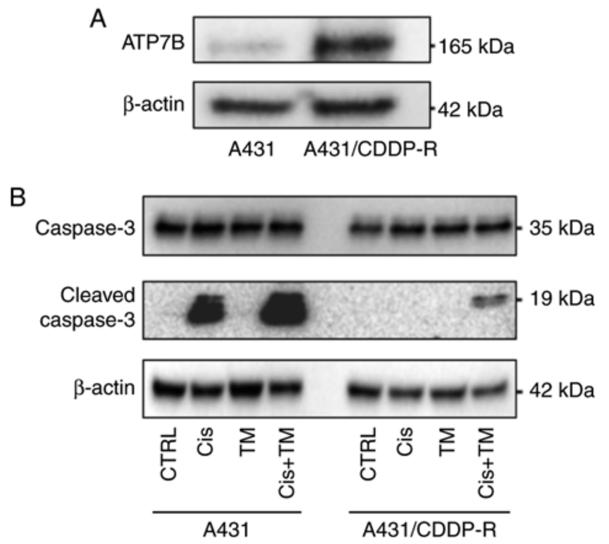

Figure 7. The role of ATP7B in cisplatin-resistant cancer cells. (A) The expression of ATP7B in the parental and cisplatin-resistant A431 cancer cells (A431/CDDP-R) was evaluated by a western blot analysis. (B) A431 and A431/CDDP-R cells were incubated with cisplatin with or without TM. Cleaved caspase-3 was evaluated with western blotting. TM, tetrathiomolybdate; ATP7B, ATPase copper transporting beta; CDDP, *cis*-dichloro-diamine-platinum-resistant.

slightly enhanced the cisplatin-induced cleaved caspase-3 expression in parental A431 cells. Treatment with cisplatin alone did not induce cell apoptosis in A431/CDDP-R cells. Notably, pretreatment with TM strongly enhanced the apoptosis effect of cisplatin in A431/CDDP-R cells (Fig. 7B).

Discussion

Copper chelators have been reported to inhibit cancer cell growth *in vitro* and *in vivo* (21-23). Copper metabolism is critical for cell proliferation, and it is strictly regulated by the copper transporters Crt1, ATP7A, and ATP7B. ATP7B is expressed in mitochondria and excretes copper from the cytoplasm to the extracellular space (15). Cisplatin is the most frequently used platinum-based alkylating agent for several cancers. Cisplatin binds to DNA and causes intra-strand crosslinking, leading to apoptosis (9). Cisplatin is transported into cancer cells via the copper transporter Ctr1 (24). Some clinical studies have indicated that the CTR1 expression in tumors was correlated with the therapeutic efficacy of platinum drugs (25,26). It was also indicated that high extracellular copper levels suppressed CTR1 expression, thereby preventing excess copper influx (27). However, the role of ATP7B in cancer cells remains unknown. The present findings are the first, to the best of our knowledge, to demonstrate that the copper chelator TM enhanced the efficacy of cisplatin in HNSCC via a decrease in the expression of ATP7B. However, the detailed mechanism underlying the decrease in ATP7B expression remains unclear. A recent study indicated that TM induces dimerization of ATP7B, leading to loss of the copper efflux transporter function (28). Furthermore, the copper chelator decreased the expression of ATP7B in liver cancer cell lines (14). This mechanism may be associated with our results. The present experiments revealed that tissues from patients with HNSCC expressed high levels of ATP7B compared to normal oral tissue (Fig. 1). The expression of ATP7B in several HNSCC cell lines was thus evaluated. In addition, since it has been hypothesized that the copper concentration in the extracellular space mediates the expression of copper transporter proteins (26), the effect of the copper chelator TM was investigated on the expression of the copper efflux transporter ATP7B in HNSCC cell lines. The present findings are the first to demonstrate that the chelation of copper ions by TM inhibited the ATP7B expression in HSC-3 HNSCC cells. Thus, it was surmised that this effect was due to the maintenance of the copper metabolism of cancer cells.

Based on our aforementioned results, it was hypothesized that TM inhibited the cisplatin efflux from cancer cells. Then, it was evaluated whether TM accelerates the apoptotic effect of cisplatin, and it was observed that TM increased the intracellular cisplatin concentration in HNSCC, resulting in an inhibition of the cell proliferation *in vitro*. These results indicate that the copper efflux transporter was a critical mediator of cisplatin efficacy against cancer cells. The present experiments also revealed that TM did not affect the proliferation of HSC-3 HNSCC cells. We reported previously that TM did not inhibit the growth of fibroblasts, osteocytes, osteoblasts, or T cells, which is consistent with other studies (17,29). We also reported the molecular mechanism of TM in osteoclastogenesis in bone marrow (17). TM decreased the activation of LOX and the RANKL expression in osteoblasts, resulting in a decrease in osteoclast formation in the HNSCC-induced bone resorption area.

HNSCC frequently invades facial bone, which is a source of growth factors for cancer cells (30). To assess the clinical synergistic effects of copper-lowering agents and platinum agents, TM and low-dose cisplatin was administered in a mouse model of bone-destructive HNSCC, and the results indicated that treatment with either low-dose cisplatin or TM alone partially reduced the tumor growth in bone (Fig. 4A-C). Notably, the combination treatment of cisplatin+TM significantly decreased the tumor growth and bone resorption, Ki-67 expression and cleaved caspase-3 expression compared to the single treatment with either agent (Figs. 5 and 6). This additive effect was due to the suppression of HNSCC cell proliferation via an accumulation of cisplatin in HNSCC cells and to osteoclast formation by TM (Fig. 4D).

In a clinical setting, the efficacy of cisplatin-based chemotherapy against cancers is limited by the occurrence of innate and acquired drug resistance, and a recent study revealed that the mechanism of cisplatin resistance is based on the copper transporter regulation in cancer cells (31). In the present study, the administration of cisplatin increased the level of the cisplatin efflux transporter ATP7B. We hypothesized that cancer cells increase the expression of ATP7B to escape from cisplatin accumulation and cell death. To evaluate the mechanism of the function of ATP7B in cisplatin resistance, (epidermoid carcinoma) A431 cells were used. Mese *et al* established cisplatin-resistant A431 cells (18,19). The expression of ATP7B in parental A431 and cisplatin-resistant A431 (A431 CDDP-R) cells was evaluated, and notably, the ATP7B expression in the A431CDDP-R cells was markedly increased compared to the expression in the parental A431 cells. It was speculated that the cisplatin-resistant A431 cells discharged cisplatin via an increase in ATP7B expression. The present experiments demonstrated that TM enhanced the caspase-3 cleavage by cisplatin. These

results indicated that the mechanism of cisplatin resistance in A431 CDDP-R cells is attributable to an acceleration of the efflux of cisplatin from cells. However, the present data revealed one possibility that ATP7B expression is associated to cisplatin resistance. ATP7B knockdown or a knock-out HNSCC cell line must be established to demonstrate this hypothesis in a future experiment.

In summary, to the best of our knowledge, the present study is the first to reveal that a copper-lowering agent could be an adjuvant to therapy with platinum agents against HNSCC, and the present findings strongly indicate that TM with cisplatin may be an effective approach for treating advanced HNSCC. A clinical study conducted at the MD Anderson Cancer Center revealed that copper-lowering agents have the potential to overcome cisplatin resistance in ovarian cancer patients by regulating the copper transporter hCtr1 (32). The present *in vitro* and *in vivo* results strongly support the notion that copper-lowering agents including TM could be a clinically effective breakthrough for overcoming cisplatin resistance. A copper-lowering agent could be an adjuvant to therapy with platinum agents against HNSCC, and the present findings strongly suggest that TM with cisplatin may be an effective approach to treat advanced HNSCC.

Acknowledgements

Not applicable.

Funding

The present research was funded by a Grant-in-Aid for Young Scientists (JSPS KAKENHI grant no. 18K17225) to TO and the Grant-in-Aid for Scientific Research (B) (JSPS KAKENHI grant no. 17H04405) to AS from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

TO conceived and designed the experiments. SR, TO, TS and KH performed the experiments. TO, SR, KK, SI, NMMH, AS analyzed and interpreted the data. SR, TO, KH, SI, YK, KA, NTTH performed the data acquisition. TO wrote the paper. KK, TS, SI, NMMH and AS revised/reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All of the animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the Okayama University Graduate School of Medicine and Dentistry (approval no. OKU-2018663).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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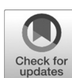
Published OnlineFirst March 1, 2019; DOI: 10.1158/1535-7163.MCT-18-0667

Small Molecule Therapeutics

Molecular Cancer Therapeutics

Inhibition of Copper Transport Induces Apoptosis in Triple-Negative Breast Cancer Cells and Suppresses Tumor Angiogenesis



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Abstract

Treatment of advanced breast cancer remains challenging. Copper and some of the copper-dependent proteins are emerging therapeutic targets because they are essential for cell proliferation and survival, and have been shown to stimulate angiogenesis and metastasis. Here, we show that DCAC50, a recently developed small-molecule inhibitor of the intracellular copper chaperones, ATOX1 and CCS, reduces cell proliferation and elevates oxidative stress, triggering apoptosis in a panel of triple-negative breast cancer (TNBC) cells. Inhibition of ATOX1 activity with DCAC50 disrupts copper homeostasis, leading to increased copper levels, altered spatial copper redistribution, and accumulation of ATP7B to the cellular perinuclear region. The extent and impact of this disruption to copper homeostasis vary across cell lines and correlate with cellular baseline copper and glutathione levels. Ultimately, treatment with DCAC50 attenuates tumor growth and suppresses angiogenesis in a xenograft mouse model, and prevents endothelial cell network formation *in vitro*. Co-treatment with paclitaxel and DCAC50 enhances cytotoxicity in TNBC and results in favorable dose reduction of both drugs. These data demonstrate that inhibition of intracellular copper transport targets tumor cells and the tumor microenvironment, and is a promising approach to treat breast cancer.

Introduction

Breast cancer is responsible for more than 250,000 new cases and 40,000 deaths among women, yearly, despite continuous efforts to improve treatment (1). Breast cancer is a biologically complex disease in its histology, molecular classification, response to therapy and mortality rates (2, 3). Five intrinsic molecular subtypes (luminal A, luminal B, HER2-enriched, claudin-low, and basal-like) have been identified using comprehensive gene expression analysis of human breast cancer tissue, cell lines and mouse models (2, 3). Triple-negative breast cancer (TNBC) lacks estrogen and progesterone receptors, the HER2, and is often associated with basal-like subtype representing a complex, clinically aggressive form of the disease (4). Although 30% to 40% of patients with early-stage TNBC benefit from treatment with anthracycline and taxane-based chemotherapy, TNBC is difficult to control if it becomes resistant to treatment and spreads to distant organ sites (5–7). No specific chemotherapy agents are able to cease metastatic spread, and most patients with TNBC die from advanced disease within 20 months post progression (8). A lack of molecular targets, the adaptive behavior of cancer cells and the microenvironment contributing to tumor progression are barriers to successful therapy. To improve patient outcomes, novel treatment approaches targeting intracellular pathways and pathways involved in cross-talk between cancer cells and the tumor microenvironment are critical.

Copper and copper-dependent proteins are emerging therapeutic targets due to their involvement in cell proliferation, survival, angiogenesis, and metastasis (9, 10). Elevated levels of copper in blood and tumor tissue of patients with cancer are correlated with disease progression (11). Copper-dependent superoxide dismutase (SOD1) is an important modulator of oxidative stress in cancer cells (12, 13), and the lysyl oxidase family of proteins require copper to stabilize the extracellular matrix, which contributes to the formation of a pre-metastatic niche (14, 15). Cells maintain a network of proteins (CTR1, ATP7A, ATP7B) that shuttle copper across membranes to regulate copper homeostasis to prevent damage from the redox-active metal, and deliver copper to secreted copper-dependent proteins (16). Intracellular transport of copper is mediated by chaperone proteins, ATOX1 and CCS, that supply copper to the copper-dependent ATP7A and ATP7B (Cu-ATPases), and to SOD1, respectively (17). CCS is also required for copper-mediated activation of HIF-1α to promote VEGF expression (18). ATOX1 has a possible role in metastasis: It promotes

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi: 10.1158/1535-7163.MCT-18-0667

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Published OnlineFirst March 1, 2019; DOI: 10.1158/1535-7163.MCT-18-0667

inflammatory neovascularization (19), wound closure (20), and breast cancer cell migration (21). Altogether, this evidence demonstrates that copper chaperone proteins, ATOX1 and CCS, are attractive targets for anti-cancer therapy.

Current approaches to targeting copper homeostasis include the inhibition of copper-dependent enzymes by global copper chelation, and the use of copper ionophores to elevate or redistribute copper and overwhelm the antioxidant capacity of cancer cells (22). Recently, the chelator tetrathiomolybdate (TM) was evaluated in a Phase II clinical trial for patients with breast cancer with high risk of recurrence. TM suppresses angiogenesis in preclinical models (23), with its activity ascribed to the inhibition of SOD1 (13, 24), LOX (25) and NF-kβ (23). TM treatment reduced serum copper levels and improved survival of patients with breast cancer, including patients with TNBC, demonstrating proof of principle for this approach (26). In a preclinical model, copper depletion with TM did not affect tumor growth, although it did reduce lung metastases. Despite the promising results of this trial, TM is insufficient to reduce the tumor burden in highly vascularized tumors, and may not inhibit tumor progression once an angiogenic switch has occurred (27).

An alternative approach to targeting cellular copper homeostasis is the inhibition of copper chaperones to impede copper transfer to copper-dependent enzymes and disrupt copper homeostasis. DCAC50 is a small molecule designed to inhibit intracellular copper transport by blocking the highly similar copper transfer interfaces of both ATOX1 and CCS, which prevents the protein–protein interactions necessary for copper transfer to the Cu-ATPases and SOD1, respectively (28). In leukemia and lung cancer cell lines DCAC50 reduced cell proliferation and tumor growth in xenograft mouse models without significant side effects (28).

Given the promising results of DCAC50 anticancer activity and the benefit of copper depletion in patients with breast cancer, we studied the impact of the inhibition of copper chaperones in a broad panel of cell lines of the most aggressive breast cancer phenotype, TNBC. We describe the effects of DCAC50 on the proliferation, viability, copper homeostasis, and redox status of TNBC cells. We also investigate the ability of DCAC50 to inhibit tumor growth and angiogenesis in breast cancer xenograft mouse models, and test its activity in combination with paclitaxel. This work illustrates the potential of copper transporters as targets for breast cancer treatment, shows the impact of the intracellular inhibition of copper homeostasis in TNBC, and investigates the mechanism of action.

Materials and Methods

Chemicals

DCAC50 was synthesized as previously reported (28) and was characterized by NMR and UV/Vis spectroscopy. Binding to ATOX1 was confirmed by fluorescence assay (28). DCAC50, ammonium tetrathiomolybdate (TM, 99.97%) and paclitaxel (Sigma) stocks were prepared in DMSO. L-buthionine-sulfoximine (BSO, 97%, Sigma) was prepared in water.

Cell culture

Breast cancer cell lines were obtained from the ATCC and were cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotic–antimycotic solution (Gibco). SUM149 cells were a gift from Dr. Perou (UNC, Chapel Hill) and were cultured in HuMEC with 5% FBS (Gibco). HMEC and HUVEC, were purchased from Lonza and cultured in MEGM or EGM-2 medium (Lonza). Cell lines were grown at 37°C and 5% carbon dioxide for less than 20 passages after thawing to conduct described experiments, tested negative for *Mycoplasma* contamination and validated for species and unique DNA profile by the provider using short tandem repeat analysis. All assays were performed according to the manufacturer's instructions and after allowing cells to adhere overnight.

Western blot

Cell lysates were collected in RIPA buffer (Sigma), sonicated (3×10 seconds) and centrifuged (10 minutes at $14{,}000 \times$ rcf). Protein concentration was determined using Thermo Scientific PierceBCA Protein Assay. Equal amount of total protein for each lysate was analyzed by SDS-PAGE and transferred to polyvinylidene difluoride membrane (ImmobilonFL, Merk Millipore). The loading controls were from the same experimental samples. All proteins were analyzed on the same blot. The membrane was scanned using Odyssey IR Scanner. Images were analyzed using Image Studio Light (LI-COR).

Cell proliferation

About 5,000 to 10,000 cells per well were plated into a 96-well plate for each drug concentration ($n = 3$–6). DCAC50, TM, BSO, and paclitaxel solutions were prepared as serial dilutions with total DMSO matched to vehicle control. Constant ratios of DCAC50 and paclitaxel were used when treated in combination. Solutions (2X concentrates) were added to the cells in media for 72 hours at 37°C and CellTiter 96-AQueous One Solution Assay (Promega) was performed. Data were fitted into a variable slope (four-parameter) model using GraphPad Prism. For paclitaxel and DCAC50 combination, fraction of viable cells was used to calculate combination index (CI) and dose-reduction index (DRI) using CompuSyn software (29). Alternatively, cell proliferation was assessed with The IncuCyte S3 Live-Cell Analysis System (Essen Instruments) using quantitative metrics derived after phase-contrast image acquisition, and presented as the percentage of confluence.

Apoptosis

Cells were plated and treated per the proliferation assay and assayed with Caspase-Glo-3/7 assay (Promega). For the AnnexinV and propidium iodide assay, the Dead Cell Apoptosis Kit (Invitrogen) was used. Cells were plated in 6-well plates, treated, collected by trypsinization, stained and analyzed with LSR-Fortessa 4–15 (Bechton and Dickenson) instrument and FlowJo software.

Inductively coupled plasma mass spectrometry

Cells were plated in 6-well plates, treated, trypsinized, washed with PBS, collected in acid-washed tubes, digested in trace metal grade concentrated nitric acid (overnight at 37°C, with shaking) and diluted to 2% nitric acid with ultrapure water. ^{65}Cu content was determined by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700x) with a germanium internal standard.

X-ray fluorescence microscopy (XFM)

A total of 10^6 cells/well were seeded in 6-well plates containing a silicon nitride membrane (1.5×1.5 mm $\times$ 500 nm, Silson, UK),

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serum-starved for 24 hours and full-serum media were added one hour before treatment. Cells were washed with PBS, fixed with paraformaldehyde (3.7%, pH 7.4, 20 minutes, room temperature), washed with trace metal grade ammonium acetate (100 mmol/L), then ultrapure water, and air-dried.

XFM was conducted at beamline 2-ID-D at the Advanced Photon Source. Samples were irradiated with a 10.1 keV beam focused through a zone plate and order-sorting aperture. X-ray fluorescence maps were generated by "fly scanning" (1 second dwell, 0.5-μm steps). Fluorescent photons were collected by a Ge detector (UltraLEGe, Canberra) at 90° to the incident beam.

Analysis was performed using MAPS software (30) as described elsewhere (31). The nuclear region of the cell was defined as the region of high P and Zn content.

Immunofluorescence staining and cell imaging

Cells were plated into 8-well glass chamber slides, coated with poly-L-lysine (Sigma), treated and fixed as for XFM microscopy, washed with PBS, permeabilized (0.1% Triton-X100; Sigma) and blocked with Protein Block Goat Serum (Biogenex Laboratories), stained with primary, then secondary antibodies. Cells were analyzed using Olympus IX-83 microscope controlled by Metamorph software, Olympus UPlan-SAPO 40 × (oil, N.A. 1.25) objective, Xcite 120 LED (Lumen Dynamics) light source, Image EMX2 CCD (Hamamatsu) camera, and ImageJ software.

ROS assay

Cells were plated into 10-cm^2 dishes, treated, collected by trypsinization, stained with 10 μmol/L DCFDA reagent (30 minutes, 37°C) and washed with PBS. FACS analysis was performed using an LSR-Fortessa 4–15 (Becton and Dickenson) instrument and FlowJo software.

GSH:GSSG assay

A total of 20,000 cells were seeded in 96-well plates. The luminescence-based GSH/GSSG-Glo Assay (Promega) was used to determine the GSH:GSSG ratio or to calculate total GSH levels.

SOD activity

Cells were seeded in 10-cm^2 dishes. For total SOD activity, treated cells were collected by trypsinization, washed with PBS. The cell pellet was lysed in lysis buffer (Trevigen; 30 minutes) and the supernatant was collected after centrifugation (10 minutes, 4°C, 10,000 × *g*). Total SOD activity was determined using the SOD activity assay (Sigma). For SOD1 activity, treated cells were washed, scraped into PBS and centrifuged. The pellet was suspended in three volumes of 50 mmol/L phosphate buffer (pH 7.6) and sonicated. Cell lysates were separated by native PAGE at 4°C. The gel was stained as in (32) with modifications (see Supplementary Methods).

Endothelial network formation

HuVEC (20,000 cells/cm^2) were plated into 48-well plates coated with phenol red-free Matrigel (Corning, 30 minutes, 37°C), in presence of DCAC50, TM or DMSO. Cell network-formation was imaged after 16 to 18 hours incubation at 37°C, using an Olympus IX81 inverted microscope with the Olympus Zero Drift Correction auto re-focusing system (Olympus) with a Hamamatsu Orca Flash 4.0 sCMOS camera (Hamamatsu Photonics) run by Slidebook 5.0 software (Intelligent Imaging Innovations), using a ×10 objective with adapter and Angiogenesis Analyzer for ImageJ (NIH; ref. 33).

LOX assay

Cells were seeded in 24-well plates and treated in phenol red-free media. Media was collected and centrifuged to remove debris. Equal volumes of sample and reagent from Amplite Fluorimetric Lysyl Oxidase Assay Kit (AAT Bioquest) were mixed. The reactions were incubated in the dark (37°C) and fluorescence (540 nm excitation/590 nm emission) was measured after 60 minutes.

Efficacy study and animal handling

All animals were humanely handled and monitored for health conditions according to the Institutional Animal Care and Use Committee (IACUC) approved protocols. Eight-week-old female *Foxn1nu/nu* (Harlan) mice were anesthetized via inhalation with 2% vaporized isoflurane and were unilaterally injected with 4 × 10^6 MDA-MB-468 or 3 × 10^6 MDA-MB-231 cells (100 μL, 50% Matrigel) into the fourth inguinal mammary gland at the base of the nipple. Seven days post cellular implantation, animals were randomly and blindly assigned to treatment groups (6–8 per group), and treated with intraperitoneal injections of 50 mg/kg/d DCAC50 or DMSO (vehicle control). Tumor growth and weight of the animals were monitored twice weekly. Tumor measurements were performed using calipers to calculate tumor volume using formula 1/2(Length × Width^2). The assessment was blind to treatment groups and was performed by the same person throughout the study.

Immunofluorescence staining of tumors

Frozen tumors were thawed in RPMI-1640 media containing 10 mg/mL BSA on ice, and prepared as described previously (34). Leica TCS SP8 confocal laser scanning microscope, white light laser, Leica HCX PL APO 10X/0.4 NA dry objective (2.2 mm working distance), and a SuperZ galvometric scanning stage were used for imaging.

Results

Protein levels of copper chaperones ATOX1 and CCS are elevated in breast cancer cell lines

Elevated copper levels in serum and tissues of patients with cancer (11) are a sign of altered copper homeostasis. Elevated mRNA levels of copper transport proteins have been observed in human cancers (28, 35). In breast cancer tissues *ATOX1* mRNA levels were upregulated relative to normal breast tissue, but no significant change was observed for *CCS* (35). Because mRNA levels do not always correlate with protein abundance, we studied the protein expression of ATOX1 and CCS in a panel of breast cancer cell lines. We included luminal and HER2-positive subtypes, as well as basal-like and claudin-low TNBC subtypes, and compared protein levels with normal human mammary epithelial cells (HMEC). Protein expression levels of ATOX1 were elevated in all breast cancer cells lines whereas CCS expression levels were more variable (Fig. 1A and B; Supplementary Fig. S1). The highest levels of ATOX1 were observed in luminal MCF7 (5.4-fold), claudin-low, MDA-MB-157 (6.3-fold) and basal-like MDA-MB-468 (2.1-fold) cell lines. CCS protein levels were most elevated in luminal MCF7 (4.8-fold), claudin-low HCC1395 (4.1-fold) and basal-like HCC1806 (2.0-fold) cell lines. Levels of both proteins, ATOX1 and CCS, were elevated in

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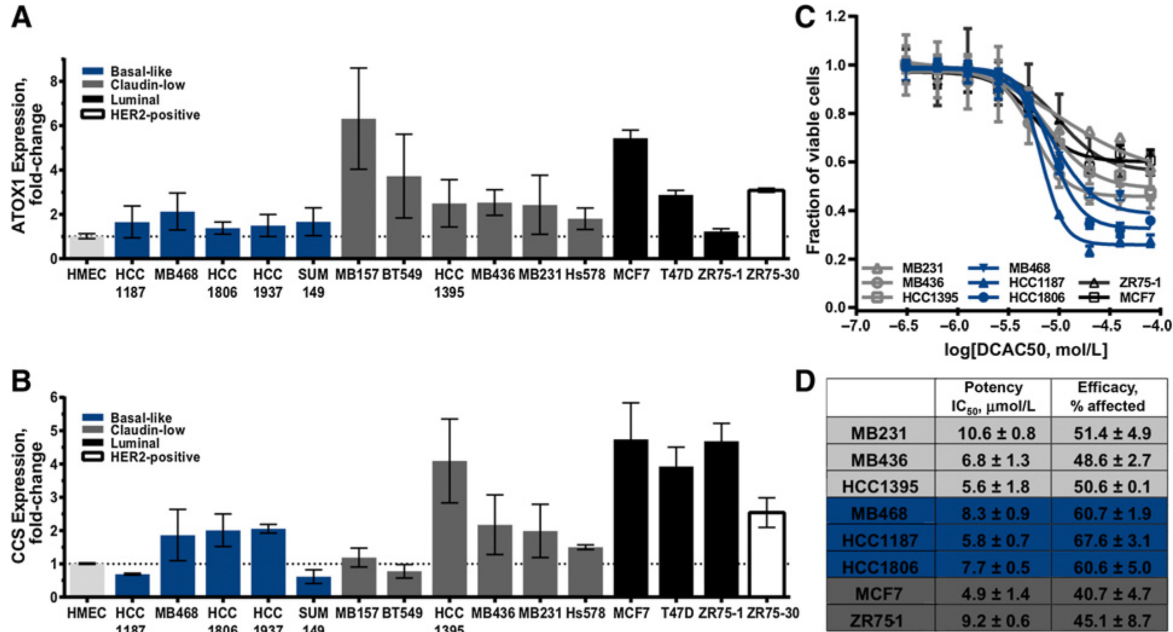

	Potency IC_{50}, µmol/L	Efficacy, % affected
MB231	10.6 ± 0.8	51.4 ± 4.9
MB436	6.8 ± 1.3	48.6 ± 2.7
HCC1395	5.6 ± 1.8	50.6 ± 0.1
MB468	8.3 ± 0.9	60.7 ± 1.9
HCC1187	5.8 ± 0.7	67.6 ± 3.1
HCC1806	7.7 ± 0.5	60.6 ± 5.0
MCF7	4.9 ± 1.4	40.7 ± 4.7
ZR751	9.2 ± 0.6	45.1 ± 8.7

Figure 1.

A and **B**, Protein levels of ATOX1 and CCS in breast cancer cell lines in comparison with normal cells (HMEC). Relative protein expression is presented as mean ± SD in breast cancer cells compared with normal cells (HMEC) and was determined using at least two biological and two technical replicates (see Supplementary Fig. S1 for representatives of full blots). Equal amount of total protein was loaded for each sample. **C,** Representative dose–response profiles of DCAC50 in breast cancer cell lines. Fraction of viable cells was determined after cell treatment with escalating doses of DCAC50 (see cell proliferation assay). Data were fitted into variable slope (four-parameter) model using GraphPad Prism to estimate the IC_{50} value. **D,** Efficacy and potency of DCAC50. The IC_{50} dose and maximum percentage of affected cells were calculated from at least three independent experiments. Data are presented as mean ± SD.

luminal (MCF7, T47D, ZR75-1), HER-2-positive (ZR75-30), basal-like (HCC1937, HCC1806, MDA-MB-468) and claudin-low (MDA-MB-231, MDA-MB-436, HCC1395) cell lines. Because DCAC50 targets the activities of ATOX1 and CCS, we continued the study with representative cell lines of different subtypes showing variable elevated levels of these proteins.

DCAC50 reduces cell proliferation and induces apoptosis in TNBC

The cytotoxicities of the intracellular copper transport inhibitor, DCAC50, and of the copper chelator, TM, previously shown to inhibit copper-dependent enzymes in breast cancer, were evaluated using a dose-response profile in breast cancer cells. To evaluate apoptosis, we measured the activity of caspase-3/7, and estimated percentages of apoptotic cells determined by simultaneous staining with propidium iodide and AnnexinV. Treatment with DCAC50 reduced cell proliferation in a dose-dependent manner in all studied cell lines. Calculated IC_{50} doses varied from 5 to 10 µmol/L in breast cancer cells, and higher efficacy was observed in basal-like cell lines (Fig. 1C and D). Next, we evaluated the ability of DCAC50 to induce apoptosis in the most aggressive breast cancer subtype, TNBC. Inhibition with DCAC50 resulted in activated caspase-3/7, and significantly increased percentages of apoptotic cells in all cell lines (except MDA-MB-231), but especially in basal-like cell lines (Fig. 2A–C). Copper chelation with TM did not affect cell proliferation in studied cell lines (except for HCC1395, IC_{50} ~60 µmol/L, and HCC1806, IC_{50} ~90 µmol/L), and did not induce apoptosis (Fig. 2A–C; Supplementary Fig. S2A). In HMEC, no significant changes in caspase-3/7 activity were observed after treatment with DCAC50; however, DCAC50 did reduce cell proliferation ($IC_{50} = 3.5 \pm 0.1$ µmol/L, Supplementary Fig. S2B–S2C). Thus, treatment with DCAC50 attenuates cell proliferation and leads to apoptosis-induced cancer cell death.

DCAC50 alters cellular copper homeostasis

Copper chaperone ATOX1, together with Cu-ATPases, are part of the copper export system that helps maintain copper homeostasis. Although targeting copper homeostasis using chelators, such as TM, is expected to reduce cellular copper levels, the inhibition of copper transfer from ATOX1 to Cu-ATPases with DCAC50 is expected to increase cellular copper content. ICP-MS analysis revealed that overall copper content in TM-treated cells is decreased, whereas copper content in DCAC50-treated claudin-low cells is significantly increased, compared with control cells (Fig. 3A; Supplementary Fig. S3A). Surprisingly, only a modest non-significant increase in copper levels was detected in basal-like cells, which are most sensitive to DCAC50. Baseline copper levels were lower in all basal-like cells (<9 pg Cu/µg protein) than in claudin-low cells (>12 pg Cu/µg protein), indicating that basal-like cells may be more sensitive to marginal copper overload.

The inhibition of copper chaperones and changes in cellular copper levels are likely to lead to changes in spatial copper distribution. To better understand the impact of DCAC50 on copper homeostasis in TNBC, XFM imaging was used to determine intracellular copper distribution (Fig. 3B). In DCAC50-

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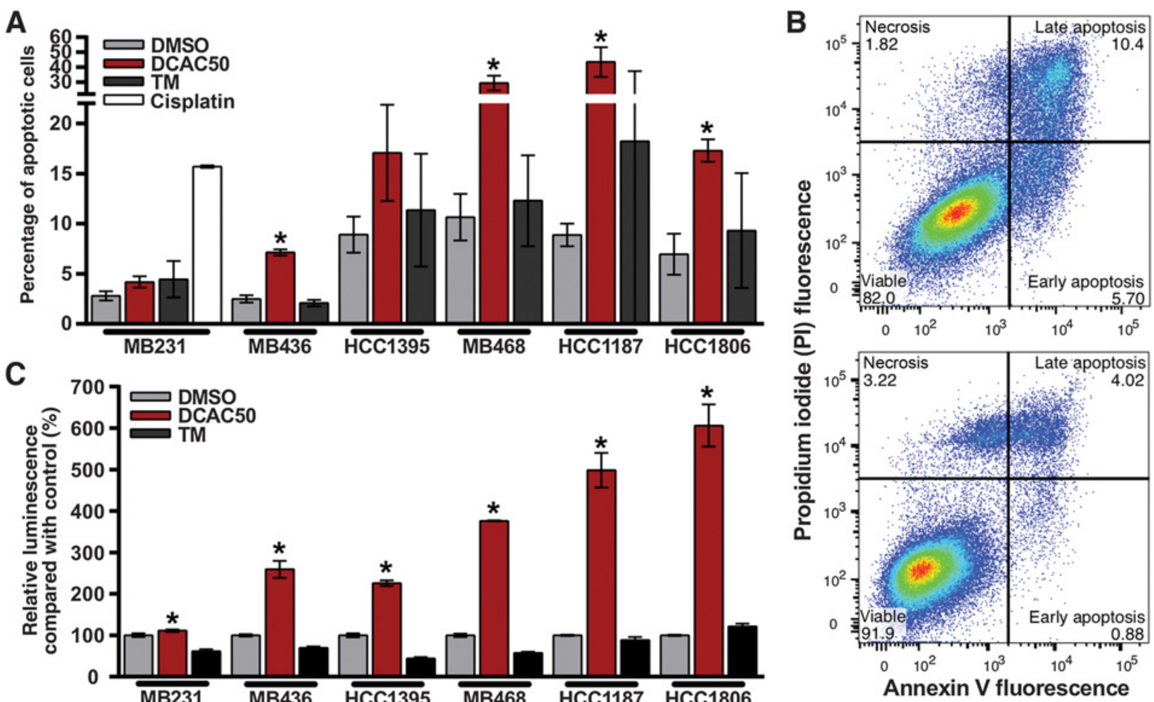

Figure 2.

Effect of DCAC50 and tetrathiomolybdate (TM) on apoptosis in TNBC cells lines. **A,** TNBC cells stained with Annexin V (AnV) and propidium iodide (PI). Percentage of apoptotic cells was calculated as the sum of early apoptotic (AnV^+/PI^-) and late apoptotic (AnV^+/PI^+) cells. Cells were treated for 72 hours with 20 μmol/L DCAC50, 30 μmol/L TM, or 20 μmol/L cisplatin, stained, and analyzed by flow cytometry. Mean ± SD of three biological replicates are reported. *, $P < 0.05$, versus DMSO control, according to the two-tailed Student *t* test. **B,** Flow cytometry analysis of HCC1806 cells treated with DCAC50 (top) or DMSO (bottom) in a representative experiment. **C,** Caspase-3/7 activity assay. The percentage of change of relative luminescence signal normalized to viability obtained from a simultaneous proliferation assay and proportional to caspase-3/7 activity after 72 hours treatment with 20 μmol/L DCAC50 in comparison with DMSO. Mean ± SD of two biological with three technical replicates are reported. *, $P < 0.05$, versus DMSO control, according to the two-tailed Student *t* test.

treated MDA-MB-231 cells, where total intracellular copper level increased with treatment, there was no significant change in the nuclear to cytoplasmic copper concentration ratio compared with control (Fig. 3B). In HCC1395 cells there was a 20% increase in the nuclear to cytoplasmic copper ratio from 1.1 to 1.3 (Supplementary Fig. S3B). In DCAC50-treated MDA-MB-468 cells, despite limited copper accumulation observed by ICP-MS, the ratio of nuclear to cytoplasmic copper concentration compared with control increased 40% from 1.2 to 1.7 (Fig. 3B). Importantly, there is no corresponding change in the localization of iron or zinc across cell lines or treatments (Supplementary Fig. S3C and S3D), indicating that the copper redistribution is not an artifact of sample preparation nor is it indicative of differences in cell cycle across treatment groups. Thus, inhibition of copper transport with DCAC50 alters copper homeostasis resulting in increased copper levels and/or increased localization of copper to the nucleus. The greatest change in copper distribution with DCAC50 treatment was observed in basal-like MDA-MB-468 cells, which is the most sensitive of the three imaged cell lines to the drug.

Inhibition of ATOX1 and CCS by DCAC50 may in turn affect the level or distribution of copper transport proteins. However, the protein levels of ATP7B, ATOX1, CCS or CTR1 were unchanged after treatment with DCAC50 (Supplementary Fig. S4A). The copper transporter ATP7B is known to translocate between the perinuclear space and the plasma membrane to metallate secreted copper proteins and maintain appropriate cellular copper levels (16). DCAC50 is designed to disrupt copper transfer from ATOX1 to ATP7B (28), and therefore may prevent ATP7B from translocating to the plasma membrane where it facilitates copper export. To test this hypothesis, we performed immunofluorescence staining of ATP7B in MDA-MB-468 cells after treatment with DCAC50. Upon treatment, ATP7B was mostly found in the perinuclear region of MDA-MB-468 cells (Fig. 3C). In contrast, we observed diffuse staining of ATP7B in DMSO-treated cells. Thus, in basal-like MDA-MB-468 cells, DCAC50 disrupts copper homeostasis altering copper distribution and ATP7B localization.

Treatment with DCAC50 results in elevated oxidative stress

To facilitate proliferation and promote survival cancer cells frequently have to adapt to high levels of oxidative stress. Copper is intimately linked with the cellular redox status via its inherent redox activity, SOD1 activity and interactions with GSH. Thus, increased copper levels and the inhibition of copper transfer to SOD1 with DCAC50 are expected to induce oxidative stress in TNBC cells ultimately leading to cellular damage. To determine the impact of DCAC50 on cellular redox status we used a cytosolic sensor of general oxidative stress, DCFDA. Treatment with DCAC50 resulted in higher DCF fluorescence intensity generated by the oxidation of DCFDA. The fluorescence signal was higher

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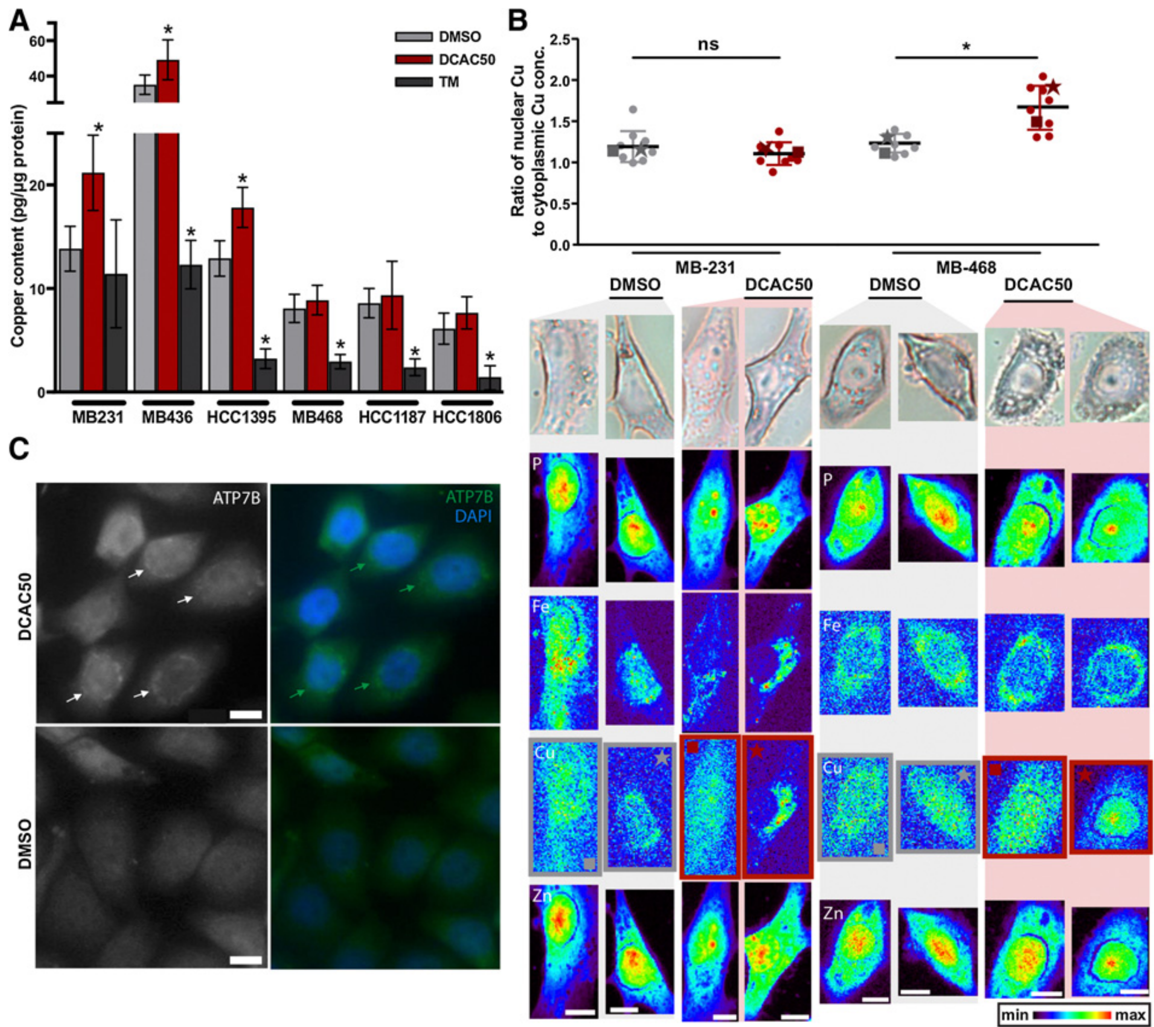

Figure 3.

Cellular copper content and distributions of copper and ATP7B in treated cells. **A,** Copper content was determined by ICP-MS of bulk cell pellets after treatment with DMSO, 20 μmol/L DCAC50, or 30 μmol/L TM for 24 hours and was normalized to protein concentration. Outliers identified by the Grubbs' test were removed. Mean ± SD of four to six biological replicates. *, $P < 0.05$, compared with DMSO control, according to the two-tailed Student *t* test. **B,** Distribution of copper (nuclear vs. cytosolic) in cells treated with DCAC50. The ratio of cytoplasmic to nuclear Cu concentration (top) in MDA-MB-231 and MDA-MB-468 cells treated with DMSO or 20 μmol/L DCAC50 for 24 hours. Visible light microscope images and XFM elemental maps (bottom) of P, Fe, Cu, and Zn are shown for representative cells within each sample, matched to the corresponding data points in the graphs by a square or star. Mean ± SD for $n = 9$–10 cells. *, $P < 0.05$, compared with DMSO control, according to the two-tailed Student *t* test; ns, not significant. A 10-μm scale bar is included in each Zn map, and the relative elemental concentration within each cell is indicated by the intensity scale. **C,** Representative fluorescent images of MDA-MB-468 cells treated with DMSO or 20 μmol/L DCAC50 for 24 hours and stained with DAPI and antibodies against ATP7B. Arrows indicate accumulation of ATP7B in the perinuclear region of cells; scale bar, 10 μm.

after 24 hours treatment with DCAC50 and by 72 hours had increased 2-fold compared with control cells (Fig. 4A and B). Copper chelation by TM had no impact on cellular oxidative stress as measured by DCF fluorescence, except for an increase observed in MDA-MB-231 cells.

Another indicator of the cellular redox environment is the oxidation state of glutathione. Lower ratios of reduced to oxidized glutathione (GSH:GSSG) were observed in all TNBC cells lines after treatment with DCAC50, indicating increased oxidative stress. Interestingly, total GSH levels at baseline were overall higher in claudin-low cells in comparison with basal-like cells, suggesting claudin-low cells possess higher antioxidant capacity and thus, may be less sensitive to the oxidative stress triggered by DCAC50 (Fig. 4C and D; Supplementary Fig. S4B). To test whether exhausting cellular antioxidant capacity by depleting GSH levels augments DCAC50's effects, we evaluated cell proliferation in claudin-low MDA-MB-436 and HCC1395 cells after treatment with DCAC50 in presence of the GSH-synthesis inhibitor, BSO. GSH levels were reduced without affecting cell proliferation after treatment with BSO, and addition of BSO to DCAC50

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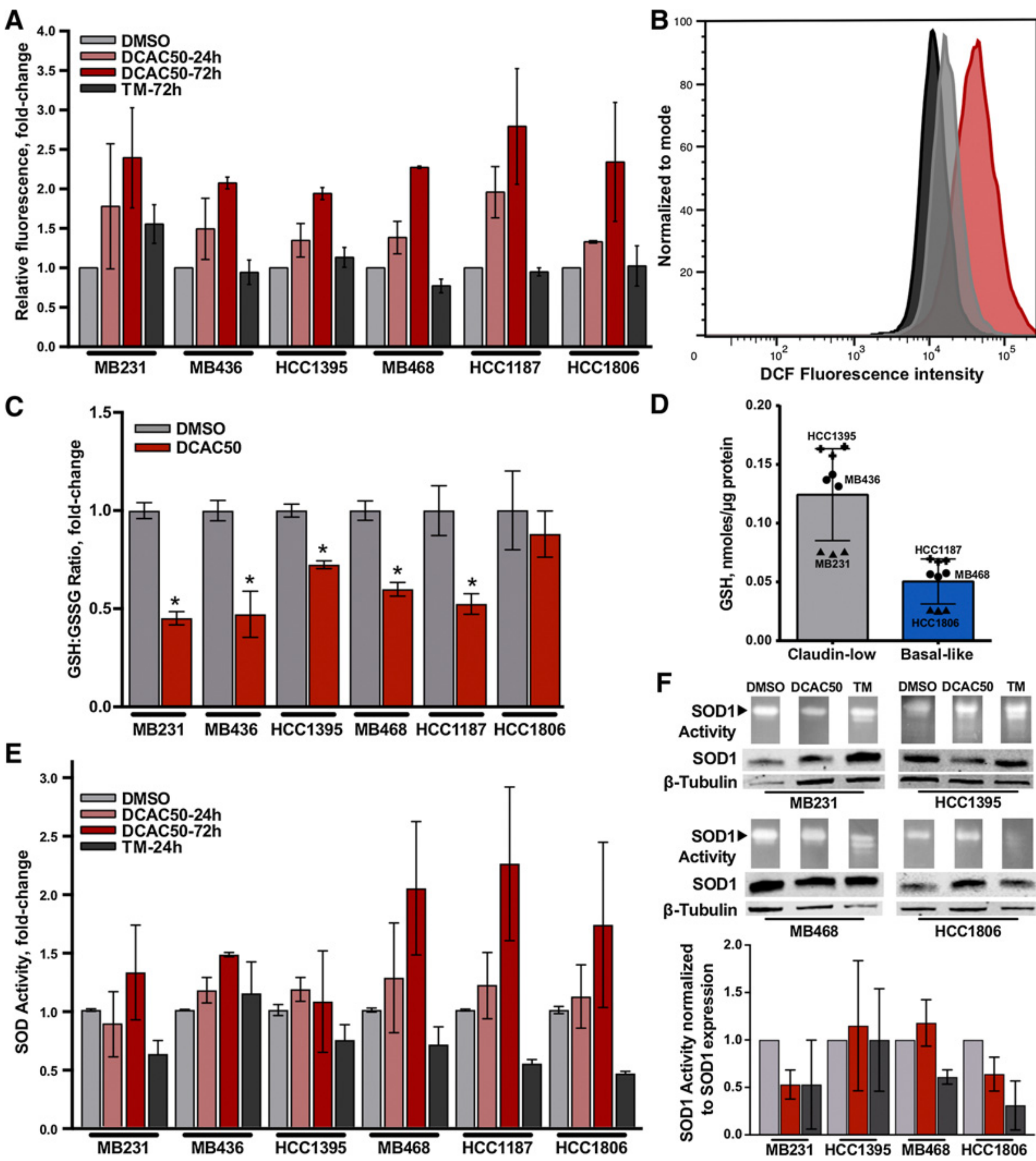

Figure 4.

Effect of DCAC50 on levels of oxidative stress. **A** and **B,** DCF fluorescence in cells treated with DCAC50 or TM. TNBC cells were treated with DMSO, 20 μmol/L DCAC50, or 30 μmol/L TM and stained with DCFDA. Data are presented as (**A**) fold-change in DCF fluorescence intensity (mean ± SD of at least two biological replicates) and (**B**) representative histogram of the flow cytometry analysis of MDA-MB-468 cells. **C,** The oxidation state of glutathione after treatment with DCAC50. GSH and GSSG levels were determined by luminescence-based assay after 24-hour treatment with DMSO or 20 μmol/L DCAC50, and presented as fold-change relative to control, with mean ± SD of three biological replicates. *, $P < 0.05$, compared with DMSO control, according to two-tailed Student *t* test. **D,** Basal glutathione levels in claudin-low and basal-like cell lines presented as mean ± SD of three biological replicates. **E,** Total SOD activity in cells treated with 20 μmol/L DCAC50 or 30 μmol/L TM, normalized to protein content and presented as the fold-change relative to control, with mean ± SD of at least two biological replicates. **F,** Semiquantitative SOD1 activity in cells treated with DMSO, 20 μmol/L DCAC50, or 30 μmol/L TM for 24 hours and normalized to SOD1 expression. Representative results of two biological replicates (see Supplementary Fig. S4 for examples of full gels and blots).

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resulted in significantly reduced cell proliferation compared with DCAC50 alone (Supplementary Fig. S4C and S4D). This finding further supports our hypothesis that lowering cellular antioxidant capacity increases susceptibility to DCAC50.

Copper-dependent SOD1 is a known regulator of cellular redox balance, and DCAC50 has been shown to inhibit SOD1 activity in lung cancer cells, presumably by blocking copper transfer from CCS to SOD1 (28). Control experiments with TM showed reduced total SOD and SOD1 activity with copper chelation (Fig. 4E and F). However, in most TNBC cells total SOD (SOD1 and SOD2) activity increased with DCAC50 treatment after 24 hours and increased further at 72 hours (Fig. 4E), perhaps in response to elevated oxidative stress. Specific SOD1 activity normalized to SOD1 expression decreased in DCAC50-treated MDA-MB-231 and HCC1806 cells (Fig. 4F; Supplementary Fig. S4E–S4G), as expected from the inhibition of copper transfer from CCS to SOD1. However, MDA-MB-468 and HCC1395 cell lines exhibited an increase in SOD1-specific activity, which may be due to the alternative pathways for copper delivery to the enzyme (36).

The increased DCF fluorescence, lower GSH:GSSG ratio and increased total SOD activity are consistent with increased oxidative stress resulting from disruption of copper transport by DCAC50. In contrast, global copper chelation with TM has no impact on DCF fluorescence and generally reduces total SOD and SOD1 activities. Taken together, these results suggest that the general copper dyshomeostasis caused by DCAC50 is a greater contributor to the observed oxidative stress than inhibition of copper transfer from CCS to SOD1.

DCAC50 suppresses angiogenic activity of endothelial cells *in vitro*

Copper has been shown to directly stimulate angiogenesis whereas copper depletion by TM inhibits angiogenesis (23). Considering the recently reported pro-angiogenic activity of ATOX1 in endothelial cells (19), we tested if blocking ATOX1 activity with DCAC50 could suppress *in vitro* network formation in HuVEC cells. DCAC50 reduced the number of nodes, junctions, segments and branches and the length of these elements in a network-formation assay. A smaller effect was observed for TM at the tested dose. The treatment also inhibited extracellular LOX activity, but no dose-dependent effect on cell proliferation or apoptosis was observed with DCAC50 in the HuVEC cells (Fig. 5A–C; Supplementary Fig. S5A–S5B). Thus, in addition to the anticancer effects, DCAC50 may also modulate the microenvironment by blocking angiogenesis, which may prevent vascular remodeling that is required for tumor progression.

DCAC50 inhibits tumor growth and angiogenesis in an MDA-MB-468 xenograft model

Taking into account the promising anticancer activity of DCAC50 *in vitro*, we evaluated the efficacy of copper transport inhibition in xenograft mouse models. In MDA-MB-468 xenografts, treatment with DCAC50 resulted in the inhibition of tumor growth and reduced tumor volumes, in comparison with the control (vehicle) group of mice (Fig. 5D; Supplementary Fig. S6A). Importantly, in addition to the inhibition of tumor growth in MDA-MB-468 xenografts, we observed significantly suppressed angiogenesis, as indicated by the reduced blood vessel area in DCAC50-treated mice, detected by staining with $CD31^+$ and $CD105^+$, vascular and neovascular endothelial cell markers, respectively (Fig. 5E–G). Reduced angiogenesis was also observed in MDA-MB-231 xenografts; although tumor volumes did not change overall at the end of treatment, slower tumor growth in 3 of 8 mice was observed (Supplementary Fig. S6B–S6D). The lower sensitivity of the MDA-MB-231 model to DCAC50 treatment is in concordance with our data observed *in vitro*. Thus, inhibition of intracellular copper transport by DCAC50 can suppress tumorigenesis by targeting both tumor cells and the tumor microenvironment, but its anti-tumor activity varies due to different sensitivities of TNBC cells to the disruption of copper homeostasis.

Inhibition of copper transport with DCAC50 in combination with paclitaxel induces synergistic cytotoxicity

Treatment of patients with TNBC relies heavily on chemotherapy, including anthracyclines, taxanes, platinum agents and their combinations. The combination of chemotherapy with targeted therapy may result in fewer adverse effects and improve therapeutic efficacy, particularly in advanced cases. To explore this concept we evaluated the benefits of DCAC50 treatment in combination with paclitaxel. We conducted multi-drug combination dose–response analysis in TNBC cells according to Chou-Talalay (ref. 29; Fig. 6A). Co-treatment of paclitaxel and DCAC50 resulted in a CI less than one, suggesting additive to synergistic cytotoxicity (Fig. 6B). In addition, we observed a favorable dose reduction for both drugs, when cell lines were treated with the above combination, as indicated by DRI values ranging from 1.60 to 3.45 for DCAC50 and 2.04 to 3.72 for paclitaxel. Although the efficacy of the treatment combination leads to the additive cytotoxicity in MDA-MB-231, MDA-MB-436, MDA-MB-468 and HCC1395 cell lines (CI ranging from 0.9 to 1.1), we observed a synergistic response in HCC1187 and HCC1806 cell lines ($CI < 0.9$). Because the cytotoxicity of DCAC50 in tumor cells is a consequence of elevated oxidative stress, we hypothesized that the greater cytotoxicity of the co-treatment could be explained by increased oxidative stress, compared with paclitaxel alone. Co-treatment of paclitaxel and DCAC50 resulted in significantly higher oxidative stress in all examined cancer cell lines, in comparison with paclitaxel alone (Fig. 6C). Oxidative stress in paclitaxel-treated cells is reduced, compared with DMSO-treated cells. Thus, combination of DCAC50 with paclitaxel increases oxidative stress in tumor cells resulting in greater cytotoxicity.

Discussion

In breast cancer, elevated levels of copper and copper-related proteins are associated with advanced disease (37–39), remodeling of the tumor microenvironment (14, 26) and resistance to chemotherapy (39, 40). Thus, copper homeostasis and the copper proteome are targets for breast cancer therapy. We have investigated an emerging approach to targeting copper-dependent cellular functions via the inhibition of intracellular copper transport in TNBC using the novel small-molecule DCAC50. We demonstrate that DCAC50 reduces cell proliferation and induces apoptosis through the inhibition of copper transport, disruption of cellular copper homeostasis, and the generation of oxidative stress in a panel of TNBC cell lines. Furthermore, DCAC50 enhances the cytotoxicity of paclitaxel *in vitro*, and inhibits tumor growth and angiogenesis in a xenograft mouse model.

The inhibition of ATOX1 and CCS activity with DCAC50 is expected to disrupt copper homeostasis and the copper proteome. In our study, significant increases in total intracellular copper levels were observed with DCAC50 treatment in claudin-low cells

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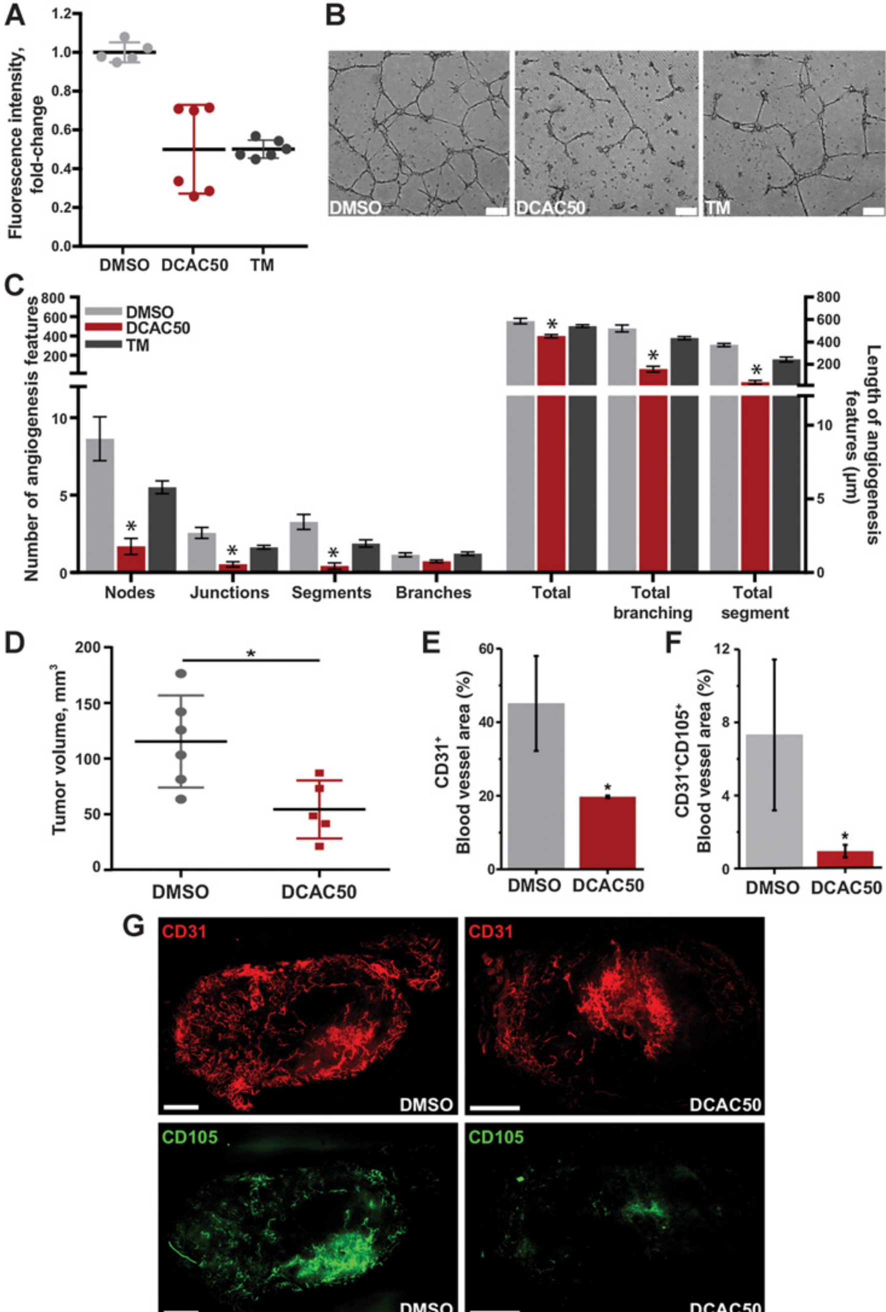

Figure 5.
Effect of DCAC50 on angiogenesis and tumor growth in MDA-MB-468 xenograft mouse model. **A,** LOX activity proportional to fluorescence intensity measured in media from HuVEC cells incubated with 20 μmol/L DCAC50, 30 μmol/L TM, or DMSO for 24 hours. Mean ± SD reported for two independent experiments, each with three biological replicates. **B,** Representative images of network formation in HuVEC cells plated on Matrigel membrane matrix with 20 μmol/L DCAC50, 30 μmol/L TM, or DMSO for 16 hours; scale bar, 100 μm. **C,** Quantification analysis of angiogenesis features in network-formation assay. Three biological replicates for each treatment were analyzed. Data reported as mean ± SD. *, $P < 0.05$, compared with DMSO control, according to the unpaired *t* test. The Holm–Sidak method was used to correct for multiple comparisons in (**C**). **D,** Tumor volumes in the MDA-MB-468 mouse model at the end of treatment. Data are expressed as mean ± SD. *, $P < 0.05$, compared with DMSO control, according to the two-tailed Student *t* test. **E** and **F,** Effect of DCAC50 on angiogenesis in the MDA-MB-468 mouse model. Quantification of $CD31^+$ (**E**) and $CD31^+CD105^+$ (**F**) angiogenic blood vessels in the 400-μm tumor macrosections for DMSO and DCAC50 treatment groups. Data are expressed as mean ± SD for three biological replicates. *, $P < 0.05$, compared with DMSO control, according to the unpaired *t* test. **G,** Z-stack projections of $CD31^+$ and $CD105^+$ angiogenic blood vessels in a representative macrosection; scale bars, 500 μm.

and modest increases in basal-like cells. Copper distribution was changed, with copper accumulating in the nuclei in MDA-MB-468 cells, to a lesser extent in HCC1395 nuclei or not at all in MDA-MB-231 nuclei. Attenuated cell proliferation and induced apoptosis were observed in all DCAC50-treated cell lines. Thus, the alteration of copper homeostasis, observed as an increase in total copper content and/or changes in copper distribution, leads to cytotoxicity. Nuclear localization of copper has previously been reported in *Atox1*$^{-/-}$ mouse embryonic fibroblasts (41), in ATP7A-deficient skin fibroblasts (42) and in *Atp7b*$^{-/-}$ mouse hepatocytes (43). However, the consequences of nuclear copper localization on cell proliferation, and the question of how copper enters the nuclei when the copper transport pathway is perturbed, remain to be elucidated. ATOX1 has been identified as a nuclear transcription factor and may undergo copper-dependent translocation to the nucleus (44). However, ATOX1 distributions were unchanged in DCAC50-treated cells (Supplementary Fig. S4A).

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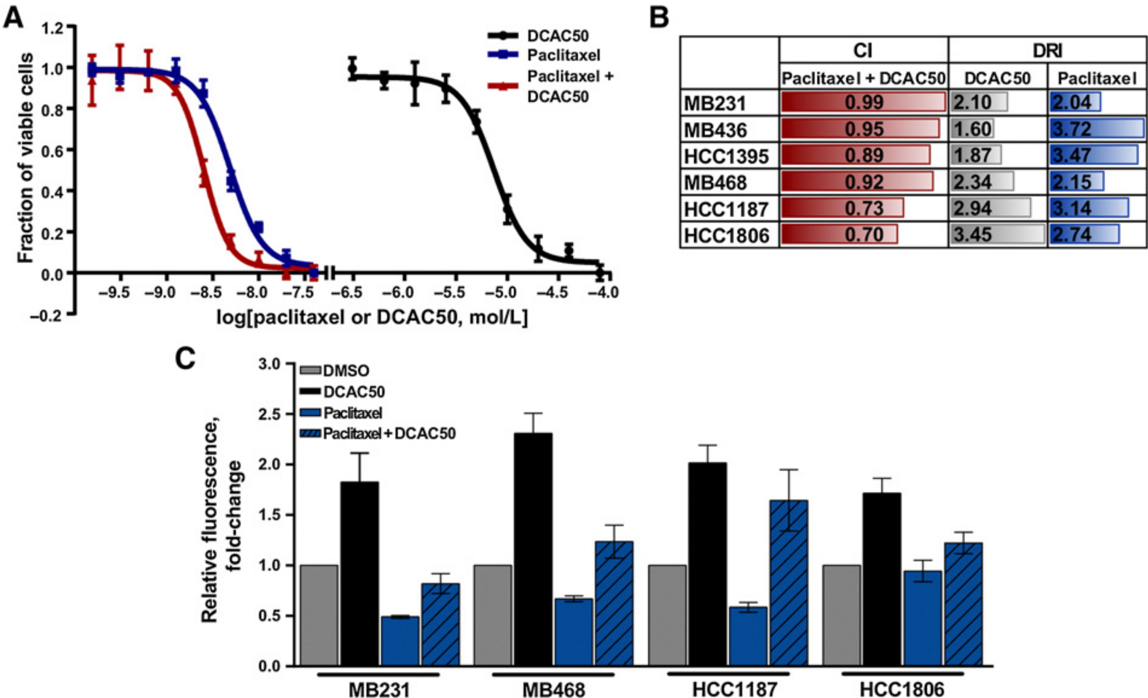

Figure 6.

Evaluation of DCAC50 in treatment combination with paclitaxel. **A,** Dose–response profile of single drugs or their combination in MDA-MB-468 cells. Cells were treated with constant ratios of paclitaxel and/or DCAC50 for 72 hours. **B,** CI and DRI calculated with CompuSyn Software. CI = [0.9–1.1], additive; CI $\leq$ 0.90, synergistic. Results of at least three independent experiments are shown. **C,** Oxidative stress in cells treated with paclitaxel, DCAC50, or their combination. TNBC cells were treated for 24 hours with DMSO, 10 nmol/L paclitaxel, 20 μmol/L DCAC50, or their combination, and stained with DCFDA. Data are presented as fold-change of DCF fluorescence intensity (mean $\pm$ SD of two biological replicates).

Various copper transporters regulate cellular copper content and its distribution. Cu-ATPases—downstream targets of ATOX1—translocate between the perinuclear space associated with TGN, where they supply copper to cuproenzymes, and the plasma membrane, to export copper from the cell (16). Copper binding is necessary for Cu-ATPase re-localization, and Cu-ATPase mutants unable to bind copper are restricted to the TGN (45). Thus, the inhibition of copper transfer from ATOX1 is expected to alter the distribution of Cu-ATPases. In MDA-MB-468 cells, DCAC50 changed the localization of ATP7B resulting in enhanced ATP7B clustering near the nuclei, which is indicative of impaired copper homeostasis. However, DCAC50 did not induce noticeable changes in protein levels of ATP7B, ATOX1, CTR1 or CCS, indicating no effect of the treatment on protein synthesis or degradation. In summary, DCAC50 causes changes in ATP7B and copper localization, but the detailed mechanism by which copper is translocated to the nucleus and how this impacts cell proliferation and survival remains unclear.

Copper homeostasis and copper-dependent proteins are intimately linked with the redox status of cells. Intracellular copper homeostasis is tightly regulated to protect cells from exposure to the redox-active metal. Free copper can induce superoxide generation and bind glutathione to create an oxidizing environment. On the other hand, copper-dependent SOD1 and total SOD (SOD1 and SOD2) activities are responsive to superoxide levels and protect cells from excessive oxidative stress. ATOX1 itself has a central role linking copper and redox homeostasis (46). Therefore, disruption of copper homeostasis and the copper proteome is expected to disrupt cellular redox status. We have observed increased oxidative stress in all DCAC50-treated cells, measured by general ROS levels and the change in the GSH:GSSG ratio. Overall, oxidative stress precedes the induction of apoptosis in cancer cells, suggesting that elevation of ROS is the reason for cytotoxicity. Inhibition of intracellular copper transport by DCAC50 is expected to inhibit the activity of copper-dependent SOD1. In our study, total SOD activity increased over time with increasing ROS levels, presumably in an effort to modulate oxidative stress. At the same time, SOD1 activity, normalized to SOD1 expression, decreased with DCAC50 treatment in some cell lines, as expected with the inhibition of copper transfer from CCS to SOD1, but increased in others. SOD1 has a half-life of more than 30 hours in mammalian cells (47) and alternative pathways exist for the delivery of copper to the enzyme (36), which may mitigate the impact of DCAC50 on SOD1 activity.

ATOX1 and CCS protein expression levels and IC_{50} values for DCAC50 were established in eight breast cancer cell lines and in normal mammary epithelial cells (HMEC). Low micromolar IC_{50} values were obtained in all cell lines, with no clear correlation between copper chaperone protein levels and sensitivity to DCAC50. Ultimately, six TNBC cell lines with variable elevated levels of ATOX1 and CCS were selected to investigate the effects of copper transport inhibition. We found that DCAC50 inhibited

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proliferation and induced apoptosis more effectively in basal-like than in claudin-low cell lines. To explain the broadly different responses of these subtypes of cells to DCAC50, we must consider cellular copper and redox statuses. High copper levels at baseline and large increases in intracellular copper levels with treatment were observed in claudin-low cells (MDA-MB-231, MDA-MB-436, HCC1395), yet DCAC50 was less effective in these cells lines. Basal-like MDA-MB-468 cells, which had lower baseline copper levels that increased only modestly with treatment, exhibited noticeable disruption to copper distribution and were particularly sensitive to DCAC50-induced apoptosis. Denoyer and colleagues (48) have argued that the sensitivity of cancer cells to ROS-generating copper ionophores may be attributed to limited antioxidant capacity rather than to high basal copper levels. Our data indicate that the ability of breast cancer cells to control ROS production is an important contributor to sensitivity to DCAC50. One of the major cellular antioxidant molecules, glutathione, binds copper and has been shown to protect cells from ROS toxicity (49, 50). In mouse embryonic fibroblasts, when glutathione levels are low, ATOX1 deficiency has been shown to disrupt copper homeostasis leading to cell death (51). This evidence suggests that when glutathione levels are low and ATOX1 is limited, cells are susceptible to copper dyshomeostasis and oxidative stress. In our study, basal-like cells (MDA-MB-468, HCC1187, HCC1806) had overall lower copper levels and lower total GSH levels at baseline, in comparison with claudin-low cells. Thus, basal-like cells may have a lower threshold for copper overload, and may be more sensitive to copper dyshomeostasis and oxidative stress when ATOX1 is inhibited by DCAC50. In contrast, claudin-low cells possess higher levels of glutathione and resist oxidative stress caused by DCAC50 treatment. Our results demonstrate that the GSH synthesis inhibitor, BSO, potentiates DCAC50 inhibition of cell proliferation in claudin-low HCC1395 and MDA-MB-436 cells by depleting glutathione levels and thus exhausting cellular antioxidant defense.

Of all the TNBC cell lines evaluated in this study, MDA-MB-231 cells are most resistant to DCAC50's cytotoxic effects, which may be a consequence of distinct genetic alterations promoting cell growth and survival. MDA-MB-231 cell line is an invasive, highly metastatic claudin-low cell line that bears activating mutations in *Kras*, *Braf*, and *Pdgfra*, which have previously been shown to upregulate cell proliferation and survival through activation of MAPK and PI3K (52, 53). Moreover, MDA-MB-231 cells can upregulate the antiapoptotic gene *Bcl2a1* upon tumor progression (54). Thus, activation of these alternative signaling pathways may contribute to the ability of MDA-MB-231 cells to resist DCAC50 treatment. In comparison with cancer cells, the disruption of copper homeostasis with DCAC50 triggered a limited response in normal cells (HMEC and HuVEC). DCAC50 attenuated cell proliferation in HMEC and inhibited network-formation in HuVEC (without reducing cell proliferation), but no induction of apoptosis was detected in either of the normal cell lines. DCAC50 has previously been shown to have a minimal effect on the proliferation of other normal human cell lines (28). The ability of cells to activate pathways to resist copper toxicity and oxidative stress, may explain heterogeneous sensitivity to DCAC50 and to the associated copper overload.

Reactive oxygen species are relevant to paclitaxel cytotoxicity, and resistance to paclitaxel relies, at least partially, on cellular total antioxidant capacity (55). Co-treatment with DCAC50 elevates oxidative stress, and is expected to enhance cytotoxicity in paclitaxel-treated cells. We observed increased production of oxidative stress and improved efficacy in cells treated by paclitaxel in combination with DCAC50, in comparison with paclitaxel alone. We hypothesize that the efficacy of the combination is due to the interference of multiple pathways involved in the defense against oxidative stress. The combination was effective to reduce treatment doses for both drugs, and may help reduce paclitaxel-associated side effects and reverse paclitaxel-associated resistance in tumor cells.

The findings presented here support our hypothesis that DCAC50 inhibits copper transport and alters copper homeostasis, disrupting the redox balance and causing cytotoxicity in cancer cells. The varying efficacy of DCAC50 reflects the heterogeneity of breast cancer cell lines and suggests that the success of drugs targeting copper homeostasis may depend on baseline cellular copper and redox statuses. Our findings also reveal the differences between targeting copper homeostasis, via the intracellular copper transport pathway, versus global copper chelation. Recent studies suggest that copper chelation is clinically important, especially for high-risk patients with breast cancer and in the prevention of angiogenesis and metastasis. In our study, TM did not have a significant effect on cancer cell growth or survival, however it did inhibit endothelial cell network-formation *in vitro*, in accordance with its known anti-angiogenic activity (23). Importantly, DCAC50 targets tumor cell viability as well as the tumor microenvironment, thus the inhibition of intracellular copper transport has the potential to become an effective strategy to treat breast cancer.

We conclude that dual effects of triggering apoptosis in tumor cells and modulating the tumor vascular environment are extremely attractive and should prompt further evaluation of the copper transport pathway as a therapeutic target in breast cancer.

Disclosure of Potential Conflicts of Interest

C. He has ownership interest (including stock, patents, etc.) in Accent Therapeutics and Epican Genetech, and is a consultant/advisory board member for Accenter Therapeutics. O.I. Olopade is a consultant/advisory board member for Tempus, CancerIQ, and HLF. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: O. Karginova, C.M. Weekley
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O. Karginova, C.M. Weekley, A. Raoul, A. Alsayed, T. Wu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O. Karginova, C.M. Weekley, A. Raoul, A. Alsayed, S.S.-Y. Lee
Writing, review, and/or revision of the manuscript: O. Karginova, C.M. Weekley, C. He, O.I. Olopade
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): O. Karginova, C.M. Weekley, T. Wu
Study supervision: O. Karginova, C. He, O.I. Olopade

Acknowledgments

Use of the Advanced Photon Source at Argonne National Laboratory was supported by the U.S. Department of Energy, Office of Science, and Office of Basic Energy Sciences, under contract DE-AC02-06CH11357. We thank the U.S. Department of Energy, under contract number DE-FG02-

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07ER15865 (to C. He), for partial support of this work. We acknowledge funding from Breast Cancer Research Foundation FP049439 (to O.I. Olopade), the National Health and Medical Research Council (Australia; to C.M. Weekley; CJ Martin Overseas Biomedical Fellowship APP1090612), and NIBIB K99 EB022636 (to S.S.-Y. Lee). We thank Dr. Andrei Karginov for helpful comments and assistance with cell imaging, and Dr. Barry Lai for his assistance with XFM imaging.

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Received June 18, 2018; revised December 18, 2018; accepted February 22, 2019; published first March 1, 2019.

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Published OnlineFirst March 1, 2019; DOI: 10.1158/1535-7163.MCT-18-0667

Molecular Cancer Therapeutics

Inhibition of Copper Transport Induces Apoptosis in Triple-Negative Breast Cancer Cells and Suppresses Tumor Angiogenesis

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Mol Cancer Ther 2019;18:873-885. Published OnlineFirst March 1, 2019.

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Metallomics

PAPER

View Article Online
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Cite this: *Metallomics*, 2018, 10, 745

Received 15th April 2018, Accepted 25th April 2018

DOI: 10.1039/c8mt00084k

rsc.li/metallomics

Tetrathiomolybdate inhibits the reaction of cisplatin with human copper chaperone Atox1†

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Cisplatin is a widely used anticancer drug in clinic, and ammonium tetrathiomolybdate ($[(NH_4)_2MoS_4]$, TM) is a copper chelator used in clinic for the treatment of Wilson's disease. Recently, TM has been found to enhance the therapeutic effect of cisplatin; however, the origin of this effect is not clear. Here we found that TM can inhibit the reaction of cisplatin with Cu–Atox1 and prevent the protein unfolding and aggregation induced by cisplatin. Although Ag(I) binds to Atox1 in a way similar to Cu(I)–Atox1, TM does not prevent the reaction of Ag–Atox1 with cisplatin. This result indicates that the formation of a Mo-centered trimeric protein cluster in the TM–Cu–Atox1 system plays a role in the inhibitory effect. This work provides new insights into the mechanism by which TM enhances the cytotoxic efficacy of cisplatin and helps to circumvent cisplatin resistance of tumor cells.

Introduction

Cisplatin is an anticancer drug widely used in clinic for cancer chemotherapy. It is well known that cisplatin induces cell apoptosis by binding to DNA; however, only a very small portion of cellular platinum is able to form DNA crosslinks in the nucleus.[1] Increasing evidence indicates that also some protein targets are involved in the mechanism of cisplatin action. In particular, proteins governing copper homeostasis were found to be associated with cellular uptake and efflux of cisplatin, and the cellular levels of these copper proteins were correlated with tumor sensitivity or resistance to cisplatin.[2] Human copper transporter 1 (hCtr1) is believed to facilitate the cellular uptake of cisplatin. Low expression of hCtr1 reduces intracellular accumulation of cisplatin and decreases drug sensitivity of tumor cells.[3] On the other hand, P-type ATPases ATP7A and ATP7B, which are associated with cellular copper efflux, can also cause efflux/sequestration of platinum drugs.[4] Thus, overexpression of ATPases increases cisplatin resistance of tumor cells,[5] and silencing of ATPase genes recovers the drug sensitivity.[6] *In vitro* assays confirmed that the metal-binding domains of these copper proteins are required to modulate resistance to cisplatin.[7] Finally, the human copper chaperone Atox1 was also found to play a role in cisplatin intracellular transport.[8]

Atox1 receives cuprous ions from hCtr1 and delivers them to copper ATPases. The CXXC copper-binding motif of Atox1 can also bind Pt(II).[9] In-cell NMR spectroscopy indicated that cisplatin reacts quantitatively with Atox1,[10] while *in vitro* assays showed that cisplatin binding can induce unfolding and aggregation of Atox1.[11] Thus, it has been proposed that Atox1 can be a candidate for cisplatin resistance by competing with DNA platination.[10] Under specific conditions, platinum transfer from Atox1 to copper ATPases has been observed *in vitro*; which could be in accord with tumor resistance also stemming from drug efflux.[12] Knockout of Atox1 was also found to decrease the influx of cisplatin and hence to reduce DNA platination.[13] Interestingly, copper coordination promotes the platination of Atox1, even though the two metal ions share the same binding site.[14] A recent study suggests that a copper–sulfur–platinum cluster can be formed in the reaction of Cu–Atox1 with cisplatin in the presence of glutathione (GSH), which could be related to the copper promoted platination of Atox1.[15]

Based on the above observations, a proposed strategy for improving the tumor sensitivity to cisplatin has been the alteration of cellular copper levels by chelating agents. Ammonium tetrathiomolybdate ($[(NH_4)_2MoS_4]$, TM) is a copper chelator used in clinic for the treatment of Wilson's disease.[16] TM also showed potency in the inhibition of tumor growth by its anti-angiogenic effects.[17] Mechanistic *in vitro* and *in vivo* investigations indicated that TM modulates copper levels by binding to copper proteins, such as serum albumin, ceruloplasmin and metallothioneins.[18] An X-ray crystal structure showed that TM binds to Atx1, the

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† Electronic supplementary information (ESI) available: Experimental details, NMR spectra and chromatographic profiles. See DOI: 10.1039/c8mt00084k

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analogue of Atox1 in yeast, and forms a stable [TM·Cu·(Cu–Atx1)$_3$] complex, containing a TM-bridged Cu cluster which corresponds to the molybdenum cluster detected in a kidney sample extracted from LPP rats (animal model of Wilson's disease) treated with TM.[19] In addition, TM can enhance the therapeutic effect of cisplatin by selectively increasing DNA platination in cancerous but not in normal tissues.[20] Pretreatment with TM was also found to significantly enhance cisplatin sensitivity by promoting p38 activation and cisplatin-induced degradation of the epidermal growth factor receptor (EGFR).[21] Moreover, it has been suggested that TM can increase the cytotoxicity of cisplatin by reducing the levels of ATP7A.[22]

Based on the role of Atox1 in cisplatin resistance and the reversing effect of TM co-administration, we hypothesized that TM could interfere with the reaction between Cu–Atox1 and cisplatin. Thus, we have investigated the influence of TM on the platination of Atox1. In addition, the investigation has been extended to the Ag-bound form of Atox1. These results help to understand the synergistic effect of TM and cisplatin.

Experimental

Protein expression and purification

The expression and purification of Atox1 protein was conducted using previous methods.[12b] Briefly, the plasmid of Atox1 containing an N-terminal His$_6$-tag was transformed into *E. coli* BL21 (DE3) gold cells. The cells were incubated at 37 °C in LB media containing 0.1 mg per ml ampicillin. The protein expression was induced by 0.4 mM isopropyl thio-β-D-galactosidase (IPTG) at OD$_{600}$ 0.8. After 5 h induction, cells were harvested by centrifugation at 4000 rpm for 20 min, and resuspended in the buffer containing 200 mM NaCl and 50 mM Tris–HCl (pH 8.0). The Atox1 protein was purified using Ni-NTA column. The His$_6$-tag of fusion protein was cleaved by tobacco etch virus (TEV) protease at 16 °C for 10 h. The protein was further purified on a Superdex 75 16/60 column (GE Healthcare) using eluent 150 mM NaCl, 50 mM phosphate (pH 7.4). The purity of Atox1 was verified using Tricine-SDS-PAGE and ESI-MS, and the concentration was determined using UV absorbance at 280 nm (ε = 2980 M^{-1} cm^{-1}). The ^{15}N labeled Atox1 was prepared with the same procedure by using M9 media containing $^{15}NH_4Cl$ (1 g L^{-1}).

Preparation of the TM–Cu–Atox1 complex

The TM–Cu–Atox1 complex was prepared using a literature method.[19] Cu–Atox1 was generated by incubation of 200 μM apo-Atox1 with 300 μM $[Cu(CH_3CN)_4]^+$ in the presence of 1 mM DTT for 15 minutes. TM–Cu–Atox1 was obtained by reaction of Cu–Atox1 with 0.4 molar equivalents of TM at 25 °C for 2 hours. The formation of the protein trimer [TM·Cu·(Cu–Atox1)$_3$] was verified by size exclusion chromatography (Fig. S1, ESI†). Consistent with literature results, the TM–Cu–Atox1 ternary system exists mainly as a trimer with small amount of monomer and oligomers. ICP-MS analyses indicate that the ratios [Atox1] : [Cu] : [Mo] are 1 : 1.29 : 0.39 in the trimer and 1 : 0.82 : 0.23 in the oligomer (Table S1, ESI†), which is in agreement with the literature results.[19] For the NMR analyses, the ^{15}N-labeled protein was used and the trimeric species [TM·Cu·(Cu–Atox1)$_3$] was separated by using size exclusion chromatography. The concentration of protein was measured using Bradford assay after ultrafiltration.

Chromatographic analyses

Analytical size exclusion chromatography (SEC) was conducted on an ÄKTA purifier system with a Superdex 75 10/300 analytical column (volume = 24 ml; GE Healthcare). The column was pre-equilibrated with 150 mM NaCl, 20 mM sodium phosphate buffer (pH 8.0). The flow rate of 0.8 ml min^{-1} was used and the chromatographic profiles were recorded with UV detection at 280 nm. Anion exchange chromatography was performed on a Source 15Q anion exchange column (4.6/100 PE; GE Healthcare) with UV detection at 280 nm. A linear gradient (0–15 min: 0–7% B) was used at a flow rate of 1 ml min^{-1} (eluent A: 20 mM Tris–HCl, pH = 8.0, eluent B: 20 mM Tris–HCl, 1 M NaCl, pH = 8.0).

ESI-MS

ESI-MS measurements were performed on Thermo LTQ linear Orbitrap XL mass spectrometer (Thermo Fisher, San Jose, CA, USA). Proteins were desalted using a hitrap desalting column and samples were directly injected. Data were recorded in positive mode in an m/z range of 200 to 2000. Spectra were processed using Xcalibur 2.0 software (Thermo).

NMR spectroscopy

^{1}H–^{15}N heteronuclear single quantum coherence (HSQC) NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer at 298 K. All samples were prepared in 50 mM sodium phosphate (pH = 7.0) containing 10% v/v D_2O. The platinated samples were prepared by incubation of Cu–Atox1 solution with cisplatin at a 1 : 1.3 molar ratio.

Circular dichroism

Circular dichroism (CD) experiments were performed on a Jasco J-720 CD chiroptical spectrometer. Far-UV CD spectra were collected using a 1.0 mm quartz cuvette at 25 °C under nitrogen atmosphere. Spectra were recorded in the range of 190–260 nm with a scan speed of 100 nm min^{-1}. All measurements were conducted on 50 μM protein samples and were repeated three times.

Electrophoresis

Electrophoretic analyses were performed using Tricine-SDS-PAGE with 15% gel. A voltage of 80 V was applied for 30 min and then increased to 130 V. Proteins were stained using Coomassie Brilliant Blue R-250. Gels were destained and images were recorded on a Tanon-1600 digital gel image analysis system.

Results and discussion

TM inhibits the reaction of cisplatin with Cu–Atox1

The formation of a TM-mediated trimeric cluster of Cu–Atox1 was verified by size exclusion chromatography and electrophoresis

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analysis on native gel (Fig. S1, ESI†). The effect of TM on the reaction of cisplatin with Cu–Atox1 was analyzed using anion exchange chromatography (Fig. 1A). Cu–Atox1 appears at an elution volume of ~10.5 ml and the reaction with TM does not alter the retention volume. Incubation of Cu–Atox1 with cisplatin resulted in a new peak at ~6.5 ml elution volume, corresponding to the platination of Cu–Atox1. This peak increased in a time dependent manner and reached ~50% of total protein in 5.5 h. On the contrary, incubation of TM–Cu–Atox1 with cisplatin does not lead to formation of new species (no new peak observed). This result indicates that the formation of the TM–Cu–Atox1 adduct prevents the reaction with cisplatin.

To confirm the effect of TM, the products of the above reactions were analyzed by ESI-MS. Cu–Atox1 alone showed three peaks corresponding to apo-Atox1 (1), Cu–Atox1 (2) and [Cu–Atox1 + 2Na$^+$] (3) (Fig. 1B). The presence of the apo-Atox1 peak is due to the dissociation of the copper ion from the protein during the ionization process, a phenomenon often observed in ESI-MS measurements.[14] For the same reason (dissociation of the complex), no peak of ternary complex was observed in the measurement performed on the TM–Cu–Atox1 cluster.

The reaction of Cu–Atox1 with cisplatin generated two major platination adducts, [Atox1 + Pt(NH$_3$)$_2$] (4) and [Atox1 + Pt(NH$_3$)$_2$(H$_2$O)] (5). This result is consistent with previous observations.[14] In contrast, no platination adducts were detected in the reaction of the TM–Cu–Atox1 cluster with cisplatin (Fig. 1B, [TM–Cu–Atox1] + Pt). In order to rule out the possibility that free TM could mask the appearance in ESI-MS of platinated Atox1 adducts, the adducts formed in the reaction of Cu–Atox1 with cisplatin were analyzed after addition of TM. The results showed that the peaks of the platination adducts (peaks 4 and 5) were not affected by the presence of TM (Fig. 1B, [Cu–Atox1 + Pt] + TM). This latter result confirms that, while the binding of TM to Cu–Atox1 inhibits the platination of the protein, TM cannot disrupt the pre-formed platinated adducts of Atox1.

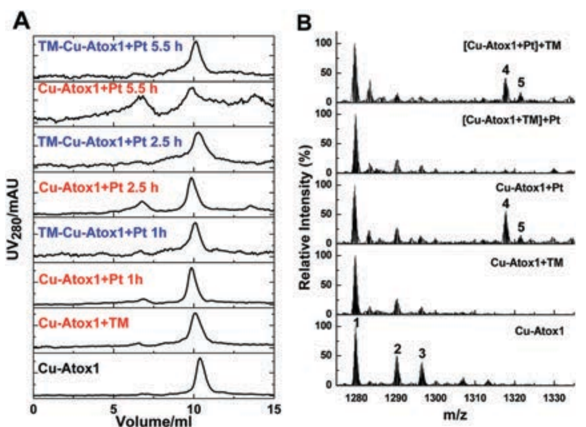

Fig. 1 TM inhibits the reaction of cisplatin with Cu–Atox1. (A) Time-dependent anion exchange chromatography profiles of Cu–Atox1 (200 μM) or TM–Cu–Atox1 (200 μM) incubated with 1.3 eq. of cisplatin at 25 °C for different times ranging from 1 to 5.5 hours. Sample and reaction time are labeled in each chromatogram. (B) ESI-MS analysis of Cu–Atox1 (50 μM) or TM–Cu–Atox1 (50 μM) treated with 1.3 molar equivalents of cisplatin at 25 °C for 7 hours. Only the +6 charged peaks are shown.

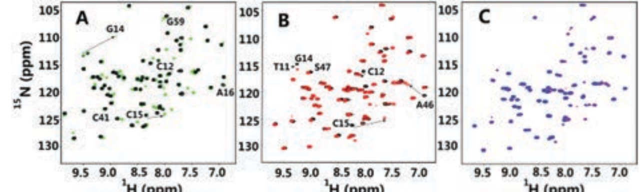

Fig. 2 Effect of TM on cisplatin binding to Cu–Atox1 monitored by ^{1}H–^{15}N HSQC spectroscopy. (A) Spectra of Cu–Atox1 before (black) and after (green) reaction with cisplatin (1.3 eq. for 2 h). (B) Spectra of Cu–Atox1 in the absence (black) and in the presence of 0.4 eq. of TM (red). (C) Spectra of TM–Cu–Atox1 before (red) and after (blue) incubation with cisplatin (1.3 eq., 2 h incubation). 0.5 mM concentration of ^{15}N-labeled protein was used in all these NMR experiments.

2D ^{1}H–^{15}N HSQC NMR spectra allow detection of all amidic groups, so that the alteration of each protein residue can be monitored. The spectra of Cu–Atox1 treated with cisplatin showed that a number of peaks undergo a shift, the most significant changes occurred around the copper binding site (Cys12 and Cys15, see Fig. 2A). These changes are consistent with literature results.[15] The reaction of TM with Cu–Atox1 was also monitored with 2D HSQC spectroscopy. While the majority of the peaks of Cu–Atox1 remained unchanged, the signals around the copper binding site (including Cys12, Cys15 and Ala16) shifted upon reaction with TM (Fig. 2B). This observation is consistent with the crystal structure of the [TM·Cu·(Cu–Atx1)$_3$] complex,[19] showing that TM coordinates to the copper ions bound to Atx1 molecules, while the overall structure of the protein is not perturbed. In addition, the broadening of NMR signals of Cu–Atox1 in the presence of TM is consistent with the reduced tumbling rate of the trimeric protein complex (Fig. S2, ESI†). Unlike the case of Cu–Atox1, the spectra of TM–Cu–Atox1 did not change after incubation with cisplatin for 8 h (Fig. 2C and Fig. S3, ESI†). These results confirm that TM binds to Cu–Atox1 and this binding inhibits the reaction of the protein with cisplatin.

TM binding prevents the aggregation of Cu–Atox1 induced by cisplatin

It has been reported that the binding of cisplatin to Atox1 promotes protein unfolding.[8b,11] Therefore, we used CD spectroscopy to investigate the effect of TM upon protein unfolding induced by cisplatin. Consistent with the above NMR results and literature reports,[11] the CD spectrum of Cu–Atox1 is nearly identical to that of apo-Atox1 (Fig. 3A). Also the reaction with TM has very little influence on the secondary structure of Cu–Atox1, indicating that the Mo-centered protein trimer does not alter the protein folding in solution. Incubation with cisplatin clearly disrupts the folding of Cu–Atox1, causing a decrease of the negative band at 220 nm. More protein unfolding is observed with increased concentration of cisplatin. On the contrary, negligible change was observed incubating TM–Cu–Atox1 with cisplatin, even at high cisplatin concentrations. These results indicate that cisplatin disrupts the secondary structure of Cu–Atox1, but not that of TM–Cu–Atox1.

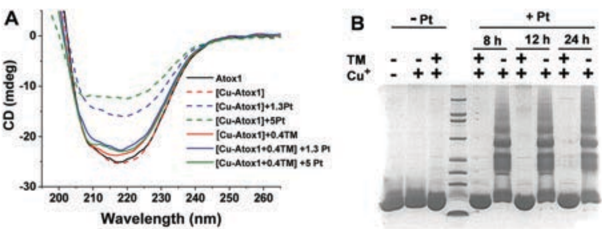

Fig. 3 Protein folding and aggregation analyses. (A) Far-UV CD spectra of 50 μM Cu–Atox1 (dashed lines) or 50 μM TM–Cu–Atox1 complex (solid lines) incubated with 1.3 or 5 molar equivalents of cisplatin at 25 °C for 5 hours. The presence of TM prevents the structural changes of Cu–Atox1 induced by cisplatin. (B) Tricine-SDS-PAGE analyses of 300 μM Cu–Atox1 or TM–Cu–Atox1 incubated with 1.3 molar equivalents of cisplatin at 25 °C for different times (incubation time labeled in Fig. 3B).

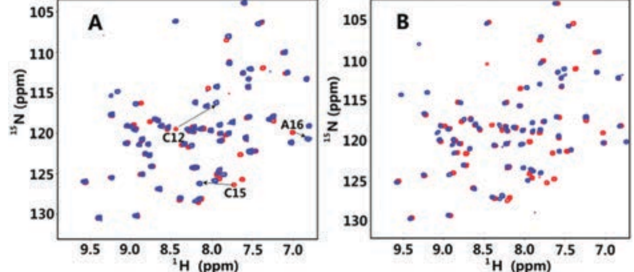

Fig. 4 Reaction of Atox1 with Cu(I) and Ag(I) monitored by ^{1}H–^{15}N HSQC spectroscopy. (A) Spectra of Atox1 before (red) and after (blue) reaction with 1.5 eq. of Cu(I). (B) Spectra of Atox1 before (red) and after (blue) reaction with 1.5 eq. of Ag(I).

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The unfolding of Atox1 induced by cisplatin can also lead to protein aggregation.[23] Tricine-SDS-PAGE analysis showed that both apo-Atox1 and Cu–Atox1 exhibit a single band on the gel, corresponding to the protein monomer. Although TM–Cu–Atox1 forms a complex containing three Atox1 units, it still appears as a monomer on this denaturing gel (Fig. 3B). Reaction with cisplatin of Cu–Atox1 leads to aggregation with formation of oligomer bands in the gel; in contrast, no aggregation is detected by incubating TM–Cu–Atox1 with cisplatin, in which case only a monomer band is observed even after 24 h incubation time (Fig. 3B). To be noted that cisplatin also causes aggregation of apo-Atox1 in the same experimental condition (Fig. S4, ESI†). Taken together, these results confirm that the formation of the TM–Cu–Atox1 cluster effectively suppresses the reaction of Cu–Atox1 with cisplatin.

TM does not inhibit the reaction of Ag–Atox1 with cisplatin

All the above results indicate that formation of the TM–Cu–Atox1 complex prevents the reaction of Cu–Atox1 with cisplatin. Similarly to the case of Atx1, the analogue of Atox1 in yeast, where the crystal structure showed that the protein forms a trimer with a Mo-centered tetra-copper cluster ([TM·Cu·(Cu–Atx1)$_3$]),[19] it can be hypothesized that a similar cluster mediated by a TM–Cu core is formed also in the case of Cu–Atox1. This TM–Cu–Atox1 cluster may prevent the access to the surrounding three Atox1 molecules of other reactive substrates, such as cisplatin investigated in this work. It has been recently reported that, in the presence of sulfur-containing reducing agents, such as GSH or DTT, Cu–Atox1 can react with cisplatin, forming copper–sulfur–platinum clusters, which can be the intermediate species in the platination of Atox1.[15] However, analogous copper–sulfur–platinum intermediates cannot form in the case of TM (also a sulfur donor) which, instead, generates the [TM·Cu·(Cu–Atox1)$_3$] cluster where the three Atox1 molecules are prevented from reacting with cisplatin.

To highlight a specific role of copper, we performed a similar study on Ag–Atox1, to be compared with the behavior of Cu–Atox1. It has been reported (X-ray absorption spectroscopy) that Ag(I) can also bind to Atox1 at the copper binding site.[24] Here the reaction of Ag^+ with apo-Atox1 was analyzed using HSQC NMR spectroscopy. As a consequence of Ag^+ binding, the signals of Atox1 shifted in a way very similar to that of Cu–Atox1, which supports a similar coordination of Ag(I) and Cu(I) to Atox1 (Fig. 4). However, TM affected differently the reaction with cisplatin of Ag–Atox1 and Cu–Atox1. Tricine-SDS-PAGE experiments showed that, similarly to the case of Cu–Atox1, cisplatin can react with Ag–Atox1 and cause protein aggregation (Fig. 5). However, unlike the case of Cu–Atox1, TM did not prevent the reaction of Ag–Atox1 with cisplatin, and the platination of Ag–Atox1 caused protein aggregation (Fig. 5). This result was also confirmed by ^{1}H–^{15}N HSQC experiments showing that cisplatin can cause significant changes of the Ag–Atox1 signals both in the absence and in the presence of TM (Fig. S5, ESI†). The different effect of TM upon Cu–Atox1 and Ag–Atox1 can be explained by the different coordination properties of Cu(I) and Ag(I). Cu(I) can readily form a tetrahedral four-coordinated structure that allows Cu–Atox1 to further react with TM and generate a protein trimer. In contrast, Ag(I) forms preferentially linear two-coordinated geometries that do not allow Ag–Atox1 to further react with TM and generate a protein trimer.[25] Size exclusion chromatography confirmed that, differently from Cu–Atox1, Ag–Atox1 does not form protein trimers or other oligomers in the presence of TM (Fig. S6, ESI†). The different effect of TM on the reaction of Cu–Atox1 and Ag–Atox1 with cisplatin further indicates that the formation of the TM–Cu–Atox1 cluster is crucial for the inhibition of the Atox1 platination.

A number of investigations indicate that TM can enhance efficacy of cisplatin and circumvent drug resistance;[20] this

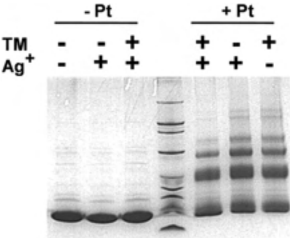

Fig. 5 Effect of TM on the aggregation of Ag–Atox1 promoted by cisplatin. The reactions were conducted on 300 μM Ag–Atox1 or [TM + Ag–Atox1] and 1.3 molar equivalents of cisplatin. The sample of [TM + Ag–Atox1] was prepared by incubation of 300 μM Ag–Atox1 with equimolar TM for 2 hours. The samples were analyzed by Tricine-SDS-PAGE analyses after 12 h incubation time. The results show that TM does not inhibit the cisplatin-induced aggregation of Ag–Atox1.

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effect was proposed to be correlated to the copper chelation ability of TM. It is well-known that cisplatin-resistance is the consequence of multiple cellular events,[26] comprising the intracellular drug distribution which can be affected by copper-trafficking proteins. Atox1 was found to react with cisplatin and was proposed to be involved in the transfer of platinum to ATPases.[12] This process could lead to drug resistance by enhancing drug sequestration/efflux. TM not only can act as a copper chelating agent but also, and more significantly, can bind directly to a copper chaperone (Atx1) and form a [TM·Cu·(Cu–Atx1)$_3$] complex which can prevent the copper trafficking of Atx1. That the TM-bridged Cu cluster can also form *in vivo* was clearly shown by a Cu and Mo K-edge extended X-ray absorption fine structure (EXAFS) analysis performed on liver lysosomes and kidney samples extracted from LPP rats (animal model of Wilson's disease) treated with TM.[19,27] Here, we demonstrate for the first time that such complex can inhibit the reaction of cisplatin with Cu–Atox1, thus possibly reducing the Atox1-related drug resistance. It is worth noting that TM does not disrupt pre-formed platinated adducts of Atox1 and reacts specifically with Cu–Atox1 while has no effect on Ag–Atox1. These observations indicate that the formation of the TM–Cu–Atox1 cluster can indeed be a key factor in the overcoming of the Atox1-dependent cisplatin resistance.

Conclusion

In summary, the effect of tetrathiomolybdate (TM) on the reaction of Cu–Atox1 with cisplatin has been investigated in detail. The results indicate that TM inhibits the platination of Cu–Atox1 and prevents protein unfolding and aggregation induced by cisplatin. Interestingly, TM only inhibits the platination of Cu–Atox1, while it does not prevent the reaction of Ag–Atox1 with cisplatin under the same experimental conditions. These findings suggest that the formation of a hindered Mo-centered trimeric protein cluster (in the TM–Cu–Atox1 ternary system) can play a key role in overcoming the Atox1-dependent resistance to cisplatin of cancer cells.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported by the National Key R&D Program of China (2017YFA0505400) and the National Science Foundation of China (21573213, 21401065, 51503194). We thank the University of Bari, the Consorzio Interuniversitario di Ricerca in Chimica dei Metalli nei Sistemi Biologici (CIRCMSB) and Collaborative Innovation Center of Suzhou Nano Science and Technology for support.

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Received: 24 October 2019 | Revised: 4 February 2020 | Accepted: 13 February 2020

DOI: 10.1002/brb3.1596

ORIGINAL RESEARCH

Ammonium tetrathiomolybdate in the decoppering phase treatment of Wilson's disease with neurological symptoms: A case series

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Abstract

Objectives: To present our experience with ammonium tetrathiomolybdate (ATTM) in the decoppering phase treatment of Wilson's disease (WD) with neurological symptoms.

Methods: An uncontrolled longitudinal study was carried out to describe a case series of five patients diagnosed of WD with neurological symptoms in our hospital over the last 5 years and receiving ATTM for 8 (or 16) weeks. Unified Wilson's Disease Rating Scale (UWDRS), Global Assessment Scale (GAS) for WD and the Brewer-adapted Unified Huntington's Disease Rating Scale (UHDRS) for WD, magnetic resonance imaging, and monitoring for potential adverse effects were carried out in all patients before starting ATTM and 3 months later when ATTM was stopped and zinc treatment was initiated.

Results: All five patients presented neurological clinical improvement in UWDRS, GAS, and Brewer-adapted UHDRS for WD. Neuroimaging improvement was present in 2/5 patients with brain edema reduction. Mild anemia, leukopenia, and elevation of transaminases were detected in 1 patient, with complete remission after stopping ATTM for 1 week and then restarting at a half dose.

Conclusion: ATTM could be a good treatment for the initial treatment of WD with neurological symptoms due to its high efficacy, with a lower rate of neurological deterioration than the drugs currently available, despite the potential adverse effects.

KEYWORDS
ammonium tetrathiomolybdate, efficacy, neurological symptoms, safety, Wilson's disease

1 | INTRODUCTION

Wilson's disease (WD) is an inherited autosomal recessive disorder caused by mutations in the ATP7B gene that encodes for a P-type ATPase protein involved in copper transport and excretion. Recent genetic studies reported a prevalence of 1:7,026 for two mutant pathogenic ATP7B alleles (Kieffer & Medici, 2017). While the pathogenesis of WD is related to the altered function of the

The peer review history for this article is available at https://publons.com/publon/10.1002/brb3.1596

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ATP7B copper transporter, and its accumulation, it is less clear how ATP7B mutations influence the phenotype (Medici & LaSalle, 2019).

The clinical symptoms are a result of organ dysfunction due to the direct or indirect effects of copper accumulation (Litwin et al., 2018). The severity of the symptoms varies widely and can appear anytime between early childhood and old age (Kieffer & Medici, 2017). Subsequent copper accumulation in organs, mainly liver and brain but also other organs, produces clinical manifestations that include hepatic, neurological, psychiatric, and ophthalmologic disorders (Ala, Walker, Ashkan, Dooley, & Schilsky, 2007; Kieffer & Medici, 2017; Pfeiffer, 2007).

Liver manifestations are present in 60% of patients at diagnosis and have been reported to first appear in patients aged between 9 months and over 70 years, ranging from mild elevation of liver enzymes to acute liver failure and cirrhosis (Kieffer & Medici, 2017; Litwin et al., 2018). After hepatic manifestations, neurological disorders, such as tremor, dystonia, parkinsonism, dysarthria, dysphagia, chorea, or gait disturbance (Ala et al., 2007; Kieffer & Medici, 2017; Lorincz, 2010), are the most frequent clinical symptoms of WD and may be its initial presentation in 18%–68% of patients (Czlonkowska et al., 2018). In addition to these typical and more frequent neurological symptoms of WD, other neurological symptoms may occur in the course of WD that include myoclonus, tics, headache, olfactory and taste dysfunction, neuropathies, epilepsy, restless legs syndrome, and sleep disorders (Dusek, Ltwin, & Czlonkowska, 2019). The psychiatric symptoms include anxiety, depression, disinhibition, and personality changes. The most common ophthalmological sign as a result of copper accumulation is the Kayser–Fleischer corneal ring, whereas sunflower cataracts are observed rarely, and retinal degeneration may serve as a marker of neurodegeneration (Dzieżyc-Jaworska, Litwin, & Członkowska, 2019; Kieffer & Medici, 2017).

Patients with WD may also present with renal disturbances (including tubular dysfunction and renal calculi), cardiac involvement (a recent cardiac study has shown a higher risk of atrial fibrillation and heart failure in WD patients than in non-WD patients), and osteoarticular involvement (including osteopenia, osteoporosis, and arthropathy, which may lead to bone fractures and joint problems mainly affecting knees and wrists; Daneshjoo & Garshasbi, 2018; Dzieżyc-Jaworska et al., 2019).

Other manifestations of WD include autonomic system dysfunction (but involvement is subclinical in most cases) and endocrine system disturbances (which can lead to recurrent abortions, infertility, growth disruption, and parathyroid failure). However, it is possible to become pregnant for females with mild WD symptoms and for those who are compliant with therapy), hematologic disturbances (which may include acute hemolytic anemia, leucopenia, anemia, and low platelet count), and skin affectation (including lipomas and characteristic of WD skin changes like hyperpigmentation of the legs, xerosis or azure lunulae of the nails; Dzieżyc-Jaworska et al., 2019).

For the evaluation of the neurological symptoms of WD, it has been proposed the use of few scales: the Unified Wilson's Disease Rating Scale (UWDRS; Czlonkowska et al., 2007) which evaluates the clinical motor disease, the Global Assessment Scale (GAS) for WD (Aggarwal, Aggarwal, Nagral, Jankharia, & Bhatt, 2009) which includes psychiatric symptoms and cognitive, and the Brewer-adapted UHDRS for WD (Brewer et al., 1991, 1994) which evaluates the neurological symptoms.

Firstly described as a lethal neurological and hepatic familial disease, WD now has different drug therapies (copper chelators and zinc salts). Current guidelines (EASL, 2012; Roberts & Schilsky, 2008) recommend the use of chelators as the initial treatment of symptomatic WD patients, with the suggestion that trientine is better tolerated. However, a particular adverse event that can occur with each type of treatment is paradoxical neurological deterioration, more frequently with D-penicillamine and trientine than zinc (Litwin et al., 2015). Worsening of neurological symptoms soon after starting treatment is reported in around 10% of patients, though much higher prevalence has also been described (Aggarwal & Bhatt, 2018) and it can be irreversible and extremely serious adverse effect that can even lead to death (Svetel, Sternic, Pejovic, & Kostic, 2001). Paradoxical neurological deterioration has prompted the discussion whether D-penicillamine or trientine should be used in neurological WD patients and led to the search for safer treatments such as ATTM (Brewer & Askari, 2005; Brewer, Terry, Aisen, & Hill, 1987) or a more recent new formulation of TTM (Bis-choline TTM) called WTX101 that has successfully run through a phase 2 trial (Weiss et al., 2017) and started a phase 3 trial comparing WTX101 with standard of care (chelation or zinc therapy or a combination of both chelation and zinc therapy; Brewer & Askari, 2005; Swenson, 2019).

Recent data support that patients treated with ATTM could have less probability of neurological deterioration than those treated with trientine (Appenzeller-Herzog et al., 2019), and the most frequent side effects found were reversible increases of anemia, leukopenia, and transaminases (Brewer et al., 2006).

Our objective was to present our experience with ATTM in the treatment of WD with neurological symptoms after eight weeks of treatment by using UWDRS, GAS, and Brewer-adapted UHDRS for WD scales.

2 | METHODS

2.1 | Study design and patient selection

An uncontrolled longitudinal study was carried out to describe a case series of all patients (five) who were diagnosed of WD with neurological symptoms in our hospital during the last 5 years and who were treated in the Department of Neurology with ATTM for 8 (or 16) weeks. All patients were over 18 years of age. The study was conducted in compliance with the ethical standards and was approved by the Ethics Committee for Clinical Research of Hospital Universitari Vall d'Hebron (Barcelona, Spain). Treatment with ATTM was administered as compassionate use, and all patients signed a specific informed consent to start this treatment and a specific consent to use the study data for scientific purposes.

2.2 | Procedure and assessments

Treatment with ATTM was administered as initial decoppering treatment at a dose of 120 mg per day (20 mg three times daily with meals and 20 mg three times daily between meals) for 8 weeks. After completion of the 8-week initial decoppering treatment with ATTM, the patients were changed to a maintenance treatment with zinc. The dose and regimen of ATTM used in our case series was described by Brewer in his initial work (Brewer et al., 1991), when he treated 6 patients with ATTM as initial decoppering treatment for 8 weeks and zinc as maintenance treatment afterward. As ATTM and zinc were not administered concomitantly, hospitalization was not needed in our case series.

All patients were followed weekly for clinical and analytical efficacy, with an anamnesis, a physical examination, measuring the levels of urinary copper in 24 hr and the levels of plasmatic copper, and monitoring for the presence of potential adverse effects: liver function alteration, anemia, or leukopenia. To ensure compliance with ATTM treatment, there was a close and exhaustive monitoring by phone calls and ambulatory visits.

A brain magnetic resonance imaging (MRI) and video recording were performed 2 weeks before starting ATTM and 3 months later when ATTM was stopped (which was 1 month after stopping ATTM) and zinc treatment was initiated.

Three scales were used: The GAS for WD, the UWDRS, and a short adaptation of the Unified Huntington's Disease Rating Scale (UHDRS; Young et al., 1986) used by Brewer in WD in several clinical trials (Brewer et al., 1991, 1994, 2003, 2006, 2009). The scales were carried out before starting ATTM and 1, 2, and 3 months later when ATTM was stopped and zinc treatment was initiated.

The GAS for WD contains two parts, GAS I to evaluate global disability in four areas: liver, cognition and behavior, motor, and osteoarticular, each item scoring severity from 0 to 5 (scores not to be summed up), and GAS II to evaluate neurological and psychiatric dysfunction with 14 items, each item scoring severity from 0 to 4 (scores to be summed up to a maximum of 56 points).

The UWDRS consists of three subscales representing three main features of clinical manifestations in WD with 35 items in total: UWDRS I to evaluate the level of consciousness, UWDRS II to evaluate the impact of the neurological symptoms on the daily life activities, and UWDRS III to evaluate the neurological signs found in the neurological examination. Each item scores severity from 0 (no symptoms) to 4 (severe affectation), except the first item (level of consciousness) where the maximum score is 3. Subsequent clinical evolution regarding neurological sequelae was extracted taking the highest score from UWDRS III: mild for scores 0 and 1, moderate for score 2, and severe for scores 3 and 4.

The Brewer scale was a short adaptation of the UHDRS to evaluate the neurological symptoms of WD.

2.3 | Statistical analysis

The statistical analysis was descriptive and was carried out using the SPSS statistical package v17.0 for Windows.

The values from each evaluation scale were presented descriptively for each patient, before and 3 months after starting ATTM treatment.

3 | RESULTS

We present five patients affected of WD with neurological impairment treated as compassionate use with ATTM at a dose of 120 mg per day for 8 weeks. The characteristics of the five patients are summarized in Table 1 and detailed here below.

Patient 1: Man of 21 years. At the age of 12 years following an episode of acute hepatitis, he was diagnosed of WD with exclusive liver involvement and received treatment with D-penicillamine showing good progress and remaining asymptomatic. At 18 years old, he decided, alone, to quit medication and two years later he

TABLE 1 Participant characteristics

Patient	Gender	Age	Age of clinical onset	Clinical presentation	Reason for starting ATTM treatment
1	Male	21	12	Liver disease	Progression of symptoms one month after restarting treatment with D-penicillamine
2	Female	39	15	Liver disease	Intolerance to D-penicillamine (lupus) Progression although receiving treatment with trientine
3	Female	19	18	Neurological syndrome	WD with neurological involvement from start
4	Female	24	10	Neurological syndrome	Progression of symptoms although receiving treatment with zinc
5	Male	34	34	Neurological/psychiatric	WD with neurological involvement from start

Abbreviations: ATTM, ammonium tetrathiomolybdate; WD, Wilson's disease.

TABLE 2 Results by patient

Patient	GAS I for WD pre-ATTM	GAS I for WD post-ATTM	GAS II for WD pre-ATTM	GAS II for WD post-ATTM	UWDRSI pre-ATTM	UWDRSI post-ATTM	UWDRSII pre-ATTM
1	8	4	30	12	1	0	23
2	6	4	22	12	0	0	14
3	4	2	17	10	0	0	3
4	4	2	20	15	0	0	4
5	5	3	20	11	0	0	7

Note: Postmeasurements were carried out 2–3 months after starting ATTM.

Abbreviations: ATTM, ammonium tetrathiomolybdate; GAS, Global Assessment Scale; UWDRS, Unified Wilson's Disease Rating Scale; WD, Wilson's disease.

began to have depressive symptoms and paranoid ideation. One year later, there were motor and vocal tics, with dysarthria, generalized bradykinesia, and mild dystonic posture in left upper extremity. It was then restarted treatment with D-penicillamine with initial clinical improvement but after one month presented rapidly progressive neurological impairment. Treatment with zinc was initiated, but progressive impairment continued, and it was then decided to initiate treatment with ATTM.

Patient 2: A 39-year-old woman, diagnosed at 15 years of age of WD because of clinical chronic liver disease. She was treated with D-penicillamine between the ages of 15 and 21 years, when she developed a lupus-like syndrome due to this drug which was replaced by trientine. She remained asymptomatic until the age of 37 when she began to have a slow progressive neurological impairment with dysarthria, bradykinesia, and depressive mood. Treatment was then changed to zinc salts, but the progression of neurological impairment continued. Treatment with trientine was started again, but neurological impairment continued to progress. It was then decided to initiate therapy with ATTM. There was a neurological improvement, progressive deterioration was stopped, and the patient was stabilized. It was decided to extend treatment with ATTM eight weeks more.

Patient 3: A 19-year-old woman who attended our unit with dysarthria, dystonic postures in the left distal extremities (fingers and wrist bending and twisting of the foot when walking), and bilateral parkinsonism, symptoms consistent with neurological debut of WD. No previous treatment was administered. It was decided to start treatment with ATTM.

Patient 4: A 24-year-old woman with neurological debut at the age of 10, with progressive dysarthria, dystonia, and parkinsonism. She started treatment with D-penicillamine, but after 1–2 months, there was a severe neurological deterioration. Treatment was changed to zinc salts, with mild improvement with sequelae, but at the age of 24, there was a new severe neurological deterioration. It was then decided to start treatment with ATTM.

Patient 5: A 34-year-old man, who showed an alteration in hepatic function in an ambulatory analytical control without any other manifestation or symptom at the age of 32. Diagnosed at the age of 34, when she presented neurological symptoms, dysarthria, gait disturbance, progressive parkinsonism, with lesions in MRI. Knowing the neurological deterioration risk when starting chelation with D-penicillamine or trientine, and knowing the slow effect of zinc, it was decided to start treatment with ATTM.

All five patients received zinc salts after the 8 weeks of treatment with ATTM (16 weeks for Patient 2).

All five patients presented neurological clinical improvement between before and 3 months after starting treatment with ATTM, measured with UWDRS, GAS, and adapted Brewer scales for WD, as shown in Table 2.

There was also a neuroimaging improvement in 2/5 patients (Patients 2 and 3) with brain edema reduction 3 months after starting treatment with ATTM, although chronic necrotic lesions of basal ganglia were persistent (Figure 1).

In 1/5 patients (Patient 3), a mild anemia and leukopenia together with transaminases elevation was found three weeks after starting ATTM treatment, being discontinued for 1 week and then restarted at a lower dose (60 mg/day; Table 2). There was a remission of the analytical abnormalities, and the 8-week treatment could be finished with no more incidences.

In 1/5 patients (Patient 1), there was a transitory depression and paranoid ideation for a few days resolved spontaneously while maintaining the treatment.

No other adverse effects were found in any other patient.

In 3/5 patients (Patients 3, 4, and 5), the subsequent clinical evolution regarding neurological sequelae was mild (Table 2).

4 | DISCUSSION

Wilson's disease with neurological symptoms, although being a potentially manageable alteration actually, it can be highly incapacitating if the treatment is inadequate or is started too late once the symptoms are present (Walshe, 2009).

In our study, none of the five patients evidenced a neurological clinical progression after starting ATTM treatment. Moreover, there was a significant neurological clinical improvement demonstrated by the three scales used to evaluate the neurological symptoms of WD, except for the UWDRS I subscale (evaluating the level of

UWDRSII post-ATTM	UWDRSIII pre-ATTM	UWDRSIII post-ATTM	Brewer scale pre-ATTM	Brewer scale post-ATTM	Adverse effects	Subsequent clinical evolution
8	61	20	13	9	—	Death unrelated to treatment
6	39	14	12	8	—	Moderate neurological sequelae
1	25	10	11	8	Mild transaminase elevation, anemia, and leukopenia	Mild neurological sequelae
3	15	13	7	4	—	Mild neurological sequelae
3	32	24	11	6	—	Mild neurological sequelae

consciousness). All our patients presented a normal level of consciousness (baseline score of 0) except for one case with a baseline score of 1 (mild somnolence). This patient improved after ATTM treatment, being the level of consciousness of 0.

Clinical improvement is the expected result in WD patients starting an effective chelation therapy. A study carried out by Brewer (Brewer et al., 2003), evaluating the efficacy of ATTM, found that responding patients had a sustained significant improvement even one year after starting ATTM treatment.

In our case series, we reproduced the experience presented by Brewer in his initial work where 6 patients received ATTM as initial decoppering treatment with no worsening of acute neurological symptoms (Brewer et al., 1991). In our case series, there was a neurological clinical improvement in all five patients.

Furthermore, in our study, none of the patients presented neurological clinical progression, which also supports the evidence from the Brewer study (Brewer et al., 2006) that ATTM could lead a much lower rate of neurological progression associated with the start of the treatment than penicillamine (estimated in 50%; Brewer et al., 2003) and trientine (26%; Brewer et al., 2006), although there were very few patients. While all patients from the Brewer study in 2006 received zinc treatment together with trientine or ATTM for 8 weeks, being all patients hospitalized (probably due to the complexity of both medications administered concomitantly), patients from the Brewer study in 2003 received ATTM alone for 8 weeks as per his initial work from 1991 and our case series.

In the decoppering phase treatment of WD, chelators may induce further clinical deterioration in some treated patients. This is a paradoxical situation of great concern, whose treatment is a challenge. In our study, three of the five patients (Patients 1, 2, and 4) started ATTM treatment due to progression of the neurological symptoms although receiving treatment for WD with other drugs (penicillamine, trientine, and zinc, respectively). All three patients evidenced clinical improvement after starting ATTM treatment, which could be an indirect estimation of superiority of ATTM treatment in WD with neurological presentation with respect to the previous treatments as well as an option to rescue the neurological deterioration related to those treatments.

Concerning WD cranial lesions, some authors consider that improvement in radiological lesions in the cranial MRI is a good prognosis factor in response to the treatment (Prashkanth et al., 2005). In our study, the neuroimaging improvement found in 2 of the 5 patients after starting ATTM treatment was consistent with a clinical improvement.

The most frequent adverse effects reported after starting ATTM treatment in the Brewer study (Brewer et al., 2003) were mild myelosuppression in 5 of the 22 patients and transaminases increase in 3 cases. We found similar results in our 5 patients, where only one patient presented a mild myelosuppression (with mild anemia and leukopenia) and an increase of the hepatic enzymes. Hematological disorders are usually present between weeks 3 and 6 of the treatment (Brewer et al., 2003, 2006) and have been related to a possible effect of lack of copper due to excess chelating treatment (Brewer et al., 2003). Hepatic enzymes increase usually appears also after week 3 of treatment, and the cause is still unknown, but seems related to a direct effect of the drug. Both adverse effects disappear after a rest drug period (usually one week) and restarting the drug at half dose (60 mg/day; Brewer et al., 2003). The analytical disorders in our patient were normalized after discontinuing ATTM for 1 week, with no clinical progression evidenced during this period, and the treatment could be completed for 8 weeks with no further incidences.

ATTM was administered as initial decoppering treatment for 8 weeks. In one patient, treatment was extended eight weeks more, keeping the efficacy and safety, which is important as it allows to consider ATTM as a rescue treatment, achieving neurological improvement in patients intolerant to penicillamine or trientine treatment. ATTM would also be considered for use as maintenance treatment; however, this is hindered by the stability issues of this drug together with the too cumbersome treatment schedule with multiple daily dosing. However, the difficulties of ATTM treatment could possibly be solved with a new formulation of Bis-choline TTM (Brewer & Askari, 2005; Weiss et al., 2017; Swenson, 2019).

In summary, there are very limited data in the literature on ATTM and Wilson's disease (which is dominated by the Brewer publications), and given this scarcity, the present case series may be of interest to the community. A new aspect is that in our

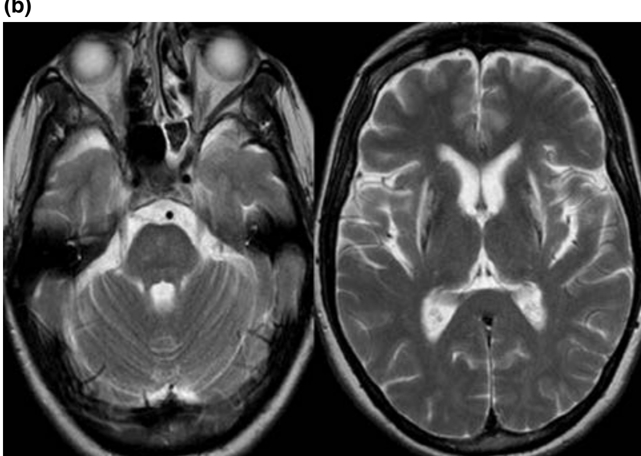

FIGURE 1 Neuroimaging improvement with brain edema reduction 3 months after starting treatment with ATTM and persistence of chronic necrotic lesions of basal ganglia. Patient 2. (a). Pre-ATTM brain MRI. Increase in signal intensity on T2-weighted images involving diffusely the pons (arrowhead), and the external border of lenticular nuclei (arrows). (b). Post-ATTM brain MRI. Observe the almost complete resolution of the diffuse pontine signal abnormalities, but persistent T2 signal abnormalities associated with atrophy, likely related to necrosis, of both putamina

experience, ATTM decoppering phase treatment for advanced neurological WD patients was effective without hospitalization and concomitant treatment with zinc. Although the study has only very few cases, it provides some more evidence to consider this drug as a good treatment for WD with neurological presentation and as rescue treatment for penicillamine, trientine, or zinc paradoxical neurological deterioration (Aggarwal et al., 2009; EASL, 2012; Roberts & Schilsky, 2008).

5 | CONCLUSION

Our data provide some more evidence to consider ATTM could be a good treatment for the initial treatment of WD with neurological symptoms and as a rescue treatment for penicillamine, trientine, or zinc paradoxical neurological deterioration, despite the potential adverse effects which, in our experience, were mild and reversible.

ACKNOWLEDGMENTS

The authors are grateful to all the patients for their participation. The authors thank Esther Pellicer for helping in writing the manuscript.

CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Oriol De Fabregues designed the study and performed organization and execution; data acquisition; analysis and interpretation of data; manuscript drafting; and manuscript revision. Jaume Viñas, Antoni Palasí, and Ignasi Cardona performed execution and data acquisition. Manuel Quintana performed descriptive analysis. Cristina Auger performed the MRI. Víctor Vargas revised the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: De Fabregues O, Viñas J, Palasí A, et al. Ammonium tetrathiomolybdate in the decoppering phase treatment of Wilson's disease with neurological symptoms: A case series. *Brain Behav*. 2020;10:e01596. https://doi.org/10.1002/brb3.1596

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Anticancer response to disulfiram may be enhanced by co-treatment with MEK inhibitor or oxaliplatin: modulation by tetrathiomolybdate, KRAS/BRAF mutations and c-MYC/p53 status

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Research

Abstract

Ammonium tetrathiomolybdate (TTM) and disulfiram (DSF) are copper (Cu) chelators in cancer clinical trials partly because Cu chelation: a) restricts the activity of Cu-binding MEK1/2 enzymes which drive tumourigenesis by KRAS or BRAF oncogenic mutations and b) enhances uptake of oxaliplatin (OxPt), clinically used in advanced KRAS-mutant colorectal carcinomas (CRC). Whereas TTM decreases intracellular Cu trafficking, DSF can reach other Cu-dependent intracellular proteins. Since the use of individual or combined Cu chelation may help or interfere with anti-cancer therapy, this study investigated whether TTM modifies the response to DSF supplemented with: 1) UO126, a known MEK1/2 inhibitor; 2) other Cu chelators like neocuproine (NC) or 1, 10-o-phenanthroline (OPT) in wt p53 melanoma cells differing in BRAF or KRAS mutations; 3) OxPt in mutant p53 CRC cells devoid of KRAS and BRAF mutations or harbouring either KRAS or BRAF mutations. TTM was not toxic against $^{\text{V600E-}\textit{mut-BRAF}}$ A375 and $^{\text{G12D-mut-}\textit{KRAS/high c-myc}}$ C8161 melanoma cells. Moreover, TTM protected both melanoma types from toxicity induced by DSF, NC and co-treatment with sub-lethal levels of DSF and the MEK inhibitor, UO126. Toxicity by co-treatment with DSF+OPT was poorly reversed by TTM in C8161 melanoma cells. In contrast to the greater toxicity of 0.1 μM DSF against mutant p53 CRC cells irrespective of their KRAS mutation, TTM did not protect $^{\text{G12V-mut-}\textit{KRAS/high c-myc}}$ SW620 CRC from DSF+OxPt compared to $^{\text{KRAS-WT/BRAF-WT}}$ Caco-2 CRC. Our results show that DSF co-treatment with: a) MEK inhibitors may enhance tumour suppression; b) OxPt in CRC may counteract impaired response to cetuximab by KRAS/BRAF mutations and c) as a single treatment, TTM may be less effective than DSF and decreases the efficacy of the latter.

Published: 08/01/2019 Received: 14/08/2018

***e*cancer** 2019, **13**:890 https://doi.org/10.3332/ecancer.2019.890

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Highlights

(a) Potentiation of melanoma antitumour toxicity of DSF by MEK inhibitor is reversed by TTM.
(b) KRAS/c-MYC dysregulation attenuates TTM reversion of melanoma toxicity by DSF + OPT.
(c) KRAS/c-MYC dysregulation increases melanoma NC toxicity reversed by TTM.
(d) BRAF mutation and lower c-MYC may attenuate toxicity by DSF ± OxPt in colorectal cancer cells

Keywords: *Cu chelation, Cu-MEK activation, KRAS/c-MYC, p53 status, oxaliplatin, tetrathiomolybdate, disulfiram*

Introduction

The copper (Cu) dependence of MEK1/2 dysfunctional signalling is an important target to inhibit tumour cells with BRAF or KRAS mutations [1, 2]. In BRAF-(V600E)-mutated melanoma, pharmacological Cu sequestration with a clinically used copper chelator, ammonium tetrathiomolybdate (TTM) inhibits MEK1/2 kinase activity and reduces mutant BRAF-driven growth in melanoma cell lines resistant to BRAF or MEK1/2 inhibitor [3]. In contrast to the initial response rates (>50%) of BRAF inhibitor monotherapy in *BRAF*^{V600}-mutant melanoma, approximately 5% of patients with *BRAF*^{V600E} colorectal cancer respond. Preclinical studies suggest that the lack of efficacy in *BRAF*^{V600E} colorectal cancer is due to adaptive feedback reactivation of mitogen-activated protein kinase signalling, often mediated by epidermal growth factor receptor (EGFR) [4]. Diminished response to treatment with anti-EGFR monoclonal antibodies is found in KRAS-mutant or BRAF-mutant colorectal cancer [5–8] but these mutations do not impair response to oxaliplatin- (OxPt) or cisplatin-based chemotherapy [9, 10]. Response to platinum (Pt)-based anti-cancer drugs involves enhancing its uptake, through the copper (Cu) transporter protein hCTR1 [10, 11] whose expression is increased by Cu chelators like TTM. The latter has been touted as an anti-cancer agent [3], [12–15] because its Cu chelation enhances uptake of cisplatin or OxPt [14, 15]. However, there are some contradictions regarding the beneficial anti-tumour effects of TTM. At low 3-µM concentration, it had no significant effect on cell viability but synergised with 10-µM cisplatin against breast cancer cells [14]. In colorectal cancer cells, 10-µM TTM increased expression of the hCTR1 protein in DLD-1 and SW620 cells but only potentiated 100-µM OxPt cytotoxicity in DLD-1 but not in SW620 cells [16]. In prostate cancer, another Cu chelator, clioquinol selectively targeted and rapidly destroyed tumour prostate lines without harming primary prostate epithelial cells but this Cu-dependent toxicity of clioquinol was abrogated by TTM [17]. In other studies, TTM above 2.5 µM inhibited the growth of some androgen-receptor prostate cancer cells [18]. In the same study, the most disappointing finding was that TTM treatment also inhibited the growth of non-neoplastic prostate epithelial RWPE-1 cells, concluding that TTM chelation by itself was not a viable therapeutic option for prostate cancer [18]. However, the same authors found that androgens enhanced Cu uptake and proliferation by prostate cancer cells, but both of these changes were more effectively suppressed by disulfiram (DSF), another FDA-approved Cu ionophore, quoted as most effective when co-administered with Cu [18]. Similar benefits of DSF against prostate cancer were reported by others [19, 20]. However, TTM almost completely blocked DSF-Cu-induced cell death in SUM149 and rSUM149 inflammatory breast cancer cells, highlighting the importance of Cu binding for enhancement of DSF's cytotoxic effects [20]. Although both DSF and TTM are Cu chelators, TTM decreases intracellular Cu trafficking [22], unlike DSF which can reach other Cu-dependent intracellular proteins [19, 22]. These apparently contradictory data between TTM and DSF imply that antitumour activity is not simple Cu chelation by TTM [13–16] but rather a gain of function seen after DSF is taken up and subsequently is free to redistribute itself by cancer cells, to increase reactive oxygen species production, under the reductive intracellular environment [18–21, 23]. Hence, this report investigated the individual or combined toxicity of TTM and DSF, in combination with some other Cu chelators or with UO126, a specific MEK inhibitor [24, 25] in some human melanomas. Aiming to avoid collateral Cu toxicity, our earlier study aimed to augment Cu chelator without exogenous Cu supplementation [26] using wt p53 C8161 melanoma cells lacking the ^{V600E} BRAF mutation compared to wt p53 A375 human melanoma harbouring this typical BRAF oncogenic mutation [26]. Since then, *genetic analysis showed an atypical* ^{G464E} mutation in the BRAF P loop region, accompanied by an enhancing ^{G12D} KRAS common oncogenic mutation [27] which adds to higher c-MYC expression in C8161 melanoma compared to A375 cells [28]. We also investigated whether other Cu chelators or MEK inhibitors behaved like TTM or DSF in wt p53 melanomas differing in KRAS/c-MYC or BRAF status. Since both KRAS and BRAF mutations drive tumour cell proliferation by Cu-dependent MEK1/2 kinase activation through different responses in melanomas [4] or colorectal carcinomas

(CRC) [5], we also studied the response to TTM and/or DSF in mutant p53 CRC with mutant KRAS [29] and high c-MYC [30] compared to CRC cells with a BRAF-mutant [29] low c-MYC [30] status and another mutant p53 CRC harbouring wt BRAF and wt KRAS [29].

Materials and methods

Cells

(a) wt p33 C8161 melanoma has a G464E mutation in the BRAF P loop region, accompanied by an enhancing KRAS G12D mutation [27]. c-MYC expression was found to be six-fold greater in C8161 cells than in A375 cells [28].
(b) wt p53 A375 melanoma [CRL-1619] with a homozygous BRAF (V600E) mutation was obtained from the American Type Culture Collection [28].
(c) SW620 colorectal cancer cells harbour two p53 mutations (pR273H; P309S), a KRAS (pG12V) homozygous mutation [29] and have a 6-fold high c-MYC amplification relative to placental DNA [30].
(d) HT-29 colorectal cancer cells harbour a homozygous p53 mutation (pR273H), a heterozygous BRAF (V600E) mutation [29] and only have a two-fold high c-MYC amplification relative to placental DNA [30].
(e) Caco-2 colorectal cancer cells harbour a p53 mutation (E204X) and are wild-type for KRAS, BRAF, PIK3CA and PTEN [29]. These cells undergo enterocytic differentiation, decreasing their c-MYC expression in response to butyrate [31]

These cell lines were kept in complete Dulbecco's containing medium supplemented with 10% foetal calf serum. Although this medium practically does not have Cu supplementation, when supplemented with 10% serum, it provides sufficient copper for cell growth and survival, approx. 50–100 ng/mL since serum albumin is a physiological Cu transporter [http://www.sigmaaldrich.com/life-science/cell-culture/learning-center/mediaexpert/copper.htm]. Whenever indicated, cultures were seeded overnight at 5 × 103 cells per 96 well plates in octuplicates and treatments were added 20 hours after, for a further 24 hours.

Relative cell viability/metabolic activity

This was estimated with Alamar Blue (resazurin) by measuring intracellular redox mitochondrial activity by quantitating the cell-catalysed conversion of non-fluorescent resazurin to fluorescent resorufin [26]. Alamar Blue was added to a 10% final concentration to each one of 96 well plates after the appropriate treatment. This assay is valuable as an endpoint of proliferation or relative viability/metabolic activity. For these experiments, cells (5,000) were allowed to adhere overnight in 96 well tissue culture plates. After the corresponding treatments, Alamar Blue (BioSource, Camarillo, CA, USA) was added without removing medium containing dead cells and fluorescence measured 4 hours later in a Fluoroskan Ascent microplate reader with an excitation of 544 nm and an emission of 590 nm.

Quantitation of CRC survival by infrared fluorescence of crystal violet stained cells

Fixed cells surviving after the indicated treatments were washed, then fixed in 70% ethanol. Subsequently, the same cells were stained with crystal violet [26] and the relative ratio of surviving cells was quantitated by crystal violet infrared detection with an Odyssey CL-x infrared imaging system, using *ImageStudio Ver 5.0.21* quantitation software.

Statistical analysis

All experiments were performed in octuplicates (n = 8), t-tests were used in Alamar Blue quantitation assays, in which the criteria for statistical significance was taken as ps r st, whenever indicated by *. Analysis of Variance (ANOVA) tests with Tukkey *posteriori* analysis were used for infrared quantitation of crystal violet stained CRC cells, in which the criteria for statistical significance were also taken as ps o ta, whenever indicated by *.

Results

Toxicity induced by neocuproine ± DSF is similarly reversed by TTM in both C8161 and A375 cells

We used neocuproine (NC), another membrane permeable Cu (I) chelator, also known as 2, 9-dimethy l−1, 10-phenanthroline [32] to ask whether: a) it also competed with DSF and b) its toxicity was also antagonised by 3-μM TTM. These studies revealed that: a) 0.25-μM NC was similarly toxic to both melanoma types, b) its activity was further enhanced by 0.1-μM DSF and c) that its activity was blocked by 3-μM TTM, even when NC was added together with DSF (Figure 1a and b).

Toxicity by co-treatment with sublethal DSF and the copper (II) chelator 1, 10-phenanthroline is reversed preferentially by 2.5-μM TTM in A375 cells compared to C8161 cells

1, 10-orthophenanthroline (1, 10-OPT), a cell-permeable chelator of Cu2+ [33, 34], was also tested for its anti-melanoma activity at 2.5 μM, either by itself or in conjunction with 0.1 DSF and/or with 2.5-μM TTM. As expected, no significant toxicity was observed in metabolic activity assays or in survival crystal violet assays when C8161 or A375 cells were exposed to 0.1-μM DSF, unless it was supplemented with 2.5-μM 1, 10-OPT. However, the addition of 2.5-μM TTM once again reversed the toxicity induced by co-treatment with 0.1-μM DSF and 2.5 μM 1, 10-OPT. However, TTM attenuation of the toxicity of the other two Cu chelators was greater in $^{(V600E)\ mut\ BRAF}$ A375 cells compared to $^{G12V\text{-}mut\ KRAS\ /\ high\ c\text{-}MYC}$ C8161 cells (Figure 2a and b).

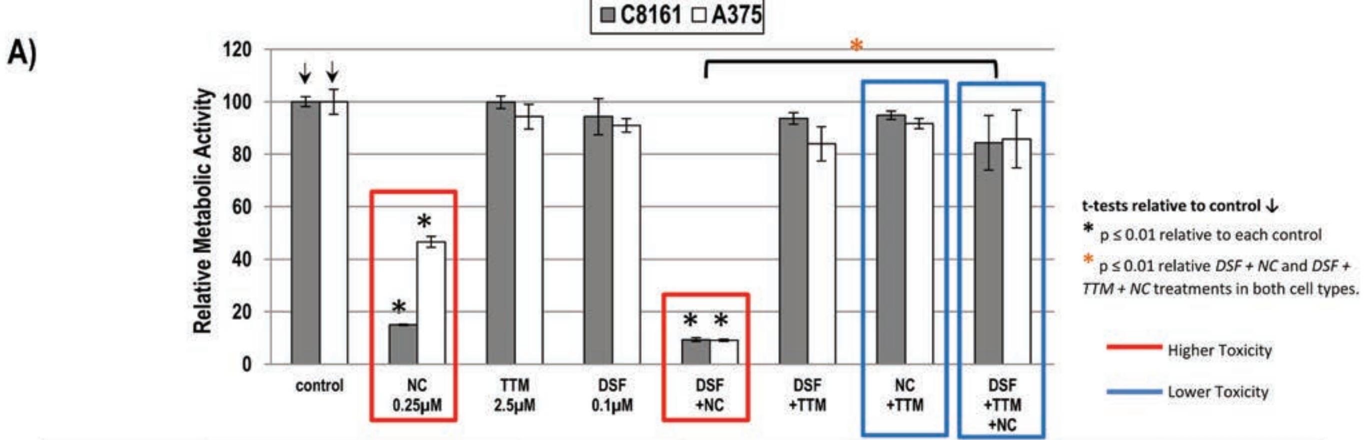
Toxicity by co-treatment with sublethal DSF and MEK inhibitor, UO126 is reversed by TTM in both C8161 and A375 cells

Since Cu chelation decreases the Cu dependence of MEK1/2 activation for KRAS [1] or BRAF [2, 3] optimal oncogenic signalling, and U0126 selectively binds and inhibits MEK-1/2 [24] but also protects from oxidative stress [25], we hypothesised that sub-toxic Cu chelation together with UO126 would diminish non-specific toxicity preserving anti-tumour activity against melanoma cells with KRAS or BRAF mutations. Using 100-nM DSF or 5-μM UO126 as single agents did not suppress metabolic activity or survival of C8161 or A375 cells. However, these cells greatly diminished metabolic activity adding together the indicated sub-lethal concentrations of these two agents, in a reaction reversed by TTM (Figure 3a and b).

Toxicity induced by co-treatment with sublethal DSF ± OxPt is attenuated by TTM in Caco-2 cells but not in SW-620 cells

Since the response to cetuximab in CRC is impaired by KRAS/BRAF mutations [4–6], these do not affect their response to OxPt [7, 8] since the latter is incorporated through the hCTR1 transporter activated by Cu chelation. Hence, three different CRC were assayed for their response to DSF ± OxPt in the presence or absence of 3-μM TTM. There was no toxicity against the three CRC tumour cells tested when using TTM as a single treatment. Survival in $^{KRAS\ (G12V)/p53\ mut}$ SW-620 cells with high c-MYC amplification by single OxPt approximated 52.9%, with DSF treatment permitting a 35% survival, which was further decreased when DSF + OxPT (24.5%) and was not counteracted by TTM (Figure 4, left). No comparably significant toxicity in response to DSF ± OxPt with or without TTM was evident in $^{BRAF\ (V600E)/\ p53\ mut}$ HT-29 cells with a lower c-MYC amplification (Figure 4, centre). In contrast, significant growth inhibition by DSF alone or when combined with OxPt (45.7 %) was partly antagonised by TTM (71.9 %) resembling the greater survival seen with OxPt treatment in $^{KRAS\ WT/\ BRAF\ WT/\ p53\ mut}$ Caco-2 cells (Figure 4, right).

Research



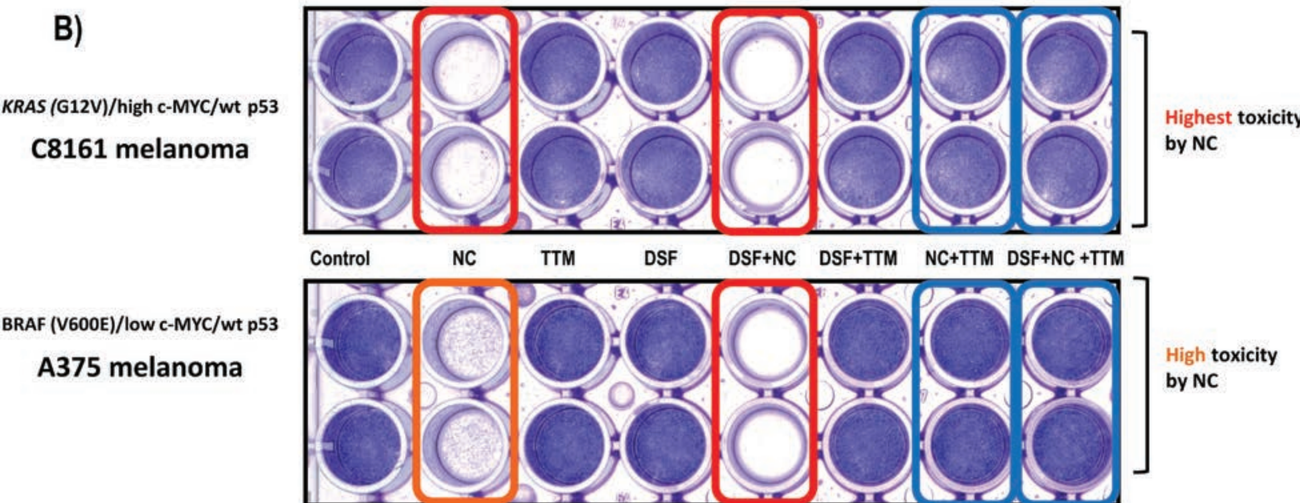
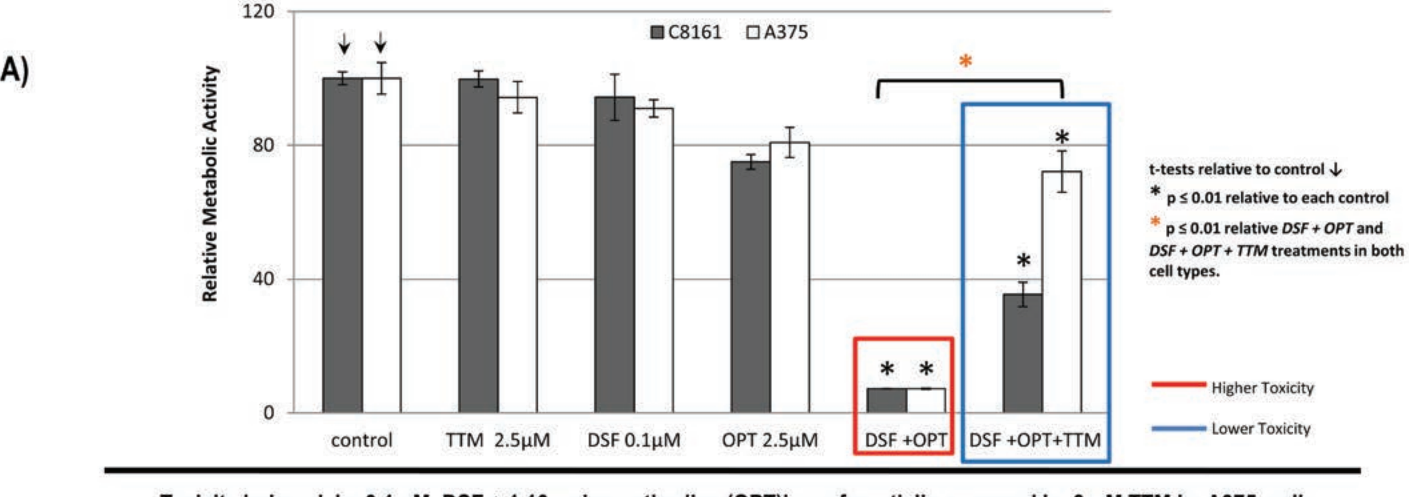


Figure 1. (a): Changes in metabolic activity/cell viability were estimated in sub-confluent cells seeded overnight followed by exposure to the treatments indicated for 72 hours in 96 well plates (*n* = 8), using the Alamar Blue resazurin/resorufin fluorometric assay described under methods. Results shown are representative of three different assays. (b): Differences in cell survival were assayed after the indicated treatments for 72 hours by fixing cells with 70% ethanol and staining with crystal violet, as described under methods.

Inhibition of metabolic activity by 0.1 µM DSF + 1,10-phenanthroline (OPT) is antagonized by 3 µM TTM preferentially in A375 cells



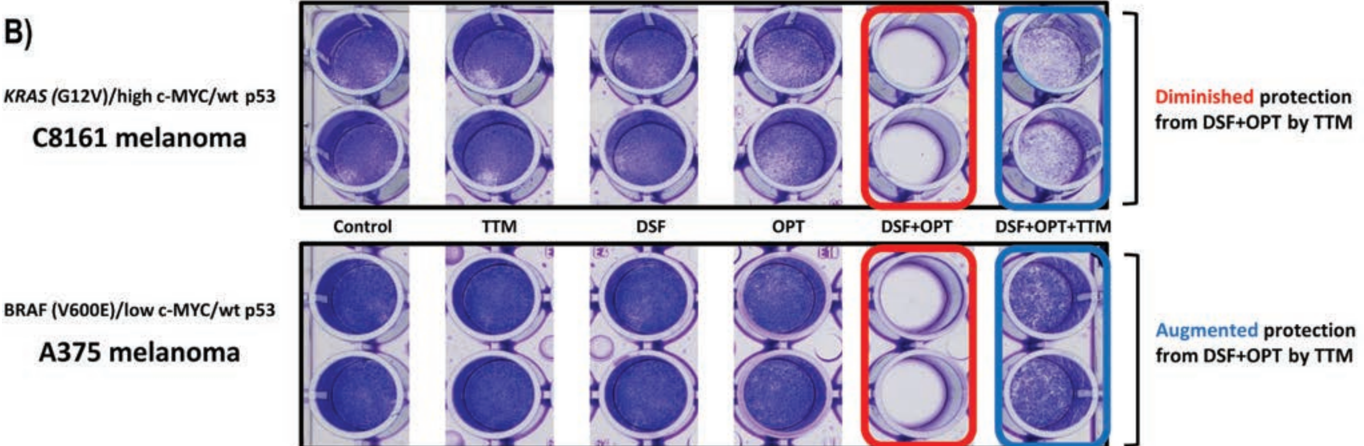


Figure 2. (a): Sub-confluent cells seeded overnight in octuplicates were exposed to the treatments indicated for 72 hours in 96 well plates (*n* = 8). Changes in metabolic activity/cell viability were then measured fluorometrically with Alamar Blue. Results shown are representative of three different assays. (b): Differences in cell survival were assayed after the indicated treatments for 72 hours by fixing cells with 70% ethanol and staining with crystal violet, as described under methods.

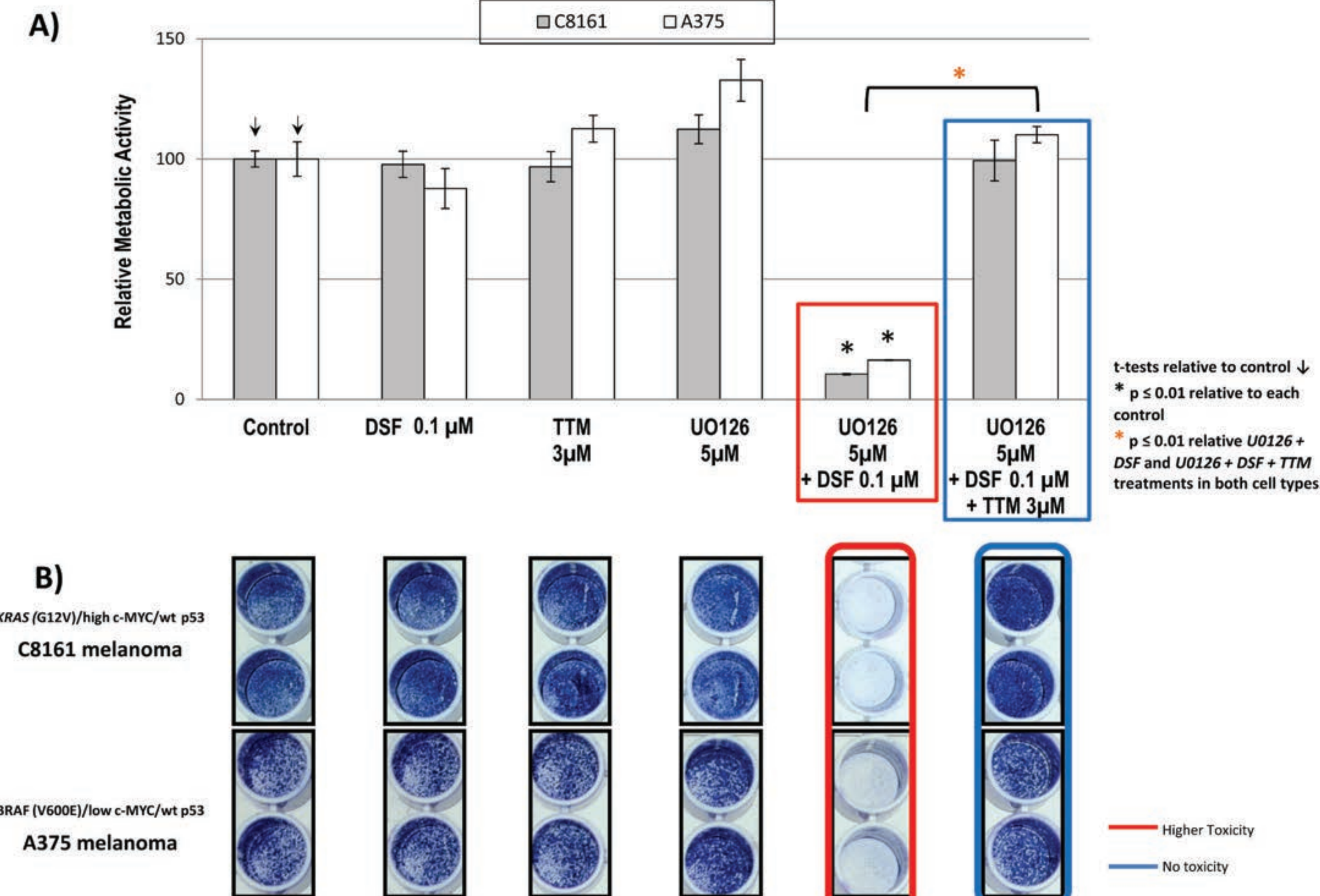


Figure 3. (a): Changes in metabolic activity/cell viability were estimated in sub-confluent cells seeded overnight followed by exposure to the treatments indicated for 72 hours in 96 well plates (*n* = 8), using the Alamar Blue resazurin/resorufin fluorometric assay described under Methods. Results shown are representative of three different assays. (b): Differences in cell survival were assayed after the indicated treatments for 72 hours by fixing cells with 70% ethanol and staining with crystal violet, as described under methods.

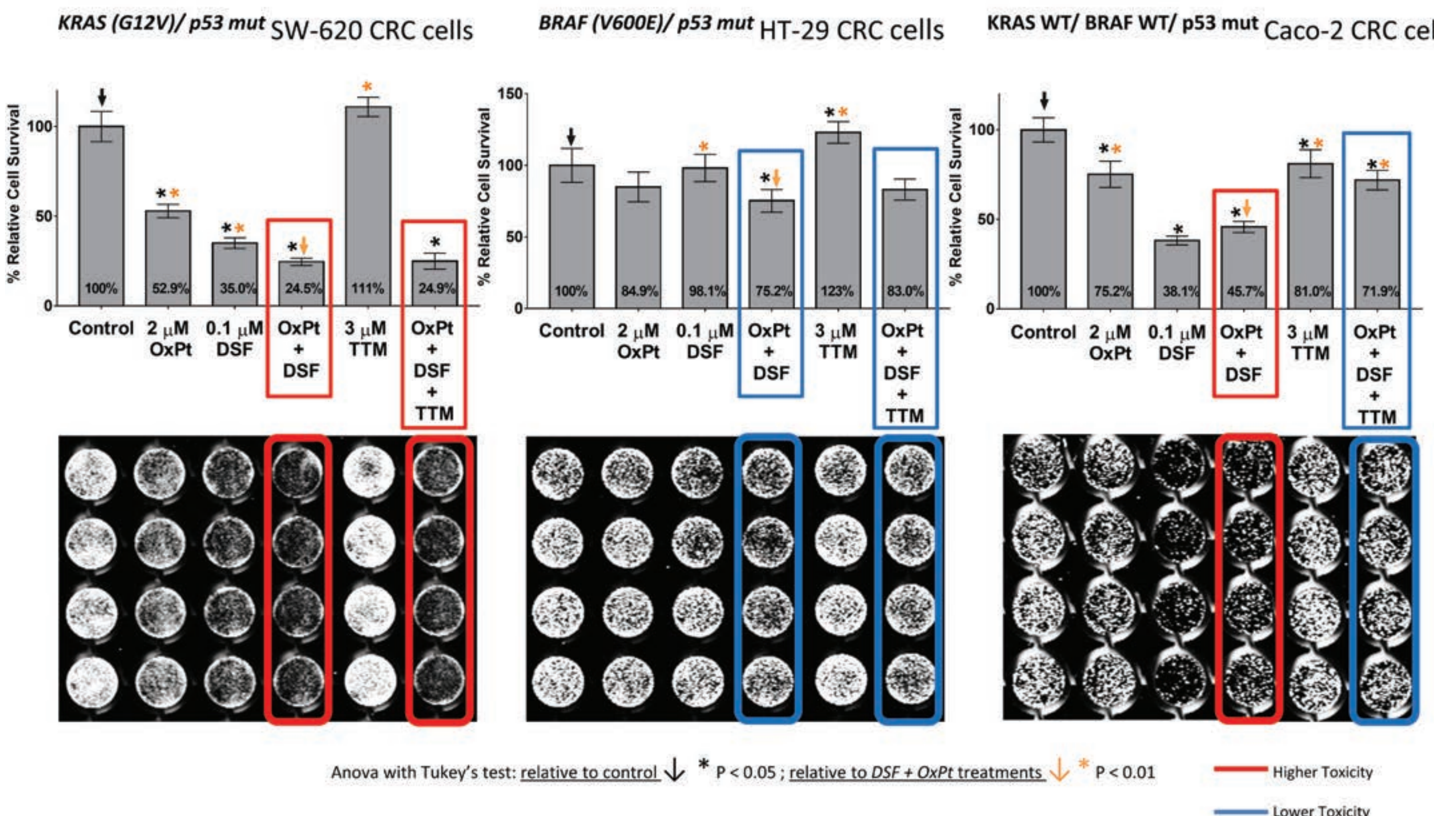


Figure 4. Differences in cell survival were estimated in colorectal cancer cells seeded to subconfluency overnight followed by exposure to the indicated treatments for 72 hours in 96 well plates (*n* = 8), followed by fixing cells with 70% ethanol, staining with crystal violet and infrared quantitation as described under methods. Results shown are representative of three different assays. Note the significant toxicity to DSF ± OxPt, which was not counteracted by TTM in *KRAS (G12V)/ p53 mut* SW-620 cells (left). Attenuated response to DSF ± OxPt with or without TTM in BRAF (V600E)/ p53 mut HT-29 cells (centre) and growth inhibition by DSF ± OxPt, partly antagonised by TTM in *KRAS WT/ BRAF WT/ p53 mut* Caco-2 cells (right).

Discussion

This study examined whether anti-cancer response was enhanced or interfered with by co-treatment between two structurally different Cu chelators currently used in clinical trials like a) TTM [A Phase II Study of *Tetrathiomolybdate* in Patients With Breast Cancer at Moderate to High Risk of Recurrence. ClinicalTrials.gov Identifier NCT00195091; study completion date: June 2020] and b) DSF [Copper Chloride, *Disulfiram*, and Copper Gluconate in Treating Patients with Metastatic Castration-Resistant Prostate Cancer. Clinicaltrials.gov Identifier NCT02963051 study completion date: August 2020] and Phase II Trial of *Disulfiram* With Copper in Metastatic Breast Cancer ClinicalTrials.gov Identifier NCT03323346; study completion date September 2020]. Since KRAS mutation and c-Myc amplification cooperate with KRAS in tumourigenesis [35–37], this report used cell lines differing in BRAF, KRAS and C-MYC status to gain insight into their modulation of response to Cu chelators. We found that 2.5-μM TTM or low 0.1-μM DSF did not suppress growth and metabolic activity in two wt p53 human melanoma cells harbouring KRAS mutation and high c-MYC (C8161) or BRAF mutation and low c-MYC (A375) (Figure 1). In contrast, 0.25-μM NC—another Cu chelator—preferentially inhibited metabolic activity to approximately 20% of its control in C8161 cells with KRAS mutation and high c-MYC expression compared to a decrease to about 40% of its control in A375 cells with BRAF mutation and low c-MYC (Figure 1a). More importantly, the synergism between two Cu chelators like NC and 0.1-μM DSF cells was notably reversed by TTM, another Cu chelator, to a similar extent in both C8161 and A375 melanoma cells (Figure 1).

However, when using another Cu chelator like 0.25-μM OPT, it preferentially killed C8161 cells compared to A375 cells. However, in C8161 cells with concomitant KRAS/c-MYC dysregulation, TTM reversion of melanoma toxicity by DSF + OPT (Figure 2) was diminished. We also show for the first time that the synergism between sub-lethal levels of DSF and the highly specific MEK inhibitor UO126 [24] against melanoma cells, irrespective of BRAF or KRAS/c-MYC status, This synergism may be partly explained by DSF chelating activity sequestering Cu [21] required as a MEK1/2 co-activator in KRAS-[1] or BRAF-mediated oncogenic signalling [2, 3] and MEK inhibitors like UO126 [24] directly binding to MEK1/2. Such synergism was also antagonised by TTM (Figure 3). Moreover, TTM not only inhibited the ability of 0.1-μM DSF to synergise with other Cu chelators (Figures 1–3) but also when DSF used at the toxic 0.3-μM concentration against C8161 and A375 cells (Figure S1). The different response between Cu chelators like DSF and TTM to MEK inhibitors or OxPt may be partly linked to the latter decreasing intracellular Cu trafficking [22], whereas DSF promotes Cu intracellular redistribution and greater bioavailability [19]. Although exogenous Cu supplementation has been widely used by others to augment the efficacy of TTM [3, 12–15] and DSF [18, 20, 21], we reported that DSF was much more effective than TTM as an anticancer agent even without Cu supplementation to avoid collateral Cu toxicity [26]. Another study investigating if elevated copper enhances the efficacy of the anti-cancer drug, imatinib (ITB), also showed that DSF was more effective than high Cu (II) as an adjuvant to ITB [37], confirming our belief that restrained manipulation of copper level in tumour may lead to a more selectively targeted killing of tumour cells and diminished collateral toxicity. DSF also showed greater efficacy against SW-620 and Caco-2 CRC cells, compared to the relatively greater resistance to DSF ± OxPt in $^{BRAF\ (V600E)/\ p53\ mut}$ HT-29 CRC cells. It was also noteworthy that TTM attenuated toxicity by DSF ± OxPt preferentially in $^{KRAS\ WT/\ BRAF\ WT/\ p53\ mut}$ Caco-2 cells compared to $^{KRAS\ (G12V)/\ p53\ mut/\ high\ c\text{-}MYC}$ SW-620 cells (Figure 4). Although this study is still at an early stage, our results suggest that mutant KRAS/high c-MYC amplification [38] may change the anti-tumour response to some specific Cu chelators in wt p53 C8161 and A375 melanoma, which are compatible with others who found that c-MYC deregulation without elevated expression cooperates with KRAS^{G12D} mutation to accelerate tumourigenesis [35, 36, 38]. *However, the cooperation between mutant KRAS and extent of c-MYC amplification may be different in the CRC cells harbouring a mutated tumour suppressor p53 gene, used in these studies* (Figure 5). Taken together, our findings are the first to show that Cu sequestration may be necessary but not sufficient for anti-cancer activity, given that TTM which binds but does not release intracellular Cu [19, 22] did not significantly inhibit any of the tumour cells tested but rather suppressed the inhibition caused by other Cu chelators.

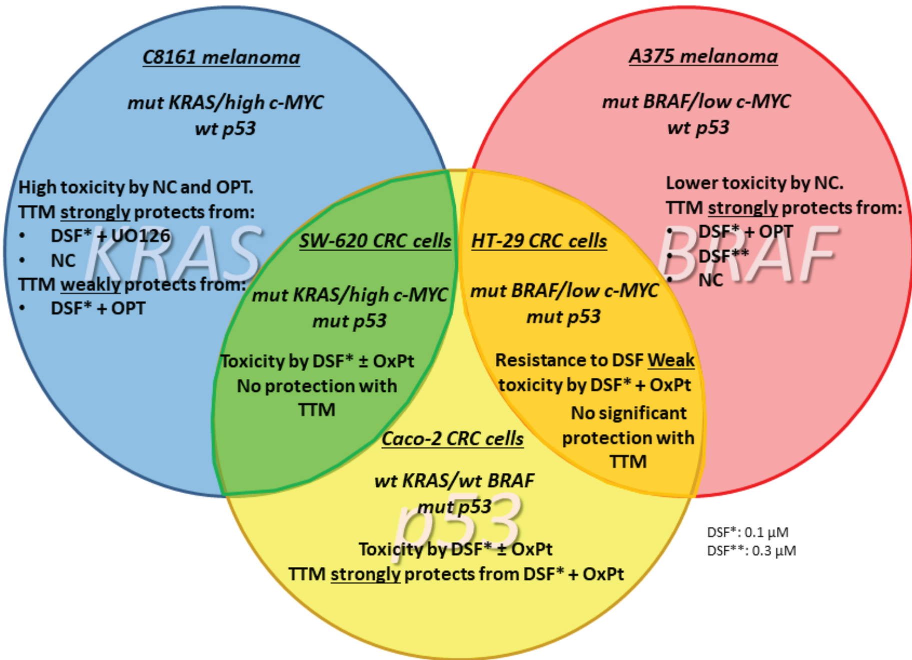


Figure 5. Summary. KRAS/c-myc dysfunction influences the anticancer response to DSF, TTM and OxPt.

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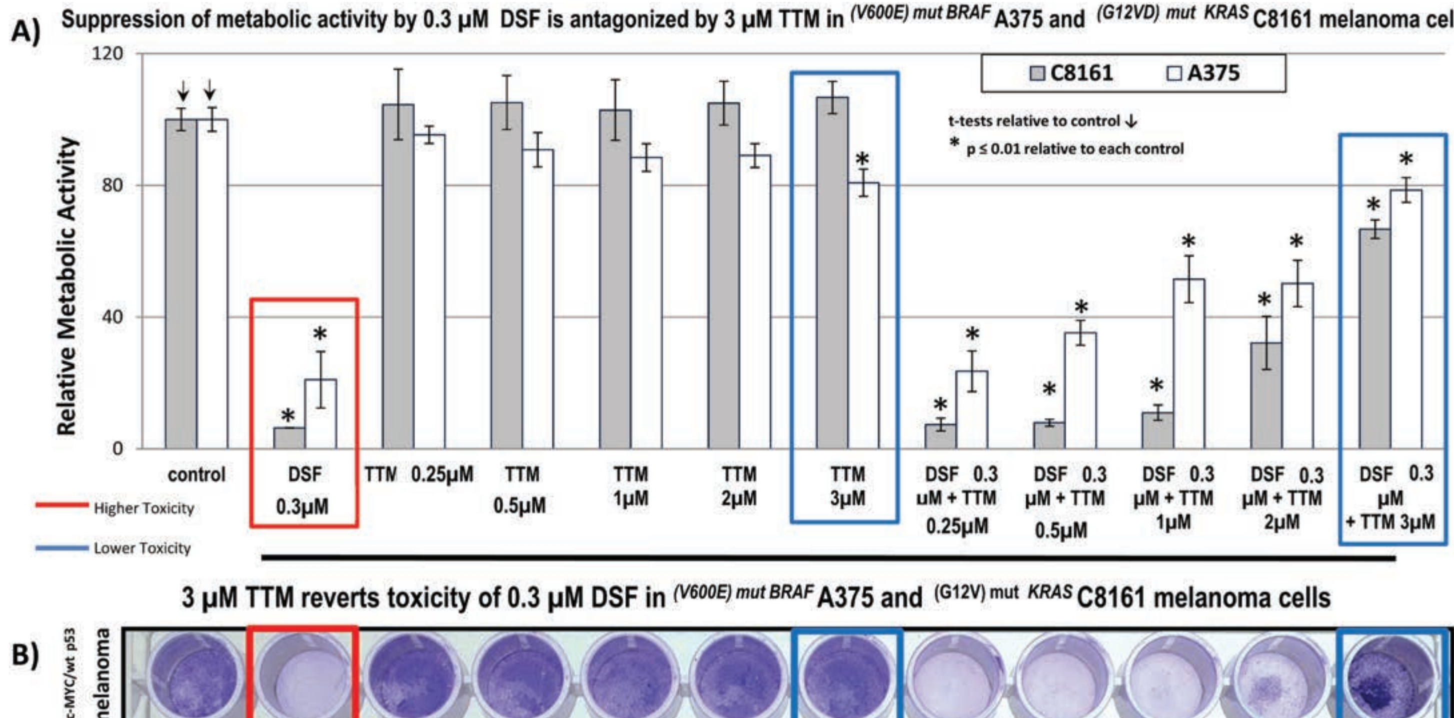

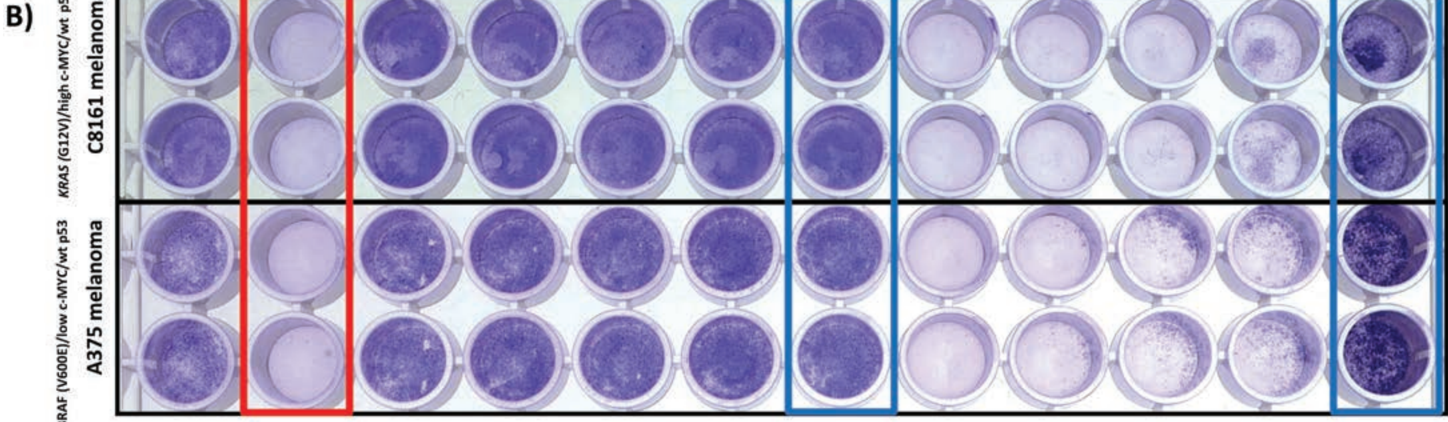

Figure S1. (a): Suppression of metabolic activity by 0.3-µM DSF is antagonised by 3-µM TTM in ***(V600E) mut BRAF*** **A375 and** ***(G12VD) mut KRAS*** **C8161 melanoma cells. (b). 3-µM TTM reverts toxicity of 0.3-µM DSF in** ***(V600E) mut BRAF*** **A375 and (G12V-)mut** ***KRAS*** **C8161 melanoma cells.**

Conclusion

In summary, our studies imply that DSF may be the most clinically promising anti-cancer Cu chelator. DSF re-purposing from its long-term clinical use against alcoholism to an anti-cancer drug is based on its ability to act as an ALDH1A1/ALDH2 inhibitor [39], a property not shared by other Cu chelators like TTM. ALDH enzymes are implicated in the breakdown of acetaldehyde to acetate, an obligatory step in alcohol metabolism. DSF inhibition of ALDH1A1/ALDH2 activity also prevents removal and increases cellular acetaldehyde accumulation, and selective DNA damage, both in proliferating and slower replicating cancer stem cells, which frequently have genomic instability and high oxidative stress [39, 40] in contrast to their normal cell counterparts.

Author contributions statement

Ali Calderon-Aparicio carried out the melanoma experiments, cooperated with Alejandro Cornejo in the CRC experiments and helped in the Discussion. Alejandro Cornejo carried out the statistical analyses, designed the Graphic Abstract and helped to improve the Discussion. Andrea Orue participated in the Discussion and final revision of this manuscript. Manuel Rieber designed, supervised, provided funding and wrote the final version of this paper.

Financial disclosure

This research was funded by Fonacit-Misión Ciencia sub-proyecto Sistema Publico Nacional de Salud 4-Cancer grant to Manuel Rieber at Instituto Venezolano de Investigaciones Cientificas. The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

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Ammonium tetrathiomolybdate enhances the antitumor effects of cetuximab via the suppression of osteoclastogenesis in head and neck squamous carcinoma

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Received September 21, 2017; Accepted January 4, 2018

DOI: 10.3892/ijo.2018.4242

Abstract. Head and neck squamous cell carcinoma (HNSCC) poses a significant challenge clinically where one of the mechanisms responsible for the invasion into facial bones occurs via the activation of osteoclasts. Copper has been demonstrated to play a key role in skeletal remodeling. However, the role of copper in cancer-associated bone destruction is thus far unknown. Lysyl oxidase (LOX) is a copper-dependent enzyme that promotes osteoclastogenesis. In the present study, we investigated the effects of copper on HNSCC with bone invasion by the copper chelator, ammonium tetrathiomolybdate (TM) *in vitro* and *in vivo*. We demonstrate that TM blocks the proliferation of HNSCC cells, inhibits LOX activation and decreases the expression of the receptor activator of nuclear factor-κB ligand (RANKL) in osteoblasts and osteocytes, subsequently suppressing bone destruction. These findings suggest that copper is a potential target for the treatment of HNSCCs associated with bone destruction.

Introduction

Head and neck squamous cell carcinoma (HNSCC) frequently invades the facial bones, and this invasion is a prognostic factor for poor clinical outcomes (1,2). Bone resection is a treatment that often leads to the post-operative disruption of speech and swallowing functions, and thus poses a significant challenge to the quality of life of patients with HNSCC presenting with facial bone invasion. Cancer cells have been demonstrated to secrete significant amounts of growth factors, which promotes osteoclastogenesis (3). Therefore, it is critical that novel approaches should be evaluated for the treatment of bone destruction in advanced HNSCC. Copper is known as a key factor for cellular metabolism, neuronal transmission and bone remodeling (4,5). Copper metabolic disorder induces Wilson's disease, a rare inherited disorder that causes copper to accumulate in the liver, brain and other vital organs (6). The copper chelator, ammonium tetrathiomolybdate (TM), is used for the treatment of copper metabolic disorder and Wilson's disease. In addition, recent research has revealed that copper chelators exert an antitumor effect against several cancer types, such as breast cancer with lung metastases and head and neck cancer (7,8).

Copper is a factor that binds to selected enzymes and functions to increase their activation. For example, Lysyl oxidase (LOX) is the prototypical member of copper-dependent enzymes whose documented function is to oxidize primary amine substrates to reactive aldehydes (9). The most well-characterized role of LOX is in the remodeling of the extracellular matrix (ECM) through the oxidative deamination of peptidyl lysine residues in collagens and elastin to facilitate covalent cross-linking (10). It has also been reported that LOX is essential for bone remodeling via the regulation of receptor activator of nuclear factor-κB ligand (RANKL) expression on bone marrow stromal cells (11,12). Cancer cells release significant amounts of LOX (13). This copper-dependent LOX activation may promote bone resorption; however, the role of copper in bone resorption in HNSCC remains unclear and thus requires clarification.

In the present study, we thus aimed to determine the role of copper in bone resorption in HNSCC. To the best of our knowledge, we are the first to provide evidence that the copper chelator, TM, exerts an anti-bone destruction effect against cancer-induced bone resorption and that TM enhances the antitumor effect of a clinically validated anticancer agent on HNSCC associated with bone invasion.

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Key words: copper, head and neck squamous cell carcinoma, bone invasion, osteoclastogenesis

Materials and methods

Cell lines and culture conditions. The human HNSCC cell lines, HSC-2 (#JCRB0622), HSC-3 (#JCRB0523) and SAS (#JCRB0260), were obtained from the Human Science Research Resources Bank (Osaka, Japan). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Primary fibroblasts were obtained from Cosmo Bio (#SCR2620, Tokyo, Japan).

Primary osteoblasts, osteocytes, bone marrow cells (obtained as described below), and fibroblasts were cultured in alpha-modification of minimum essential medium (α-MEM). T cells (obtained as described below) were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin, 10 mM 2-mercaptoethanol (Sigma, St. Louis, MO, USA) and Pyruvic acid (Wako, Osaka Japan). All of the above-cited cell lines were characterized by genotyping at cell banks. All cell lines were cultured in an atmosphere of 10% CO_2 at 37˚C.

Osteoclastogenesis assay. Bone marrow cells were obtained from the femurs and tibiae of 4-week-old male C57BL/6 mice (n=2), purchased from Charles River Laboratories (Yokoyama, Japan). Under anesthesia with 0.4 mg/kg of medetomidine, 4.0 mg/kg of midazolam and 5.0 mg/kg of butorphanol, the mice were sacrificed by cervical dislocation. After the leg was cut, muscle and connective tissue was removed from the tibiae and femurs. Both ends of the femur and tibiae were then clipped with a scissors. A 5 cc syringe was filled using a 27 gauge needle with PBS, and a needle was inserted into one end and the bone marrow was flushed out the other end. The flushed bone marrow cells wre corrected in a 50 cc tube. The bone marrow cells were washed twice by centrifugation (125 x g, 10 min) in 4˚C PBS. The cells were the nincubated in α-MEM in 10 cm culture dishes in the presence of macrophage colony-stimulating factor (M-CSF) (10 ng/ml) for 24 h. Non-adherent cells were transferred to 24-well plates ($2x10^6$ cells/well). The cells were treated with vitamin D_3, 1,25-Dihydroxy (10^{-8} M) and TM (0.1, 1, 2.5 and 5 μM) for 9 days.

Purification of osteoclast progenitors. Bone marrow cells were washed twice by centrifugation (125 x g, 10 min) in 20 ml of 4˚C sterile phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin. The cell pellet was resuspended, and the cells were magnetically labeled by the addition of anti-CD11b microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The cells were then incubated for 30 min on ice and then washed by centrifugation (125 x g, 10 min) with a volume of 4˚C buffer that was 10-fold that of the labeling volume and resuspended. $CD11b^+$ cells were depleted using an MD depletion column (Miltenyi Biotec GmbH). A total of $1x10^5$ murine $CD11b^+$ bone marrow cells/well were plated in a 24-well plate. The cells were treated with RANKL (50 ng/ml) and M-CSF (30 ng/ml) and the desired amount of TM (0.1, 1, 2.5 and 5 μM) for 9 days. Following 9 days of incubation, the cells were fixed and stained for tartrate-resistant acid phosphatase activity using the acid phosphatase, leukocyte (TRAP) kit (#A386A, Merck KGaA, Darmstadt, Germany), and the number of TRAP-positive multinucleate cells (i.e., a nuclear number >3) in each well was then counted.

Cell proliferation assay. The HSC-2, HSC-3 and SAS cells were each plated in 6-well plates at a density of $5x10^3$ cells per well and treated with the TM (1, 5 μM) or with an equivalent volume of the diluent (DMSO) as a control for 5 days. Osteoblasts, osteocytes and fibroblasts were each plated in 6-well plates at a density of $1x10^4$ cells per well and treated with TM (1, 5 μM) for 48 h. $CD4^+$ T cells were isolated by the magnetic sorting system. The cells from the homozenized spleens of two C57BL/6J mice were incubated with CD4 antibody-conjugated microbeads (eBioscience, San Diego, CA, USA) and sorted with MD depletion column (Miltenyi Biotec). The cells were stimulated with anti-CD3 monoclonal antibody (Cat. no. 16-0031, 5 μg/ml), CD28 antibody (Cat. no. 16-0281, 2 μg/ml) (Affymetrix, Santa Clara, CA, USA) and TM (1, 5 μM) for 48 h. The cell number was then counted with trypan blue assay. Osteoblasts were obtained following the method of Teramachi *et al* (14). After flushing the bone marrow from the tibiae of 3 C57BL/6J mice, the tibiae were cultured in αMEM for 7-10 days in 60-mm dishes until the cells growing out of the bones formed a confluent monolayer. The original bone was removed, and the cells grown out of the the bone were treated with 0.25% trypsin and 0.05% EDTA for 10 min at 37˚C. These cells were used as primary osteoblas without further passage. Osteocytes were obtained following the method of Shah *et al* (15). After flushing the bone marrow from the tibiae of the 2 C57BL/6J mice, the bones were dissected into 1-2-mm sized sections. The bone sections were then incubated for 25 min in collagenase (300 units/ml) and 0.25% EDTA (5 mM) and the collagenase was removed and discarded. These processes were repeated 8 times. The cells from the bone sections were cultured as osteocytes without further passage. These cells were classified as osteocytes by measuring dentin matrix acidic phosphoprotein 1 (DMP-1) expression by western blot analysis (data not shown).

Copper concentration measurement assay. The copper concentrations in the culture medium and serum, collected from the tail vein of the mice inoculated with the cancer cells at the time of sacrifice were measured using the Metallo Assay Copper Assay kit (Funakoshi, Tokyo, Japan). The samples, buffer and chelate color solution were mixed and incubated for 10 min at room temperature. Subsequently, the absorbance was read at a 580 nm wavelength using a microplate reader (SH-1000, Hitachi, Tokyo, Japan).

LOX activity assay. The HSC-2 and SAS cells were cultured in DMEM with increased copper ion (10 μM) in the presence or absence of TM for 24 h. Conditioned medium and serum collected from the mice inoculated with the cancer cells were tested using a LOX activity kit (Cat. no. ab112139, Abcam, Cambridge, MA, USA). The samples and LOX reaction mix solution were mixed and incubated for 30 min at room temperature. Subsequently, the fluorescence increase was measured on a microplate reader (Gemini EM microplate reader, Molecular Devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 550 and 600 nm. These conditioned media were used in the following experiment.

Western blot analysis. The osteocytes and osteoblasts were cultured in the above-mentioned conditioned media (30%)

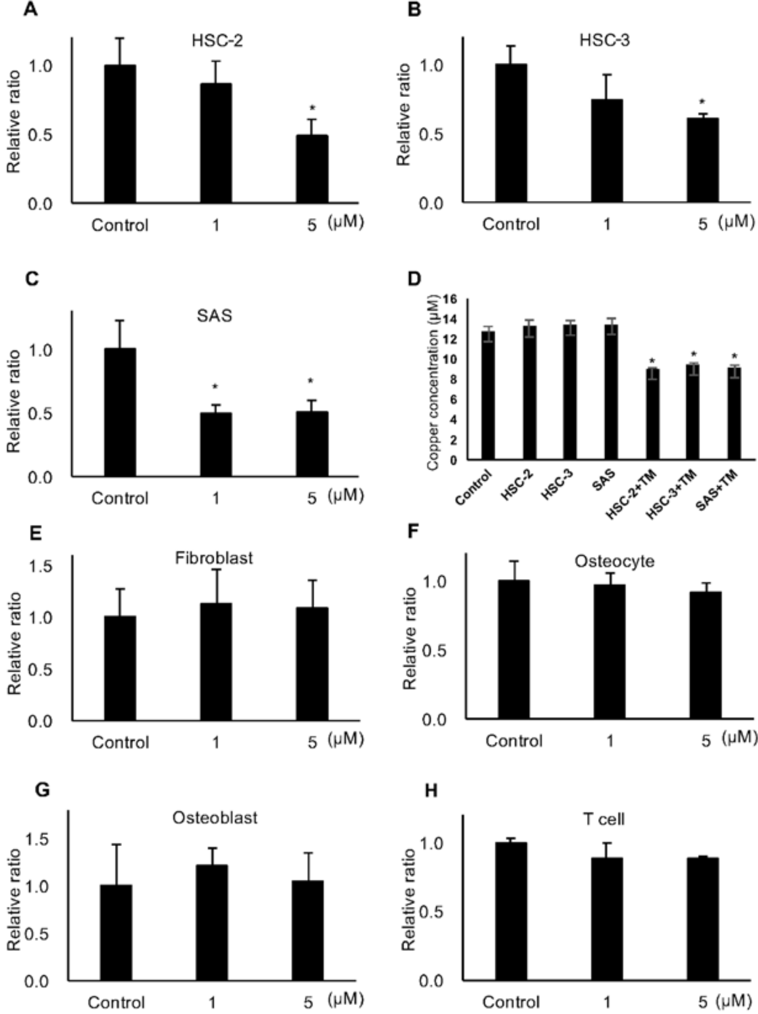

Figure 1. Effects of ammonium tetrathiomolybdate (TM) on the proliferation of HNSCC and bone microenvironment cells *in vitro* (A) HSC2 cells, (B) HSC3 cells, (C) SAS cells, (E) osteoblasts, (F) osteocytes, (G) fibroblasts, and (H) T cells were plated in triplicate and treated with 0, 1, or 5 μM TM for 48 h. Error bars indicate the means ± SD; $^{*}P<0.05$ vs. control. (D) Copper concentration in culture medium from HNSCC cells treated with TM (5 μM) for 48 h. Error bars indicate the means ± SD; $^{*}P<0.05$ vs. untreated cells.

for 24 h. The cell culture conditioned media (25 μl) were mixed with 4X Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) and boiled at 95˚C for 5 min. The samples were electrophoresed in 4-12% SDS-PAGE gels and the proteins were transferred onto membranes (Immobilon-P; Millipore, Bedford, MA, USA). The membranes were incubated with primary and secondary antibodies according to the ECL chemiluminescence protocol (RPN2109; Amersham Biosciences, Buckinghamshire, UK) to detect secondary antibody binding. Antibodies against RANKL (Cat. no. sc-377079, 1:1,000, Santa Cruz Biotechnology (Dallas, TX, USA) was used as a primary antibody and HRP-conjugated anti-mouse antibody (Cat. no. 7076, 1:2,000, Cell Signaling Technology, Danvers, MA, USA) was used as the secondary antibody.

Immunohistochemical analysis. The tibial bone and soft tumor was fixed in 10% formalin, decalcified and then embedded in paraffin. Serial sections were then prepared (3-μm-thick). For immunohistochemical analysis, the specimens were incubated with antibody (CD-31, 1:50, Cat. no. ab28364, Abcam), (Ki67, 1:400, Cat. no. 9129, Cell Signaling Technology), (EGFR, 1:50, Cat. no. 4267, Cell Signaling Technology), (p-EGFR, 1:200, Cat. no. 3777, Cell Signaling Technology), (RANKL, 1:100, Cat. no. sc-377079, Santa Cruz Biotechnology) overnight at 4˚C, followed by 3 washes with TBS. The slides were then treated with a streptoavidin-biotin complex [EnVision System labelled polymer, horseradish peroxidase (HRP); Dako, Carpinteria, CA, USA] for 60 min at a dilution of 1:100. The immunoreaction was visualized by using a DAB substrate-chromogen solution (Dako Cytomation Liquid DAB Substrate Chromogen System; Dako). The cells were counted using a microscope and evaluated.

Animal experiments. Mouse models of bone invasion by human oral squamous cell carcinoma were established in 5-week-old female BALB/c nude mice (each group, n=5; total, n=20, mean body weight, 19.5 g; Charles River Laboratories) by the inoculation of $1x10^5$ HSC-2 cells into the bone marrow space of the left tibial metaphysis. At 7 days after tumor cell inoculation, the mice were divided into 4 groups (control, cetuximab-treated, TM-treated, and TM- and cetuximab-treated). The cetuximab group was treated with an intraperitoneal injection of 100 μl of

a solution containing cetuximab (1 mg/kg) in PBS or PBS alone twice a week for 5 weeks. The TM group was orally administered a 100 μl solution containing TM (1 mg) in distilled deionized water (DDW) or DDW alone 5 times a week for 5 weeks. Under anesthesia with 0.4 mg/kg of medetomidine, 4.0 mg/kg of midazolam and 5.0 mg/kg of butorphanol, the hind limb long bones of the nude mice that had been injected with the cancer cells were excised, fixed in 10% neutral-buffered formalin. Osteolytic bone destruction was assessed on radiographs. The bones were placed against films (22x27 cm; Fuji industrial film FR: Fuji Photo Film Co. Ltd., Tokyo, Japan), and exposed to soft X-rays at 35 kV for 15 sec by the use of a Sofron apparatus (Sofron, Tokyo, Japan). The radiolucent bone lesions were observed microscopically (IX81, Olympus, Tokyo, Japan), and the areas were quantified with Lumina Vision/OL (Mitani, Tokyo, Japan). A micro-CT image was obtained with SKYSCAN (Bruker Japan, Kanagawa, Japan). Subsequently, the bone was decalcified and embedded in paraffin. Serial sections (3 μm-thick) were cut cross-sectionally, and the sections were stained with TRAP stain.

Human oral squamous cell carcinoma xenografts were established in 5-week-old male BALB/c nude mice (each group, n=5; total, n=20, Charles River Laboratories) by the inoculation of $1x10^6$ HSC-2 cells into the dorsal flank. At 7 days after tumor cell inoculation, the mice were divided into 4 groups (control, cetuximab-treated, TM-treated, and TM- and cetuximab-treated). The mice were treated with an intraperitoneal injection of 100 μl of solution containing cetuximab (1 mg/kg) in PBS or PBS alone twice a week for 5 weeks. The mice were treated with orally adminisitration of 100 μl of a solution containing TM (1 mg) or DDW, 5 times a week for 5 weeks. The tumor volume (cubic mm) was calculated using the following equation: $4\pi/3xr1/2xr2/2^2$, where r1 is the longitudinal diameter, and r2 is the transverse diameter. At 5 weeks after tumor cell inoculation, all the mice were then sacrificed, and the volume of tumors was measured.

All the animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the Okayama University Graduate School of Medicine and Dentistry with the following approval numbers: OKU-2016055 (for human oral squamous cell carcinoma xenografts), OKU2016060 (isolation of bone-related cells) and OKU-2016056 (inoculation of cancer cells into bone marrow).

Statistical analysis. The experiments were performed in quadruplicate. Data were analyzed using an unpaired Student's t-test for the analysis of two groups, and one-way ANOVA with Bonferroni and Dunnett's post hoc tests for the analysis of multiple group comparisons using SPSS statistical software. The results are expressed as the means ± SD. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

TM suppresses the growth of oral squamous cell carcinoma cells. To examine the antitumor effects of TM against oral squamous cell carcinoma *in vitro*, we performed a trypan blue staining assay. As shown in Fig. 1A-C, TM significantly reduced the number of viable HSC-2, HSC-3 and SAS cells proportionately with the increasing concentrations 5 days after treatment. In the same experiment, the concentrations of copper ion in the conditioned media of the HSC-2, HSC-3 and SAS cells treated with 5 μM TM for 72 h was decreased by approximately 30% (Fig. 1D). By contrast, TM did not affect the proliferation of fibroblasts, osteoblasts, osteocytes and T cells, which are components of the bone microenvironment (Fig. 1E-H).

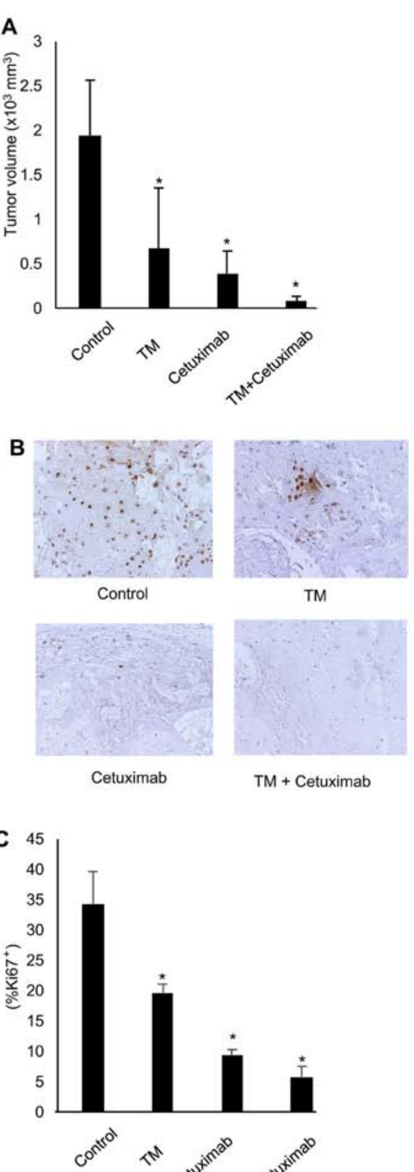

Figure 2. Effects of TM and cetuximab on oral squamous cell carcinoma cells grown in mice. (A) Tumor growth is expressed as the mean tumor volume ± SD; $^*P<0.05$ vs. control. (B) Immunohistochemical analysis of Ki67 in xenograft tumors. (C) The percentage of Ki67-positive cells in each section; $^*P<0.05$ vs. control.

To examine the antitumor effects of TM *in vivo*, we established an HNSCC xenograft tumor derived from HSC-2 cells in nude mice. The mice were treated with TM (1 mg; 5 times a week) and/or cetuximab (1 mg/kg; twice a week) for 5 weeks beginning at 7 days after tumor cell inoculation, and the tumor volume was measured at day 35. As shown in Fig. 2A, the HSC-2 xenograft tumor volumes were significantly

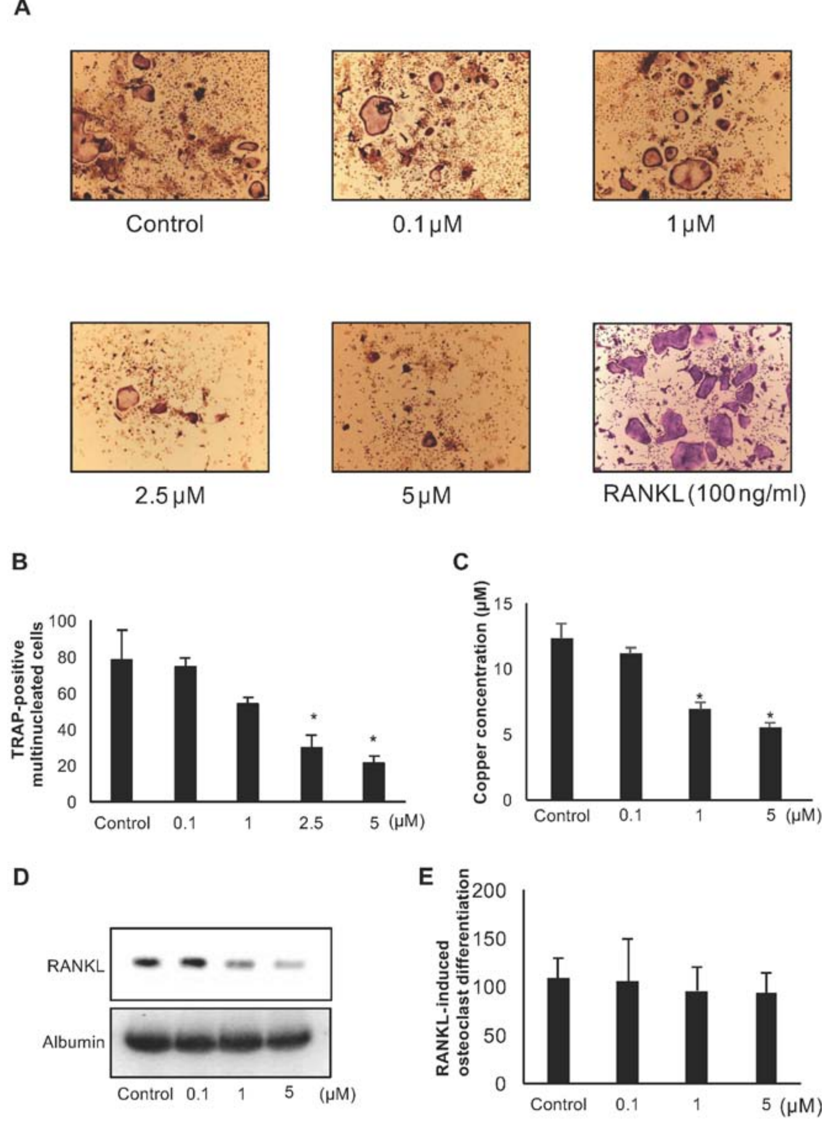

Figure 3. Effect of ammonium tetrathiomolybdate (TM) on osteoclastogenesis. (A and B) Total bone marrow cells were cultured with TM (0, 0.1, 1, 2.5, or 5 μM) in the presence of vitamin D_3, 1,25-Dihydroxy (10_{-8} M) in a 24-well plate for 9 days. (B) The numbers of TRAP-positive multinucleated cells (nuclear number >3) were counted as osteoclasts (y-axis). Data are the means ± SD; $^*P<0.05$ between the indicated groups. (C) Copper concentration in bone marrow cell cultured medium in the presence of vitamin D_3, 1,25-Dihydroxy (10^{-8} M) with or without TM (0.1, 1, 5 μM) for 3 days. Data are means ± SD; $^*P<0.05$ vs. control (D) RAKNL expression in bone marrow cell culture medium in the presence of vitamin D_3, 1,25-Dihydroxy (10^{-8} M) with or without TM (0.1, 1, 5 μM) for 3 days. (E) CD11b-positive pre-osteoclasts were cultured with TM (0, 0.1, 1, 5 μM) in the presence of 30 ng/ml M-CSF and 50 ng/ml RANKL in a 24-well plate for 9 days. TRAP-positive multinucleated cells (nuclear number >3) were counted as osteoclasts. Data are the means ± SD.

decreased in the TM-or cetuximab-treated mice compared to the untreated mice. No significant toxicity was observed during the treatment period. At sacrifice after the treatment period with TM or cetuximab, the tumors were excised and examined histologically. Immunohistochemical analysis revealed a significant decrease in the number of Ki67-positive tumor cells in the HSC-2 tumor sections from the TM-treated and cetuximab-treated mice (Fig. 2B and C). TM treatment led to a positive trend by enhancing the antitumor effects of cetuximab (cetuximab single treatment vs. combined treatment, P=0.057). TM or cetuximab did not cause any body weight loss at the end of experiment [mean body weight (g): control, 23.01; TM, 23.15; cetuximab, 24.15; and TM + cetuzimab, 22.8]. None of the animals experienced >20% decrease in body weight during the experiment. However, no statistically significant differences were observed between single treatment and combination treatment with TM and cetuximab as regards by tumor volume in this soft tissue tumor model. These results suggest that antitumor effects of TM require further evaluation in soft tissue HNSCC models in order to definitively evaluate its direct anticancer activity.

TM suppresses osteoclast formation. Copper ions are known to play a key role in bone remodeling (5); however, the effects of copper chelating on bone cancer remain unknown. To examine the effects of TM on osteoclast formation, we treated murine total bone marrow cells harvested from mouse tibias with vitamin D_3 ($1x10^{-8}$ M) in the presence or absence of TM for 5 days. TM inhibited the number of TRAP-positive multinucleated osteoclasts in a dose-dependent manner (Fig. 3A and B).

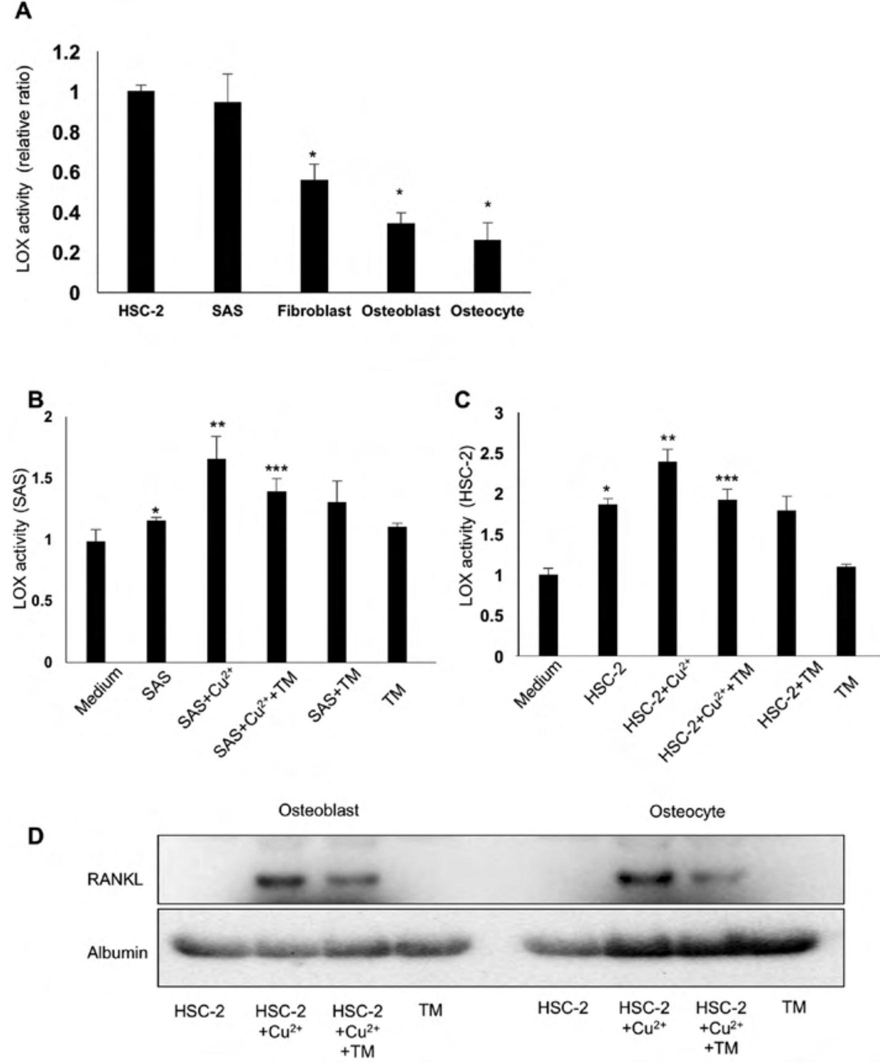

Figure 4. Effect of ammonium tetrathiomolybdate (TM) on LOX activity in cancer cells and the expression of RANKL in cells of the bone microenvironment. (A) LOX activation in conditioned media of HNSCC cells and bone microenvironmental cells. LOX activation is shown as the relative ratio. HSC-2 cells were used as a control (1.0). Data are the means ± SD; *P<0.05 vs. HSC-2 cells. (B) LOX activation in (B) SAS or (C) HSC-2 cell conditioned media with increased concentrations of copper ion (10 μM) with or without 5 μM TM. LOX activation is shown as the relative ratio. Medium was used as the control (1.0). Data are the means ± SD; *P<0.05 vs. medium, **P<0.05 vs. cancer CM, ***P<0.05 vs. Cu^{2+}-added cancer CM (D) Osteoblasts and osteocytes were treated with the previously prepared HSC-2 conditioned medium for 24 h. RANKL expression in the osteoblasts and osteocytes was evaluated by western blot analysis.

The copper concentration in the cell conditioned media and RANKL expression in bone marrow cells were decreased by TM treatment in a dose-dependent manner (Fig. 3C and D). By contrast, TM did not affect RANKL-induced osteoclast differentiation on CD11b-positive bone marrow cells (Fig. 3E). These results indicate that copper chelating by TM suppresses osteoclast formation via indirect osteoclast differentiation, such as that represented by RANKL expression in osteoblasts.

TM suppresses cancer cell-derived LOX activation via copper chelating. The enzyme activity of LOX was activated by copper binding to pro-LOX. Fig. 4A illustrates soluble LOX activation increased by copper ion (10 μM) from the conditioned media of the cancer cells and bone microenvironment cells. Both the SAS and HSC-2 cells released a large amount of activated LOX compared to the bone microenvironment cells, i.e., the fibroblasts, osteoblasts and osteocytes. As expected, TM suppressed LOX activation in the conditioned medium of HNSCC cells via copper chelating (Fig. 4B and C).

TM suppresses RANKL expression in osteoblasts and osteocytes in vitro. To examine the effects of copper-induced LOX activation on RANKL expression in bone marrow cells, we treated osteoblasts and osteocytes with HSC-2 conditioned medium and cultured the cells in increased copper ion concentrations with or without TM for 24 h. The HSC-2 conditioned medium and the presence of increased copper ions promoted RANKL expression in the osteoblasts and osteocytes. TM treatment decreased this effect via copper chelating (Fig. 4D).

TM decreases the copper levels and cancer-induced LOX activity in vivo. To build on these findings, we evaluated the effects of TM on bone destruction and resorption induced by the injection of HSC-2 cells into mouse tibiae. The results

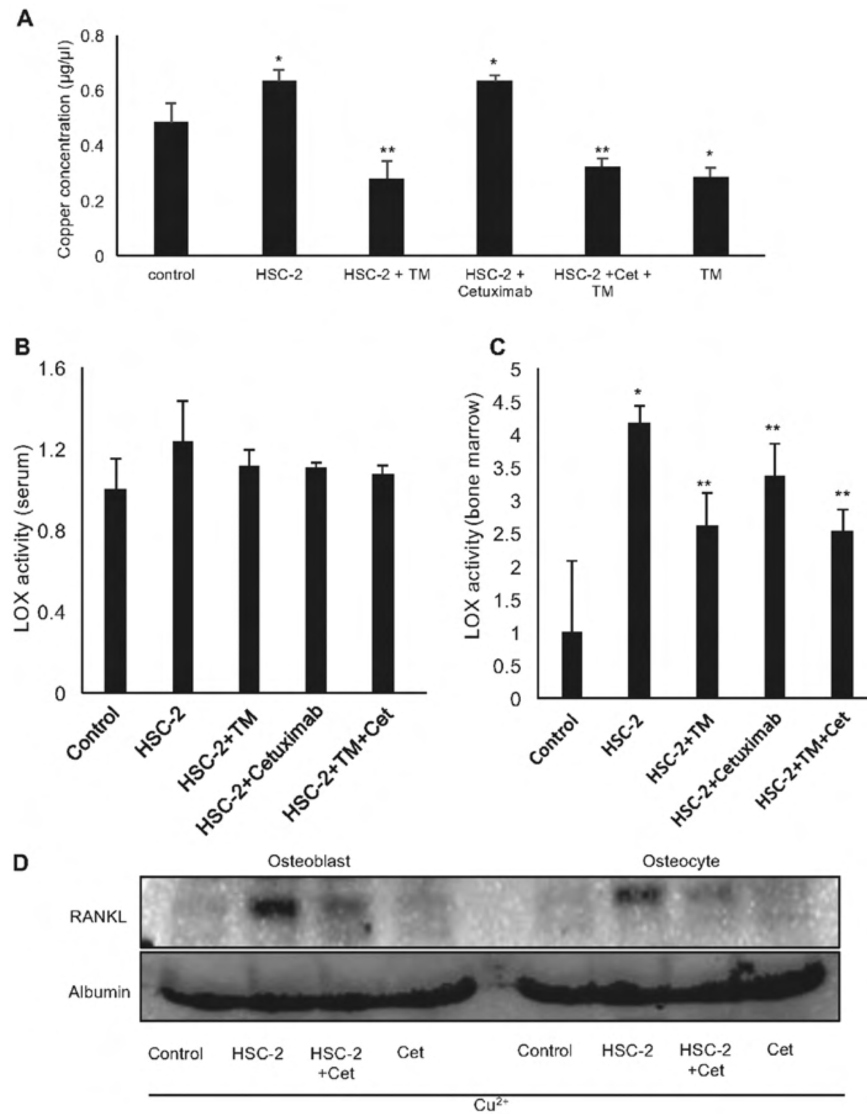

Figure 5. Effect of ammonium tetrathiomolybdate (TM) on the copper levels and LOX activity in free blood serum from mouse models of HSC-2-induced cancer bone invasion. At 7 days after tumor inoculation into he tibial bone marrow, mice were orally administered TM (1 mg/body) or DDW 5 times a week for 5 weeks. Mice were intraperitoneally administered cetuximab (1 mg/kg) or PBS twice a week for 5 weeks (n=5/group). Copper levels in free blood and LOX activation in whole blood serum and bone marrow serum were measured in mice injected with the HSC-2 cells into their tibiae following treatment with TM or cetuximab alone or in combination. (A) Copper levels in free blood serum of the mouse model of bone invasion. Data are the means ± SD; *P<0.05 vs. controls, **P<0.05 vs. HSC-2 tumor cell-injected mice. (B) Analysis of LOX activity in whole blood serum. (C) Analysis of LOX activity analysis in bone marrow serum. LOX activation is shown as the relative ratio. Data are the means ± SD; *P<0.05 vs. control, **P<0.05 vs. HSC-2 tumor cell-injected mice. (D) Osteoblasts and osteocytes were treated with the HSC-2 culture medium with increased concentrations of copper ion in the presence or absence of cetuximab for 24 h. RANKL expression in osteoblasts and osteocytes was evaluated by western blot analysis.

demonstrated that the serum copper levels were increased in the mice injected with the HSC-2 into the tibiae, and this was suppressed by treatment with TM (Fig. 5A).

As expected, HSC-2 cell inoculation into the tibiae increased LOX activity in bone marrow, and treatment with TM and cetuximab significantly decreased this activity (Fig. 5C). However, TM did not affect the whole serum LOX activity (Fig. 5B). These data thus demonstrated that TM and cetuximab suppressed the local LOX activation in bone by chelating copper ion.

Cetuximab reduced LOX activation in bone marrow *in vivo* (Fig. 5C). We also evaluated the effects cetuximab treatment on RANKL expression in osteoblasts and osteocytes *in vitro*. Surprisingly, conditioned medium from the HSC-2 cells treated with cetuximab markedly decreased RANKL expression in osteoblasts and osteocytes (Fig. 5D). Cetuximab decreased the number of HNSCC cells *in vitro* and thereby cetuximab may suppress the amount of LOX in media.

TM enhances the anticancer effects of cetuximab and prevents bone resorption in vivo. We examined the *in vivo* effects of TM on osteolytic bone destruction induced by oral squamous carcinoma by conducting soft X-ray and micro-CT examinations. As shown in Fig. 6A and B, the osteolytic lesions were clearly visible in the tibiae of mice with bone invasion induced by HSC-2 cells treated with the vehicle only. Surprisingly, few destructive lesions were detected in the tibiae of mice treated with TM. The total area of radiographic osteolytic

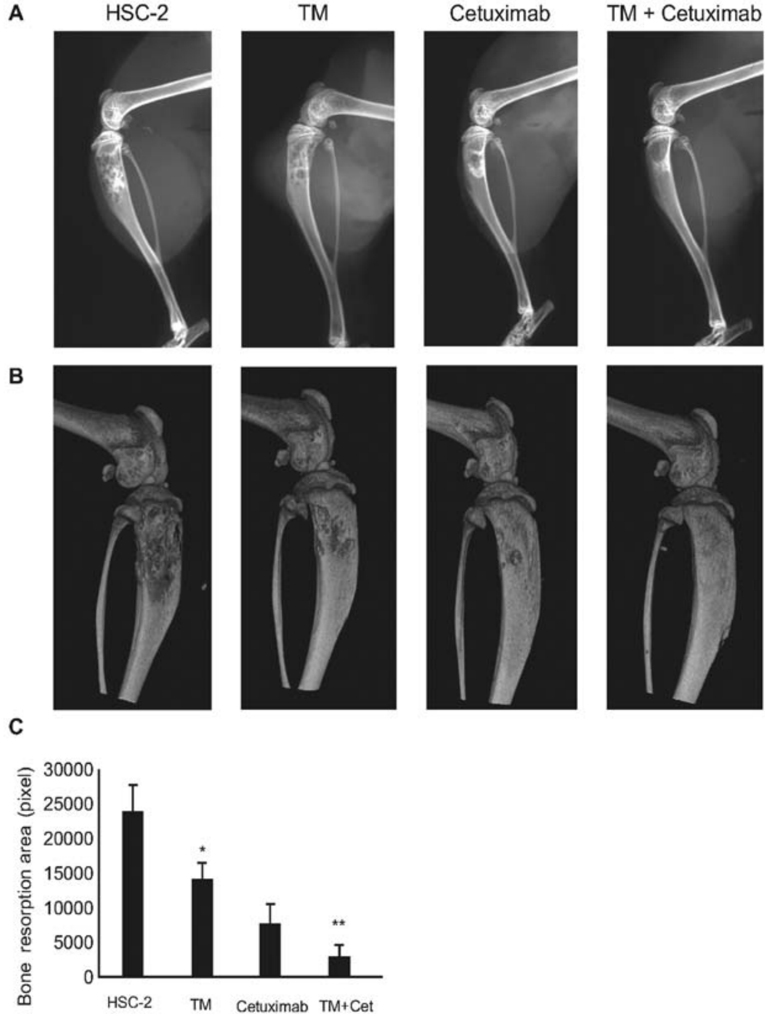

Figure 6. Effects of ammonium tetrathiomolybdate (TM) on mouse models of cancer-associated osteolysis. (A and B) Representative radiographs and micro-CT of tibia from mice with bone invasion following the injection of HSC-2 cells and treated with TM or cetuximab alone, or their combination (n=5/group). (C) Osteolytic lesion area in tibia from mouse. The osteolytic area. Data are the means ± SD; $^{*}P<0.05$ vs. HSC-2-injected mice; $^{**}P<0.05$ vs. mice treated with cetuximab or TM alone.

lesions from all tibiae was significantly suppressed by TM treatment compared to the controls (P<0.05). Cetuximab is a standard of care agent for the treatment of human head and neck cancers (16), and it is well known to suppress HSC-2 cell growth. Surprisingly, TM enhanced the antitumor effects of cetuximab in bone (Figs. 6C and 7A). Treatment with both TM and cetuximab alone decreased cancer cell proliferation in bone marrow. Furthermore, combination treatment with TM and cetuximab intensively suppressed tumor cell proliferation in bone marrow compared to treatment with each agent alone.

In addition, the numbers of RANKL-positive cells and TRAP-positive osteoclasts were significantly decreased in the tibiae of the mice treated with both agents compared with the mice treated with single treatment (TM or cetuximab only) (P<0.05; Fig. 7B-D). To build on these findings, we examined the activity of cetuximab in this model. Cetuximab suppressed EGFR levels and phosphorylated EGFR expression in these bone marrow tumors. In addition, combined treatment with cetuximab and TM enhanced the suppressive effects on the epression of EGFR, phosphorylated EGFR, CD31 and Ki67 compared with single treatment (Fig. 8A).

These results suggest the following: i) TM significantly suppresses oral squamous cell carcinoma via the suppression of osteoclastogenesis and angiogenesis in osteolytic bone destruction that is associated with the invasion of oral squamous cell carcinoma; and ii) TM may enhance the effectiveness of cetuximab.

Discussion

Copper chelators have been previously reported to inhibit cancer cell growth *in vitro* and *in vivo* (7,16,17). LOXs are copper-dependent enzymes. Copper ion binding to pro-LOX is necessary for LOX activation (9,18). The most well-studied roles of LOX enzymes are in the remodeling of the ECM and angiogenesis. It has been reported that cancer-derived LOX induces bone destruction in HNSCC and other malignancies (19-21); however, the role of copper ion involvement in bone destruction induced by HNSCC is not yet well understood. To the best of our knowledge, the present findings are the first to demonstrate that the chelation of copper ions by TM, inhibited LOX activation from HSC-2 head and neck

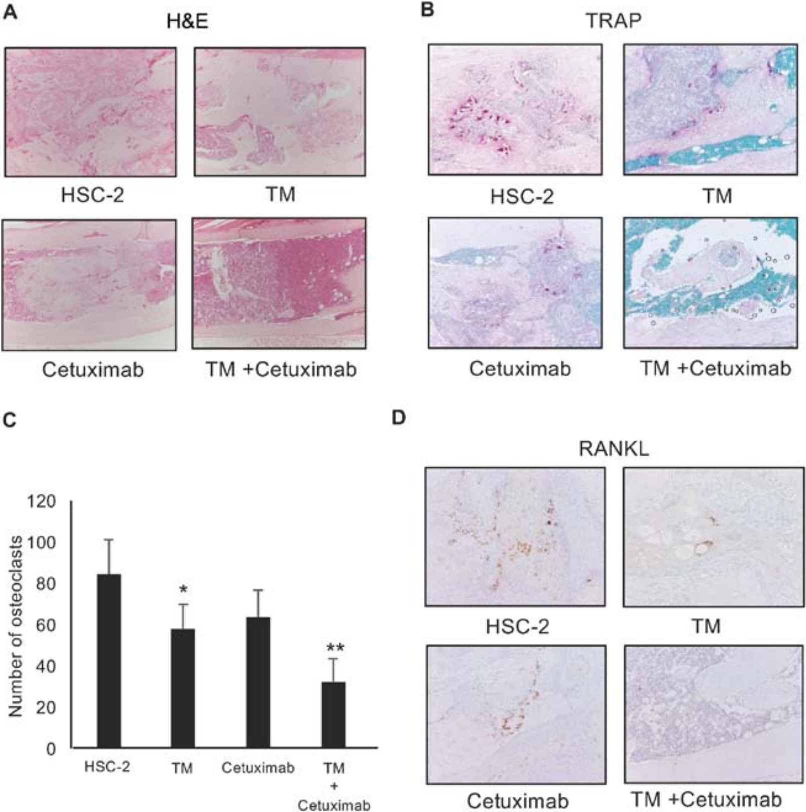

Figure 7. Histological and histomorphometric analysis of the bone marrow in the mouse model of bone invasion. The sections of previous-experiment mice tibial bone marrow were stained with (A) hematoxylin and eosin (H&E), (B and C) TRAP and (D) RANKL (n=5/group). The numbers of osteoclasts in bone marrow are presented as thee means ± SD; *P<0.05 vs. control, **P<0.05 vs. treatment with ammonium tetrathiomolybdate (TM) or cetuximab alone.

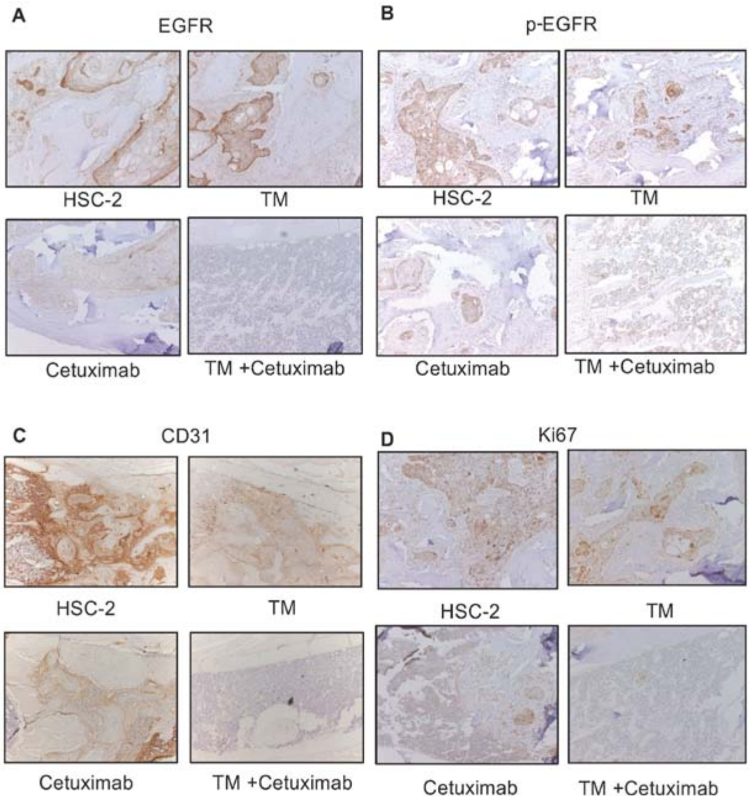

Figure 8. Immunohistological analysis of the bone marrow in the mouse model of bone invasion. Representative sections of mice tibial bone marrow stained with (A) EGFR, (B) p-EGFR, (C) CD31 and (D) Ki67 (n=5/group).

cancer cell models. In an earlier study, LOX was shown to increase RANKL expression in osteoblasts and consequently promote osteoclastogenesis (11). In this study, we observed that copper chelation by TM inhibited RANKL expression in osteoblasts and osteocytes via LOX suppression, resulting in an inhibition of the bone destruction associated with HNSCC invasion. These results indicate that copper ions are a critical mediator of osteolytic bone destruction in the bone tumor microenvironment.

Our experiments revealed that the oral squamous cell carcinoma HSC-2 and SAS cells were potently and effectively inhibited by TM at the level of proliferation. TM inhibited HSC-2 and SAS cell growth at an IC_{50} of 1-5 μM, whereas TM did not inhibit the growth of the fibroblasts, osteocytes, osteoblasts or T cells at the IC_{50} of 5 μM (Fig. 1D-G), which is consistent with previous findings (22). The discrepancy in IC_{50} results may be due to differences in the cellular systems, and cancer cells may be more sensitive to copper metabolism than normal cells. We also observed that the administration of TM exhibited efficacy in the HSC-2 xenograft model, where TM inhibited tumor growth *in vivo*. Furthermore, TM demonstrated a positive trend by enhancing the antitumor effects of cetuximab, which is an EGFR receptor inhibitor used to treat head and neck cancer patients (cetuximab single treatment vs. combined treatment, P=0.057). However, there no statistically significant differences were observed between single treatment and combined treatment with TM and cetuximab as regards tumor volume in this soft tissue tumor model. These results suggest that the antitumor effects of TM require further evaluation in soft tissue HNSCC tumor models *in vivo*.

In our investigation of the molecular mechanisms of action of TM in osteoclastogenesis in total bone marrow cells and CD11b-positive bone marrow cells, the data indicated that TM inhibited osteoclastogenesis from total bone marrow cells induced by vitamin D_3 (Fig. 3A and B). However, TM did not affect RANKL-induced osteoclast differentiation from CD11b-positive cells (Fig. 3E). These data indicated that TM did not have a direct effect on osteoclast precursor cells, but did have an effect on other cells of the bone microenvironment.

TM reduced LOX activation in the HSC-2 and SAS cells following copper ion treatment. It has been previously reported that LOX induces RANKL expression in osteoblasts (11). Consistently, the present findings demonstrated that the suppression of LOX downregulated RANKL expression in osteoblasts and osteocytes, resulting in the suppression of the differentiation of osteoclasts. The HSC-2 and SAS head and neck cancer cells released significant amounts of LOX (Fig. 4A). Copper ions increased LOX activation and TM suppressed it. These data indicated that TM may have a potent antitumor effect in bone-invasive HNSCC cells by not only suppressing tumor growth, but also by suppressing bone resorption by osteoclasts. To test this hypothesis, we created an HNSCC bone destruction mouse model, and treated the mice with TM. As expected, the serum copper levels in the mice injected with HSC-2 cells in their tibiae that were treated with TM were decreased compared to those of the untreated mice. The intratibial LOX activation in these mice was decreased similar to the serum copper levels. By contrast, there were no differences in LOX activation in blood serum. These data indicated that pro-LOX from cancer cells and copper ions are necessary for LOX activation and bone destruction.

Studies have reported that copper and LOX promote EGFR activation (22,23). In the present study, TM inhibited EGFR and phosphorylated EGFR expression in HSC-2 tumors *in vivo*. This mechanism may result in reduced Ki67 expression and consequent tumor growth suppression. To test the effects of TM *in vivo*, we treated the mice in the bone-destructive HNSCC model with TM, cetuximab or both, and the results indicated that single treatment with TM and single treatment with cetuximab reduced tumor growth in the bone (Fig. 6), and the combination treatment significantly decreased tumor growth and bone resorption compared to single treatment with either agent alone. This additive effect was due to the suppression of osteoclast formation, angiogenic potential and EGFR activity (Figs. 7B-D, and 8A-C).

In conclusion, copper may be a target for the treatment of bone osteolysis induced by HNSCC, and our findings strongly suggest that the single use of TM or combination treatment with TM and approved agents, such as cetuximab requires further evaluation as a potential novel therapy for the treatment of advanced bone invasive HNSCC.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research (B) (JSPS KAKENHI grant no. 17H04405) to A. Sasaki and a Grant-in-Aid for Research Activity start-up (JSPS KAKENHI grant no. 16H06992) to T. Okui from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Competing interests

The authors declare that they have no competing interests.

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www.impactjournals.com/oncotarget/ Oncotarget, 2016, Vol. 7, (No. 51), pp: 84439-84452

Research Paper

Ammonium tetrathiomolybdate treatment targets the copper transporter ATP7A and enhances sensitivity of breast cancer to cisplatin

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Keywords: *cisplatin, breast cancer, ATP7A, copper, resistance, sequestering*

Received: *August 23, 2016* ***Accepted:*** *October 12, 2016* ***Published:*** *October 31, 2016*

ABSTRACT

Cisplatin is an effective breast cancer drug but resistance often develops over prolonged chemotherapy. Therefore, we performed a candidate approach RNAi screen in combination with cisplatin treatment to identify molecular pathways conferring survival advantages. The screen identified ATP7A as a therapeutic target. ATP7A is a copper ATPase transporter responsible for intercellular movement and sequestering of cisplatin. Pharmaceutical replacement for ATP7A by ammonium tetrathiomolybdate (TM) enhanced cisplatin treatment in breast cancer cells. Allograft and xenograft models in athymic nude mice treated with cisplatin/TM exhibited retarded tumor growth, reduced accumulation of cancer stem cells and decreased cell proliferation as compared to mono-treatment with cisplatin or TM. Cisplatin/TM treatment of cisplatin-resistant tumors reduced ATP7A protein levels, attenuated cisplatin sequestering by ATP7A, increased nuclear availability of cisplatin, and subsequently enhanced DNA damage and apoptosis. Microarray analysis of gene ontology pathways that responded uniquely to cisplatin/TM double treatment depicted changes in cell cycle regulation, specifically in the G1/S transition. These findings offer the potential to combat platinum-resistant tumors and sensitize patients to conventional breast cancer treatment by identifying and targeting the resistant tumors' unique molecular adaptations.

INTRODUCTION

Breast cancer affects approximately one in eight women in Western countries. In the U.S., more than 200,000 new breast cancer cases are diagnosed each year, with about 5% of which is caused by mutations in breast cancer associated gene 1 (*BRCA1*) and breast cancer associated gene 2 (*BRCA2*) [1–4]. Platinum drugs such as cisplatin (*cis*-[$PtCl_2(NH_3)_2$], *cis*-diamminedichloroplatinum(II), cDDP), carboplatin and oxaliplatin serve as conventional treatment for breast cancer and other solid tumors [5–7]. Cisplatin is a crosslink-inducing DNA-damaging agent that causes cell death primarily via adduct-formation through adjacent guanine residues [8–10]. Cisplatin may also induce cell death by damaging cytoplasmic proteins, inducing apoptosis at the execution phase level [8, 9, 11].

Platinum agents are highly effective in combating *BRCA1*-associated breast cancer because there is defect in the homology-directed DNA repair capability of these tumors that contributes to genomic instability [12, 13]. Unfortunately, resistance to platinum agents often develops, through cellular adaptations that result in reduced drug uptake, increased efflux and sequestering, and enhanced detoxification, contributing to metastasis

and overall treatment failure [14, 15]. Previous studies also indicate that altered gene expression, DNA copy number changes, and substantial genomic instability contribute to cisplatin resistance [9, 15, 16]. This underscores the need for identification of alternative and ameliorative treatments that re-sensitize cells to platinum agents.

In this study, we conducted an RNAi library screening combined with cisplatin treatment in human and mouse breast cancer cell lines to identify potential therapeutic agents. The copper transporting P-type ATPase, ATP7A [17], was one of the candidates that emerged from our screen. Herein, we describe how ATP7A specifically contributes to cisplatin resistance in breast cancer, and how combining cisplatin and ammonium tetrathiomolybdate (TM), which degrades ATP7A, to sensitizes breast tumor cells to cisplatin.

RESULTS

Candidate RNAi screen revealed ATP7A as a target for inducing cisplatin sensitivity

To identify specific gene and pathway targets that confer to cisplatin sensitivity upon knockdown, we utilized RNAi library screen combined with cisplatin treatment in human breast cancer cell lines. This screen was carried out in three human breast cancer cell lines, MDA-MB-231, T47D and MCF7, respectively. These cell lines were determined to be resistant to cisplatin by National Cancer Institute (NCI) *In Vitro* Cell Line Screening Project (IVCLSP). Cells were treated with cisplatin alone at its IC_{50} (50% lethality) dose of 10 µM for MCF-7 and 36 µM for MDA-MB-231 and T47D, or in combination with a human siRNA siGENOME library (Thermo Dharmacon). Our RNAi library comprised of siRNA against 55 custom-selected genes (Supplementary Table S1), including genes identified in the common genomic gain regions found in the cisplatin resistant breast cancer cells and associated with poor prognosis in breast cancer, which are located on chromosomes 6p12, 6p21, 11q13, 20q13.2 and several regions of 14q [18–21]. Additionally, we included siRNA targeting genes related to stem cell maintenance, such as *SOX2* and *OCT3/4*, as well as drug detoxifying enzymes, and transporters involved in drug and metal flux.

In our RNAi screen, 14 out of 55 (25.5%) of the candidates exhibited synergy in cell killing when combined with cisplatin at corresponding cisplatin IC_{50} dose (Figure 1A), some of which were reported to contribute to cisplatin resistance when overexpressed, such as *STAT3*, *MDR1* and *ATP7A* [22, 23]. To validate the RNAi result, the human breast cancer cell lines T47D, MDA-MB-231 and MCF7, as well as the *BRCA1*-mutant mouse breast cancer cell line 69, were treated with cisplatin alone or in combination with *ATP7A* siRNA respectively. Indeed, significantly enhanced cytotoxicity was achieved with combined treatment of *ATP7A* siRNA and cisplatin in all cell lines (Figure 1B). Increased protein levels of ATP7A or ATP7B (both are copper export pumps) were reported to correlate to cisplatin resistance a in several human cancer cell lines examined [24, 25]. Studies also showed that ATP7A sequesters cisplatin into cell vesicles (such as lysosomes) [26, 27]. Therefore, we chose to study the role of ATP7A in cisplatin resistance.

Ammonium tetrathiomolybdate treatment sensitizes breast cancer cells to cisplatin

We first tested three metal chelating agents: neucoprine ([$C_{14}H_{12}N_2$], 2,9-dimethyl-1,10-phenanthroline), ammonium tetrathiotungstate ([$(NH_4)_2WS_4$], TT), and ammonium tetrathiomolybdate ([$(NH_4)_2MoS_4$], TM). While neucoprine and TT treatment alone or in combination with cisplatin on the four cell lines under investigation did not have significant effect on cell survival, our results indicated that double treatment with TM and cisplatin significantly sensitized breast cancer cells to a level comparable to that attained with *ATP7A* siRNA (data not shown). Furthermore, we plotted a dose response curve of TM in the MB-MDA-231 human breast cancer cell line at its cisplatin IC_{30} dose of 10 µM, or in the absence of cisplatin. A 20% decrease in overall cell survival was observed after cisplatin/TM treatment as compared to the predicted additive curve (Figure 1C). It is noteworthy that the synergy between TM and cisplatin occurred at very low TM concentration, which had virtually very low or no effect on cell viability (Figure 1C).

TM is designated an orphan drug in the U.S., and was first used therapeutically in the treatment of copper toxicosis and Wilson's disease [28, 29]. TM serves as an attractive anti-cancer compound on the basis of its ability to act as both an angiogenesis inhibitor and copper trafficking protein inhibitor [30, 31], and is currently being tested in clinical trials in combination with doxorubicin and alone for the treatment of metastatic breast cancer [32]. We hypothesized that TM exerts distinct ameliorative effects in combination with conventional platinum chemotherapy independent of its effects on tumor vascularization.

Cisplatin and TM synergistically inhibit tumor progression through inhibition of cancer stem cells accumulation and proliferation

Afterwards, we tested the effect of cisplatin/TM double treatment *in vivo* utilizing athymic nude mice implanted with breast cancer cells. For this experiment, 1 × 10^6 *BRCA1*-mutant mouse breast cancer cells were implanted in the bilateral 4th mammary fat pads of athymic nude mice at 6–10 weeks of age. Tumors became visible 7–14 days post-implantation, and drug administration was initiated when tumor reached 200 mm^3. Mice were

intraperitoneally (IP) injected with cisplatin at 6 mg/kg body weight with or without oral administration of TM at 0.030 mg/mL in drinking water. Tumor volume of cisplatin/TM double treated mice (Cis/TM) was significantly smaller than untreated (Untr) and mono-treated (TM or Cis) groups in 69 allograft model (Figure 2A). Mice treated with cisplatin alone had smaller tumor volume and longer survival than the mice treated with TM alone (data not shown). Similar tumor progression profile was observed in MDA-MB-231 xenograft mice (Figure 2B).

We have previously shown that prolonged treatment of cisplatin induces cisplatin resistance that is accompanied by accumulation of cancer stem cells (CSCs) [13]. This was observed in cisplatin mono-treated 69 allograft tumors harvested at 45 days post-treatment, as seen by the stark rise of tumor growth curve (Figure 2A). Thus, we refer to this tumor as the cisplatin mono-treated resistant tumor (CisR) hereafter. Flow cytometry analysis of 69 allograft tumor-dissociated cells revealed an elevation of $CD24^{hi}$ $CD49f^{hi}$ CSC population in cisplatin mono-treated resistant tumors (CisR) as compared to untreated tumors (Untr), whereas a decline of CSC population was observed in cisplatin/TM double treated tumors (Cis/TM) (Figure 2C).

Using these 69 allograft tumors, we analyzed cell proliferation using Ki67, a common biomarker used for the diagnosis of aggressiveness of many types of cancers, including breast cancer. Immunostaining of 69 allograft mouse tumor sections showed increased Ki67 antibody staining on untreated mouse tumor (Untr) and cisplatin mono-treated resistant tumors (CisR), as compared to cisplatin/TM double treated tumors (Cis/TM) (Figure 2D). Quantification of Ki67 staining depicted over 20% reduction in Ki67 staining in cisplatin mono-treated resistant tumors (CisR) as compared to cisplatin/TM double treated tumors (Cis/TM) (Figure 2E). Furthermore, the increase in Ki67 staining in cisplatin mono-treated resistant tumors (CisR) as compared to untreated tumors (Untr) in this experiment (Figure 2E) suggested that acquisition of cisplatin resistance correlated to increased cell proliferation. Conversely, cisplatin/TM double treated tumors had retarded tumor growth, less CSCs and lower

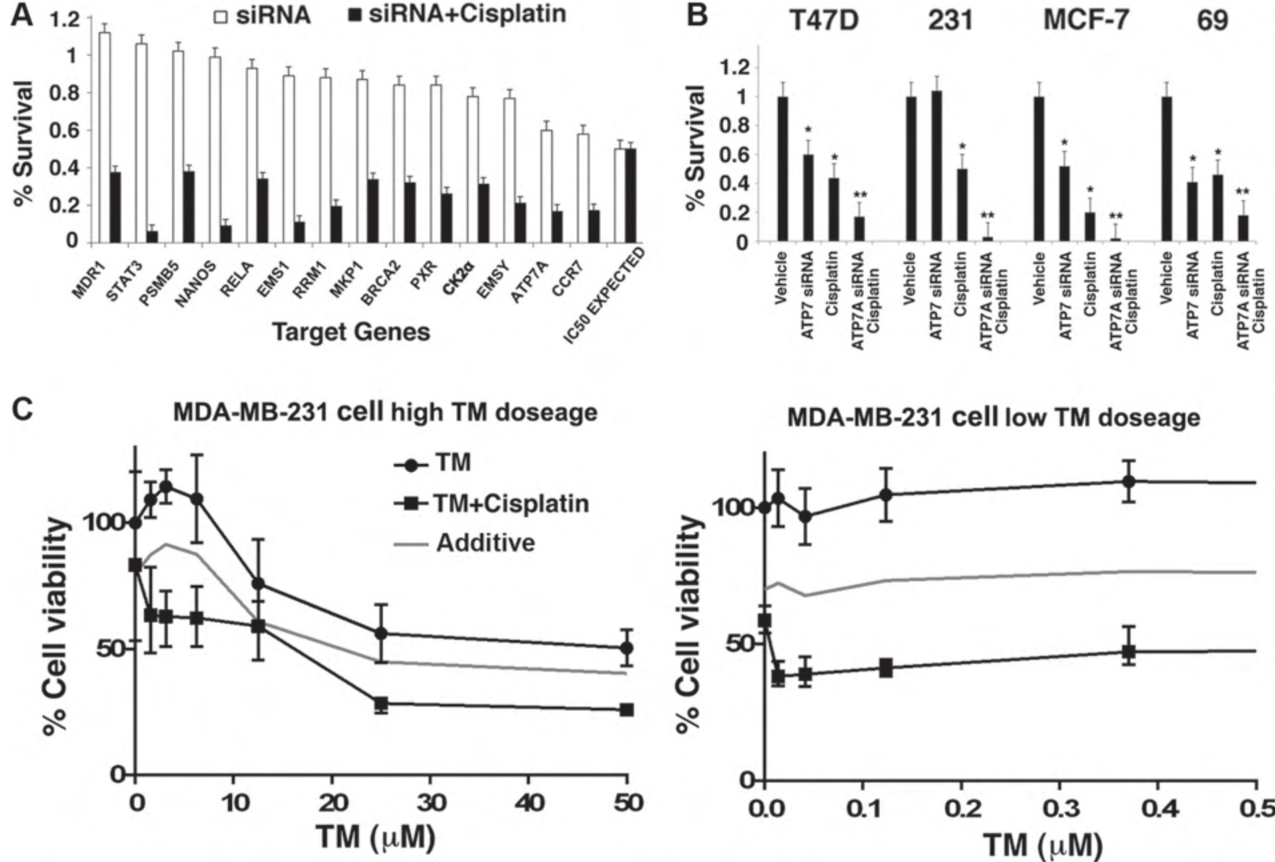

Figure 1: Identification and validation of a target pathway that affects cisplatin response in human breast cancer. (**A**) 14 out of 55 candidate gene siRNAs that exhibited synergistic effect with cisplatin upon double treatment in T47D cells were shown. Cell survival rate was measured by ATP release assay after treatment with 36 μM (IC_{50}) cisplatin with or without siRNA, and normalized to both the non-targeting siRNA and vehicle control. (**B**) Validation of synergistic effect of cisplatin and *ATP7A* knockdown in three human breast cancer cell lines T47D, MDA-MB-231 and MCF-7, and one mouse breast cancer cell line 69 using RNAi pools distinct from the initial candidate screen. (**C**) Dose response curve of MDA-MB-231 upon TM treatment in the absence or presence of 10 μM cisplatin. MTT assay was used to determine cell survival rate after 3 days drug treatment.

Ki67 levels. Taken together, our allograft model suggested that TM overcomes cisplatin resistance through inhibition of cancer stem cells accumulation and cell proliferation.

Aside from CSC accumulation and cell proliferation, we also performed terminal dUTP nicked end labeling (TUNEL) assay and immunohistochemical staining of antibodies against γH2AX and 53BP1, in order to assess apoptosis through DNA fragmentation and DNA damage. Results showed that cisplatin mono-treatment resulting in resistance emergence (CisR) led to more TUNEL-positive staining and γH2AX foci as compared to untreated mouse allograft tumors (Untr), whereas further elevation was observed in cisplatin/TM double treated mouse allograft tumors (Cis/TM) (Figure 3A), suggesting that TM promotes apoptosis and DNA damage. Quantification of the stained sections showed that the number of TUNEL-positive cells (Figure 3B) in cisplatin/TM double treated tumors (Cis/TM) was positively correlated to the percentage of γH2AX-positive cells (Figure 3C) and increased γH2AX foci (Figure 3D), as compared to cisplatin mono-treated resistant tumors (CisR) and untreated tumors (Untr). Consistent with this, elevated foci of 53BP1, which is recruited to sites of aberrant fork structures to suppress homologous recombination and facilitate non-homologous end joining [33], was observed in cisplatin/TM double treated tumors (Cis/TM) as compared to cisplatin mono-treated resistant tumors (CisR) and untreated control (Untr) (Figure 3A). Taken together, these data suggested that TM enhances tumor apoptosis synergistically with cisplatin and overcomes cisplatin resistance by increasing platinum-induced DNA damage and cell death in breast cancer.

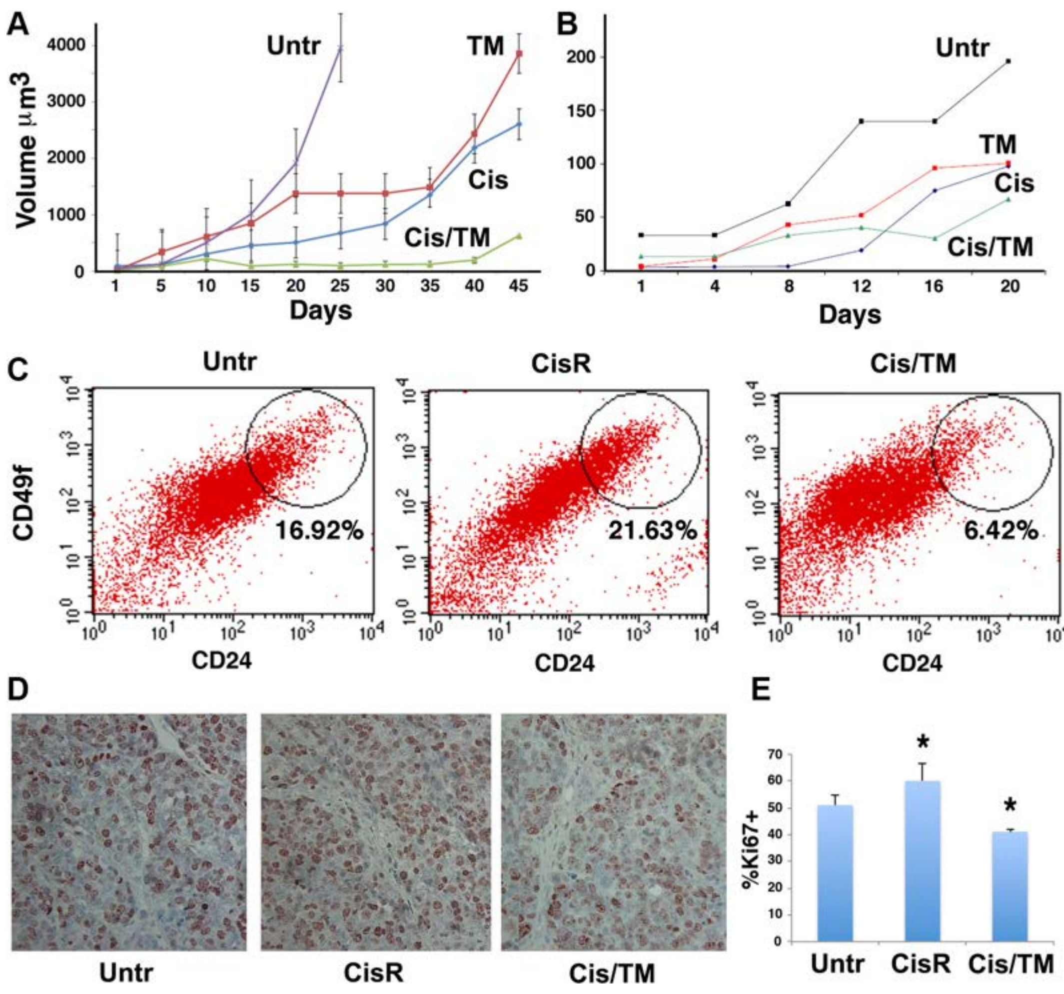

Figure 2: Cisplatin/TM combined therapy inhibits cancer growth and cancer stem cell accumulation *in vivo*. (**A**, **B**) Average tumor volumes were measured in athymic nude mice implanted with allografts of *BRCA1*-mutant mouse 69 breast cancer cells (A) or xenografts of human MDA-MB-231 breast cancer cells (B). Mice received cisplatin (Cis), TM, cisplatin/TM (Cis/TM), or no treatment (Untr) (N = 5 per group). (**C**) Percentage of $CD24^{hi}$ and $CD49f^{hi}$ cells was profiled using combination of CD24 and CD49f antibodies. Flow cytometry analysis of 69 allograft tumor-dissociated cells revealed an elevation of $CD24^{hi}CD49f^{hi}$ CSC population in cisplatin mono-treated resistant tumors (CisR) as compared to untreated tumors (Untr), whereas a decline of CSC population was observed in cisplatin/TM double treated tumors (Cis/TM) (**D**, **E**) Ki67 positive cells were detected by immunohistochemical staining with an antibody against Ki67 (D) and shown as percentage of total cells (E). *$P < 0.05$ by Student's T-test.

Microarray analysis reveals changes in gene expression unique to cisplatin and TM combinatorial treatment

To study the mechanism underlying synergistic action between cisplatin and TM on tumor growth, we compared changes in gene expression using microarray and conducted pathway analysis to investigate changes in gene expression profiles in MDA-MB-231 breast cancer cells under treatment with TM, cisplatin and cisplatin/TM. Our data revealed that cisplatin treatment for 12 hours and 24 hours induced expression change of 5,884 genes and 4,127 genes, respectively. TM treatment at these two time points induced expression change of 3,539 and 2,119 genes respectively, while cisplatin and TM double treatment induced expression change of 5,665 and 2,088 genes at 12 hours and 24 hours post-treatment respectively (Supplementary Table S3). Next, we used the Venn diagram to identify genes whose expression was altered at both 12 hours and 24 hours under each treatment condition. Data indicated that 3,156 genes (2,355 upregulated and 801 downregulated genes) overlapped at both time points upon cisplatin treatment (Figure 4A); 1,836 genes (1,449 upregulated and 387 downregulated genes) overlapped at both time points upon TM treatment (Figure 4B); and 1,576 genes (1,143 upregulated and 433

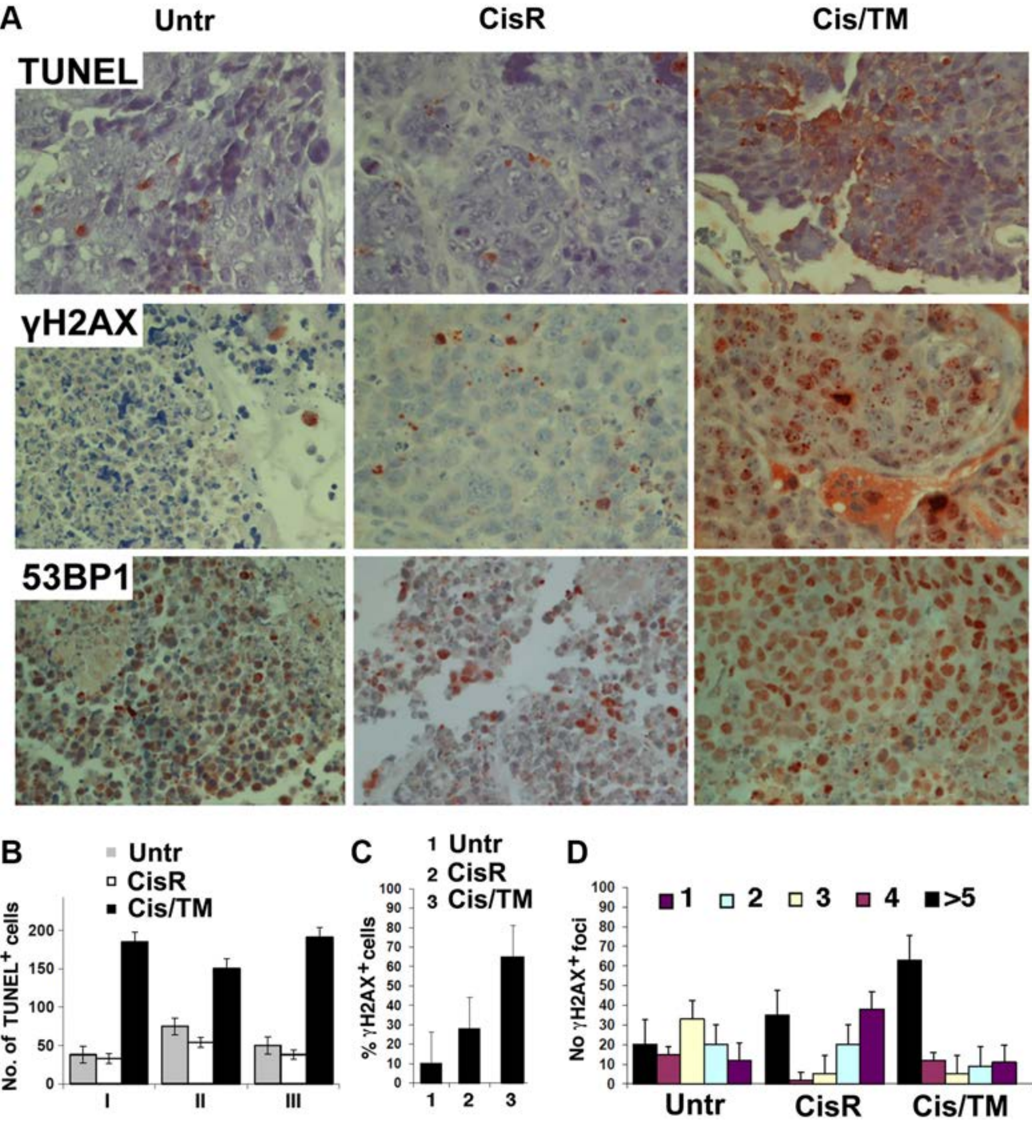

Figure 3: TM enhances cisplatin sensitivity in cisplatin/TM combined therapy in athymic nude mouse models through enhancement of DNA damage and apoptosis. (**A**) Apoptosis and DNA damage was revealed by immunohistochemical analysis of tumor sections from allografted mice that developed resistance to cisplatin or responded to cisplatin/TM (Cis/TM) treatment with tumor regression. (**B**) Number of TUNEL$^+$ cells per arbitral area in untreated (Untr), cisplatin resistant (CisR) and cisplatin/TM (Cis/TM) treated tumors. I, II and III represent three different cancers. (**C**) Number of γH2AX$^+$ cells per arbitral area in untreated (Untr), cisplatin resistant (CisR) and cisplatin/TM (Cis/TM) treated tumors. (**D**) Number of γH2AX foci per nucleus in untreated (Untr), cisplatin resistant (CisR) and cisplatin/TM (Cis/TM) treated tumor cells.

downregulated genes) overlapped at both time points upon cisplatin/TM double treatment (Figure 4C). Enrichment of KEGG pathway analysis of these common genes (including both upregulated and downregulated genes) identified pathways that were significantly changed in cisplatin, TM and cisplatin/TM treated tumors (Supplementary Figure S2). Inspection of the top 10 upregulated pathways under these treatment conditions identified 3 pathways in common, i.e. RNA polymerase, proteasome, and DNA replication, together with a number of pathways involved in DNA damage repair (Figure 4D–4F). Because double treatment of cisplatin and TM is most effective in triggering DNA damage, apoptosis, and inhibition of cancer growth, we first focused on the pathways changed in the double treated cells. Of note, the majority of the top 10 pathways is involved in cell cycle, DNA repair and damage response (i.e. DNA replication, RNA polymerase, homologous recombination, mismatch repair, base excision repair, nucleotide excision repair and p53 signaling), which may be accountable for the phenotypes observed in these cells (Figure 4G). Analysis of the microarray data using enrichment of biological functions highlighted changes in DNA replication, DNA damage repair, and cell cycle regulation and checkpoint (Figure 4H), which also attribute to the phenotype observed in the cisplatin/TM double treated cells. On the other hand, inspection of the top 10 downregulated pathways under cisplatin/TM treatment did not reveal such a causal relationship between drug treatment and phenotype (data not shown).

These pathways also exhibited more extensive changes (both in the number of genes and their expression levels) in the cisplatin/TM double treated cells than cisplatin or TM mono-treated cells (Figure 4G and 4H, and Supplementary Figure S3), which is consistent to the synergy between these two drugs as demonstrated in this study. Further analysis indicated that 21 genes are involved in various aspects of DNA damage (Supplementary Figure S4A), 47 in DNA damage response (DDR) (Supplementary Figure S4B), 26 in cell cycle regulation and checkpoint (Supplementary Figure S4C), and 33 in cell death (Supplementary Figure S4E). Real-time quantitative PCR (RT-qPCR) validation confirmed expression patterns of these genes (Figure 4I). Thus, cisplatin and TM double treatment significantly enhances DNA damage, activates DDR and multiple cell cycle checkpoints, leading to cell cycle arrest allowing cells to repair DNA damage, or cell death if the DNA damage cannot be repaired. Taken together, the result of microarray analysis is consistent with our observation in mouse allograft tumor.

TM promotes cisplatin localization in cell nucleus

Next, we sought to understand the mechanism of how TM enhances DNA damage in breast cancer. It was reported that TM inhibits tumor growth through its role in copper chelation and angiogenesis inhibition [30, 31, 34, 35]. We did not see an obvious connection between angiogenesis inhibition and the markedly enhanced DNA damage in the cisplatin/TM double treated cells. Instead, we believed this might be associated with nuclear availability of cisplatin upon combined treatment with TM.

To investigate this, we engrafted *BRCA1*-mutant 69 mouse breast cancer cells in athymic nude mice. Mono-treatment with cisplatin alone resulted in cisplatin resistant tumors that grew rapidly to 3,000 mm^3 in 32 days, while cisplatin/TM double treated tumors exhibited retarded growth at 1,000 mm^3 60 days post-treatment (data not shown). Freshly isolated cisplatin mono-treated resistant (CisR) and cisplatin/TM double treated (Cis/TM) mouse allograft mammary tumors were subject to comparative analyses by ImageStream multi-spectral imaging flow cytometry.

We observed key differences between cisplatin localization, and ATP7A protein expression and distribution in the mammary tumor cells. In cisplatin resistant cells (CisR), ATP7A expression was high, while cisplatin intensity (Pt) is low and is mainly excluded from the nucleus (Figure 5A). In contrast, cells isolated from cisplatin/TM double treated mammary tumors had significantly lower amounts of ATP7A and the protein was frequently observed to be localized in distinct, punctuate regions of the cell (Figure 5B). Coherently, the amount of cisplatin co-localization with nuclear DNA (DAPI) in cisplatin/TM doubled treated cells (Cis/TM) is significantly greater (Figure 5B) than that observed in cisplatin resistant cells (CisR) (Figure 5A).

Co-localization between ATP7A and cisplatin was further analyzed and plotted as normalized frequency against an arbitrary similarity scale from ”low” to ”high” using ImageStream's Quantitative Mean Similarity Score. The results showed that the cisplatin resistant tumor cells (CisR) exhibited extensive co-localization between cisplatin and ATP7A in the cytoplasm (Figure 5C), suggesting that ATP7A sequesters cisplatin and prevents it from getting into the nucleus. Cisplatin/TM double treated cells (Cis/TM) prevailed the lowest co-localization score, while untreated cells (Untr) displayed intermediate levels of cisplatin and ATP7A localization (Figure 5C). This observation supports the notion that ATP7A prevents the nuclear localization of cisplatin by sequestering and pumping it out of cells.

TM reduces ATP7A protein level but not gene transcription

TM was reported to reduce the copper transporter CTR1 in cervical and ovarian carcinoma [36, 37]. Thus, we hypothesized that TM may also decrease ATP7A protein expression level through copper chelation. Copper staining of allograft tumor sections showed high copper levels in both untreated (Untr) and cisplatin mono-treated

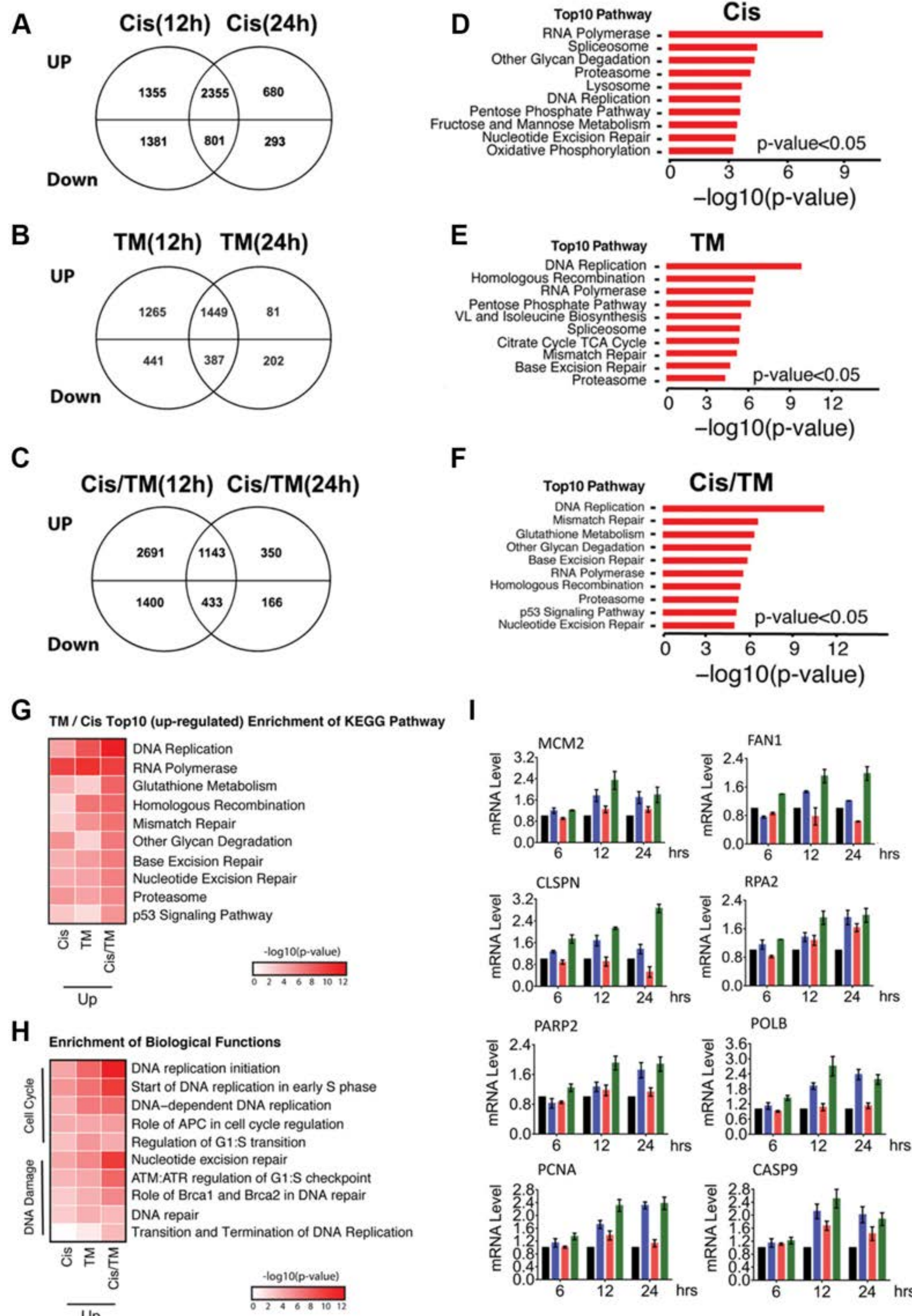

Figure 4: Microarray analysis of MDA-MB-231 cells treated with cisplatin (Cis), TM or cisplatin/TM (Cis/TM). (**A–C**) Venn diagrams depicting the number of unique upregulated or downregulated genes in cisplatin (Cis) (A), TM (B) and cisplatin/TM (Cis/TM) (C) treatment groups. (**D–F**) Bar graphs showing the top 10 enriched pathways by KEGG pathway enrichment analysis to demonstrate the difference in gene enrichment in cisplatin (Cis) (D), TM (E) and cisplatin/TM (Cis/TM) (F) treatment groups. (**G**) KEGG pathway enrichment heatmap to demonstrate the top10 upregulated pathway in cisplatin/TM treated cells and their changes in other treatment conditions. (**H**) GO function enrichment heatmap to highlight the enrichment of upregulated genes in the DNA Damage and Cell Cycle related functions under different treatment conditions. (**I**) Validation of gene expression by RT-qPCR.

resistant (CisR) mouse tumors, while cisplatin/TM double treated (Cis/TM) mouse tumors prevailed low copper levels (Figure 6A). In concert to lower copper levels, immunohistochemical staining of antibody against ATP7A on mouse tumor sections showed that cisplatin/TM double treatment (Cis/TM) reduced ATP7A protein levels as compared to untreated control (Untr) (Figure 6B).

We next asked whether ATP7A protein downregulation is due to inhibition of *ATP7A*'s gene transcription. Relative luciferase activity of ATP7A -224 HIF2 promoter was tested with cisplatin addition in the presence or absence of TM in cells with no significant changes in promoter activity in TM treated (TM) group as compared to negative control (Vehicle), whereas cisplatin (Cis), cisplatin/TM (Cis/TM) and hypoxia-inducing desferrioxamine mesylate (Dfx) treated groups showed 1.3, 1.2 and 1.4 fold increase in *ATP7A* promoter activity respectively (Figure 6C). This result suggested that TM alone or in combination with cisplatin does not significantly induce or inhibit *ATP7A*'s promoter activity.

In contrast to *ATP7A*'s promoter activity, *in vitro* treatment of cell cultures with drugs showed that addition of TM (TM) slightly reduced ATP7A protein levels 8 hours post-treatment, whereas cisplatin/TM double treatment (Cis/TM) led to ATP7A protein downregulation 6 hours post-treatment; alternatively, cisplatin treatment alone (Cis) resulted in no changes in ATP7A protein levels as compared to vehicle control (Vehicle) (Figure 6D). Therefore, this data suggested that ATP7A downregulation upon cisplatin/TM double treatment occurred at protein level. Taken together, TM reduces ATP7A protein level *in vitro* and *in vivo*, without significant effect on *ATP7A* gene transcription.

Collectively, this data supports a model that increased ATP7A sequestering of cisplatin contributes

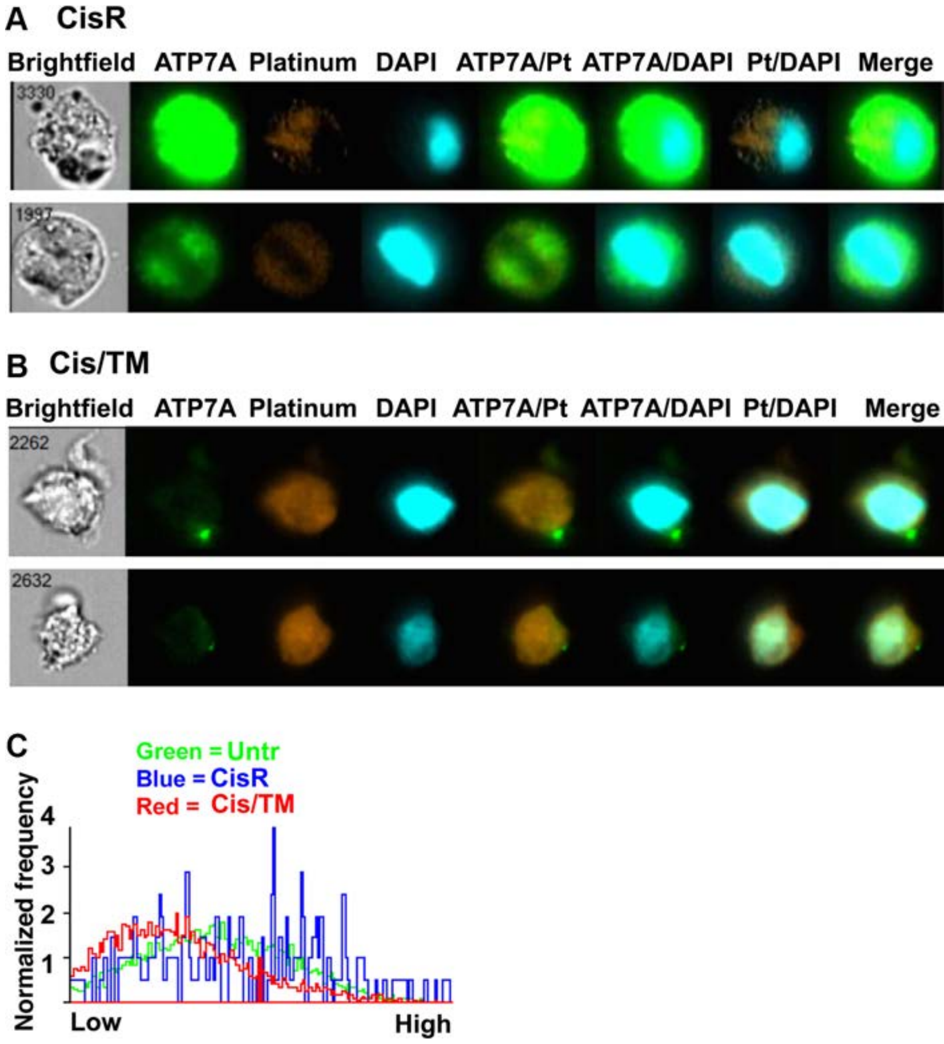

Figure 5: Co-localization between ATP7A, cisplatin and cell nucleus. (**A**, **B**) ImageStream analysis of co-localization between ATP7A, cisplatin (Pt) and cell nucleus (DAPI) in mouse 69 allograft breast cancer cells. (**C**) Frequency plot of similarity scores to demonstrate ATP7A and cisplatin co-localization in untreated, cisplatin resistant and cisplatin/TM treated mouse 69 allograft breast cancer cells after ImageStream analysis. X-axis shows arbitrary similarity scale from "low" (lower degree of co-localization) to "high" (greater degree of co-localization) between ATP7A and cisplatin. Y-axis is normalized frequency reflecting the quantity of cells scored. TM treatment significantly reduced the co-localization between ATP7A and cisplatin. Over 5,000 cells were analyzed for each group of sample.

to resistance by preventing it from reaching the nucleus, and TM treatment, which effectively reduces ATP7A, may reverse this process, leading to enhanced nuclear localization of cisplatin and consequently, DNA damage and apoptosis.

DISCUSSION

The development of drug resistance presents a major impediment to the treatment of breast cancer [7, 38, 39]. *BRCA1*-mutant breast cancers respond well to platinum agent therapy, which induces DNA crosslinking, as the tumors are deficient in DNA repair; however, cisplatin resistance often develops [12, 13, 40–43]. Platinum resistance was caused by numerous reasons, mainly covering the influx and efflux of platinum, and overcome of cytotoxicity mechanisms [9, 15, 16].

In this study, we focused on elucidation of the specific biological roles of ATP7A in cisplatin resistant breast cancer. We observed a significant increase in drug efficacy in platinum resistant breast cancer cells when we combined RNAi directed at *ATP7A* with cisplatin. In an effort to target the copper transport pathway pharmacologically, we found treatment of cells with TM could achieve comparable cytotoxicity yielded from *ATP7A* knockdown. TM is an active copper chelating agent used to treat disorders of copper metabolism, such as Wilson's disease, and serves as an anti-angiogenesis agent [28, 31]. It has been shown that TM-copper chelation has a good therapeutic effect for a number of solid tumors [34, 35].

It was previously shown that TM could increase cisplatin sensitivity and efficacy in cervical and ovarian cancer cells in a CTR-1 dependent manner [36]. However, our study revealed additional effects of TM on transport

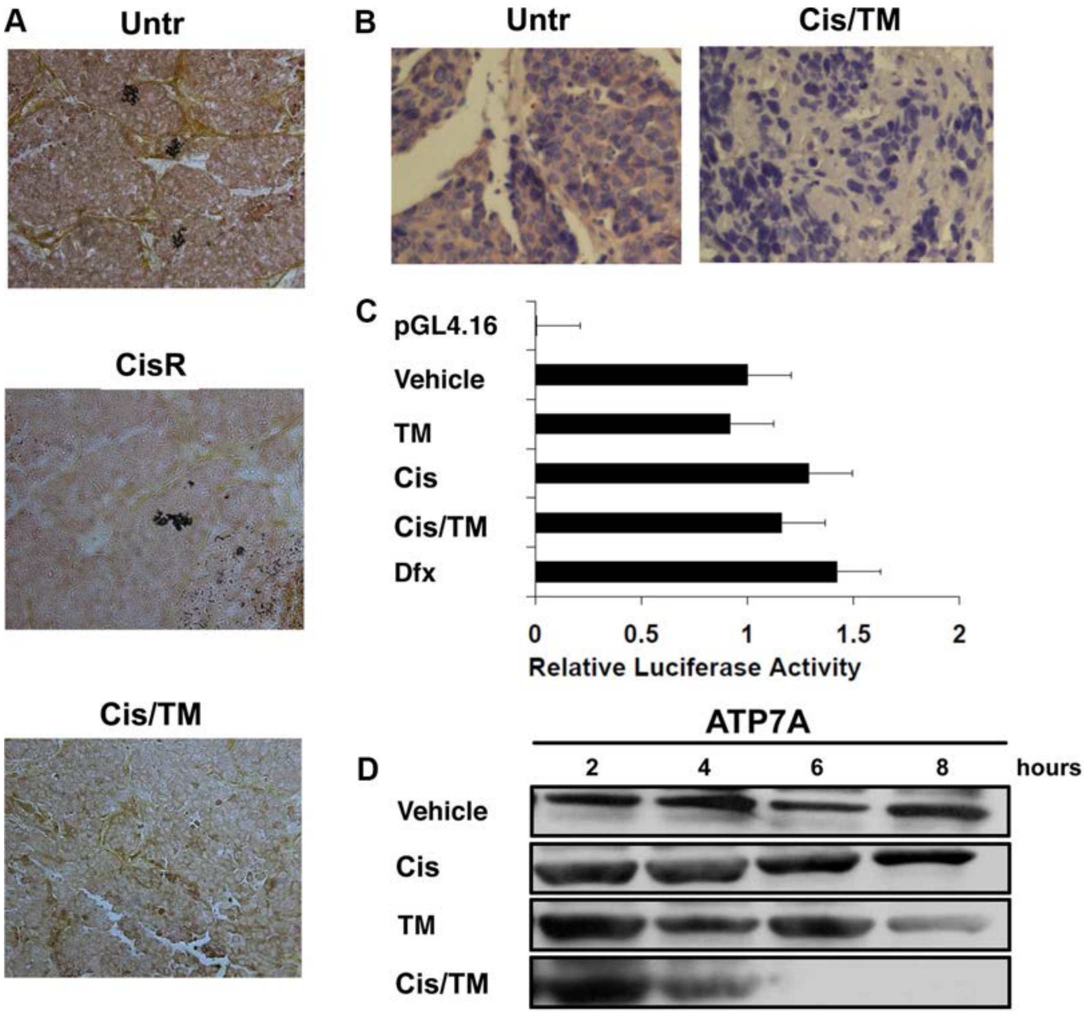

Figure 6: Investigation of ATP7A downregulation by TM in cisplatin resistant breast cancer. (**A**) TM treatment reduces copper level in cisplatin/TM (Cis/TM) treated cancers compared with untreated (Untr) and cisplatin mono-treated tumors (CisR). (**B**) Immunohistochemical staining of ATP7A in untreated and cisplatin/TM treated mouse allograft tumors showed that cisplatin/TM (Cis/TM) treated tumors had significantly less ATP7A than untreated tumors (Untr). (**C**) Relative luciferase activity assay demonstrated no significant change in ATP7A minimal promoter activity regardless of 24 hours treatment with vehicle, TM, cisplatin, cisplatin/TM, or the hypoxia-inducing Dfx. (**D**) Western blot against ATP7A in MDA-MB-231 cells under treatment with 36 µM cisplatin, and/or 10 µM TM.

downstream of CTR-1 and the exact contribution of ATP7A, a protein attributed to platinum efflux and implicated in platinum sequestering, to platinum resistance [44–46]. We demonstrated that the ability of TM to increase tumor sensitivity to cisplatin primarily through effects on ATP7A, i.e. by reducing the amount of ATP7A protein in cisplatin resistant tumor cells. We suspected that DNA damage was caused by increased nuclear localization of cisplatin. The reduction in ATP7A was also associated with reduced colocalization between ATP7A and labeled platinum, and increased colocalization between nuclear DNA and labeled platinum, suggesting that lower levels of ATP7A resulted in less sequestering of platinum.

We have previously shown that prolonged treatment with cisplatin triggers accumulation of cisplatin resistant CSCs [13]. We now found that the combination of TM and cisplatin could prevent accumulation of CSCs, accompanied by widespread DNA damage and apoptosis. To uncover the mechanism underlying the synergistic actions between cisplatin and TM, we performed microarray analysis. It revealed that combined treatment of cisplatin and TM induced changes both in gene expression level and the number of genes that are involved in DNA damage repair (DDR), cell cycle checkpoint and apoptosis at much higher frequency than the treatment of either drug alone. This finding provides the molecular basis for the synergy between these two drugs.

In summary, our data revealed that ATP7A plays a critical role in creating platinum resistance through sequestering cisplatin in the cytoplasm and pumping it out of the cell. TM treatment reduces ATP7A concentration and facilitates cisplatin localization to the nucleus in order to trigger DNA damage. This, in turn, triggers activation of DNA damage response involving ATM/ATR signaling that activates p53. The activation of p53 activates multiple cell cycle checkpoints and triggers cell cycle arrest, which allows cells to repair damaged DNA. Meanwhile, p53 also triggers apoptosis if the damage is too extensive to be repaired, resulting in reduced cell proliferation and inhibition of cancer growth (Figure 7). As cisplatin is widely used for cancer therapy and cisplatin resistance is a common problem associated with chemotherapy, our finding provides a clue to combat platinum resistant tumors and sensitize patients to conventional breast cancer treatment strategies.

MATERIALS AND METHODS

Human RNAi candidate screen

A small-scale screen of a Thermo Dharmacon human custom siRNA siGENOME library comprised of 55 hand-selected breast cancer chemotherapy resistance-related gene targets was carried out *in vitro* (Supplementary Table S1). These genes were identified in the common genomic gain regions found in the cisplatin resistant breast cancer cells and associated with poor-prognosis breast cancer, including 6p12, 6p21, 11q13, 20q13.2 and several regions of 14q [18–21]. Additionally, we added siRNA targeting genes related to stem cell maintenance to the candidate screen, such as *SOX2* and *OCT3/4*, predicting that a small cancer stem cell population maintained or evolved in cisplatin treated tumors could drive or contribute to resistance. Finally, we included several siRNAs to knock down drug detoxifying enzymes as well as drug and metal transporters.

This screen utilized several human breast cancer cell lines that were determined to be resistant to cisplatin by the National Cancer Institute (NCI) *In Vitro* Cell Line Screening Project (IVCLSP) (http://dtp.nci.nih.gov/branches/btb/ivclsp.html).

Cell transfection

For RNAi screen, T47D, MCF7 and MDA-MB-231 human breast cancer cells (ATCC) were transfected with 55 distinct siRNA pools using Dharmfect4 at 25 nM in 96-well plates seeded with 5,000 cells/well in triplicate. RNAi transfections were performed both with and without cisplatin treatment at 10 μM for MCF7 and 36 μM for MDA-MB-231 and T47D, and knockdown was validated by Western blot and quantitative RT-PCR with a consistent 88–92% knockdown efficiency achieved in three distinct human breast cancer cell lines (Supplementary Figure S1). Cells were analyzed for ATP7A expression by quantitative RT-PCR using the following primers: 5′ GCTCCTATCCAGCAGTTTGC 3′ and 5′ ACAGGGACATGCGATACACA 3′. Sensitivity to cisplatin was assayed by ATP release using the Promega Cell Titer Non-Radioactive Cell Proliferation Assay Kit and/or the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphehyl-2H-tetrazolium bromide (MTT) assay. Lamin positive controls and a non-target control were run with each assay.

For pharmacological replacement, 5,000 cells/well of the indicated cell lines was seeded into a 96-well plate in triplicate. 16 hours cultures were added with gradient concentrations of ammonium tetrathiomolybdate (TM) (Sigma-Aldrich, cat# 323446) as indicated in corresponding figures in the absence or presence of 10 μM cisplatin (Sigma-Aldrich, cat# P4394). Cells were incubated at 37°C at 5% CO_2 for 72 hours after drug addition. MTT assay was performed as instructed by manufacturer's protocol (Roche Diagnostics cat. 11465007001). All raw data was normalized to DMSO control. Predicted addictive curve was calculated by multiplying normalized mean cell viability of TM mono-treatment by mean cell viability of cisplatin mono-treatment used in this study, which is 70%. For example for doses of TM that generate 80% and 50% viability, respectively, the addictive point is 56% (70% × 80%), and 35% (70% × 50%), respectively. Graphs were plotted using GraphPad Prism 6.

Allograft and xenograft mouse models

Adherence to the NIH Guide for the Care and Use of Laboratory Animals was followed for all *in vivo* experiments. All allograft and xenograft *in vivo* experiments followed protocols described earlier [13]. Briefly, 6–10 weeks old female aythymic nude mice were implanted with *BRCA1* mutant breast cancer cells in the bilateral 4th mammary fat pads. 1 × 10^6 cells were implanted subcutaneously and tumors became visible 7–14 days post-implantation. TM and cisplatin treatment was initiated when tumors reached 200 mm^3. Mice were intraperitoneally injected with cisplatin at a dose of 6 mg/kg body weight twice per week with or without TM treatment at 0.015–0.030 mg/ml continuously in drinking water. Tumor volume was measured 2–4 times per week and compared between different treatment groups of mice (N = 10–12 tumors per group). Tumor volume was calculated using the formula: $V = ab^2/2$, where a and b is tumor length and width, respectively. *In vivo* experiments were performed in triplicate with 5–6 mice per treatment group.

Isolation and staining of mouse tumor cells for imagestream analysis

Mice were euthanized by CO_2 inhalation and tumors excised to create single cell suspensions. Tumor tissue was finely minced under sterile conditions in DMEM (Cellgro Mediatech) containing 10% FBS, 1% glutamine, 10 ng/mL epidermal growth factor (EGF) (Invitrogen), and 5 µg/mL insulin (Sigma-Aldrich), and passed through a 45 micron nylon cell strainer (BD Falcon) to create single cell suspension. Cells were pelleted and plated on 100 mm polystyrene plates (Corning) at a concentration of 3 × 10^6 cells per plate in 5 mL of DMEM media described above. Cells were left undisturbed and allowed to attach in a 37°C incubator with 5 percent CO^2 for 3 days and then passaged or frozen in liquid nitrogen. 1 × 106 live tumor cells at passage 2 or younger were incubated for 1–2 hours on ice in dark with cisplatin labelled with Kreatech Platinum Bright 570 Red/Orange Reagent (Kreatech/BioMicroSystems) in PSS buffer containing phosphate buffered saline pH 7.2, 0.5% bovine serum albumin,

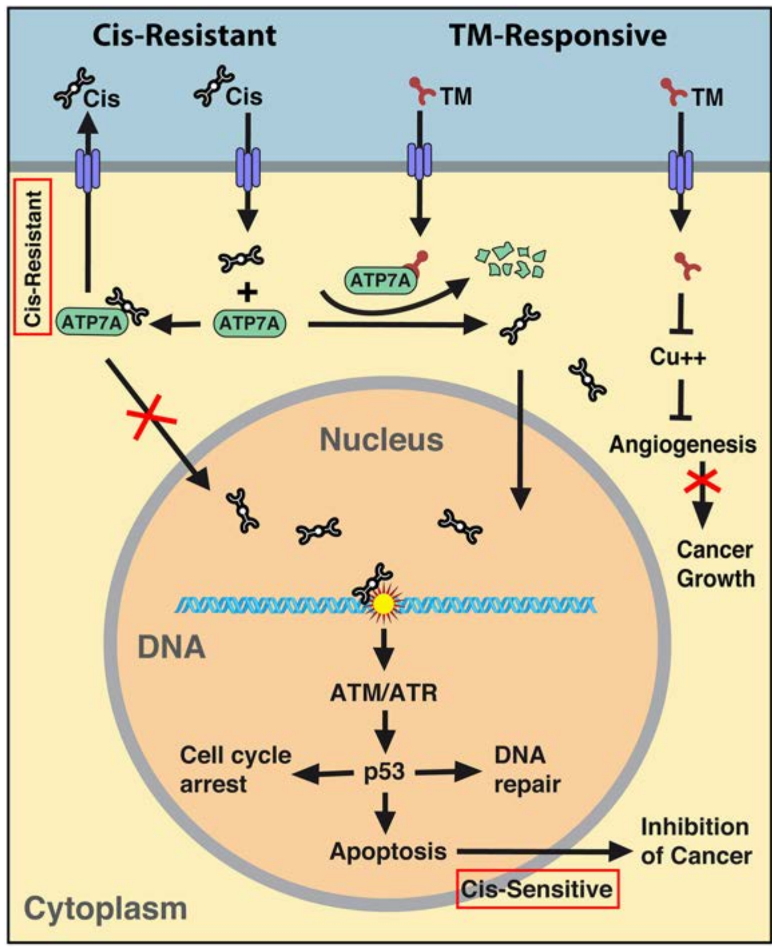

Figure 7: Summary of the mechanism regarding synergy between cisplatin and TM. Cisplatin resistant tumors express increased levels of ATP7A that sequester cisplatin in the cytoplasm to prevent its nuclear localization and also pump cisplatin out of cells. The presence of TM reduces intracellular ATP7A levels, facilitates nuclear localization of cisplatin, enhances DNA damage, hence sensitizes cisplatin resistant tumor to cisplatin therapy.

and 2 mM ethylenediaminetetraacetic acid (EDTA). The labeled cisplatin is comprised of mono-reactive cisplatin derivatives that react at the N7 positions of guanine moieties in DNA. After incubation, cells were washed again in PSS buffer and fixed in 2% paraformaldehyde and stained with anti-ATP7A primary antibody produced in chicken (Sigma-Aldrich) for 30–60 minutes on ice in dark, then an anti-chicken Alexa488 secondary antibody (Molecular Probes) for 30 minutes on ice in dark and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

Imaging flow cytometry

Localization and similarity analyses were performed on Amnis ImageStream X multi-spectral imaging flow cytometer. Alexa488-labeled ATP7A and fluorophore-labeled cisplatin were excited by a 488nm laser and collected in channels containing 470–560 nm and 560–595 nm filters, respectively. DAPI was excited by a 405 nm laser and collected in the channel containing 430–505 nm filter. 5000–10,000 cells were imaged and data were compensated and analyzed using Amnis IDEAS software. The degree of co-localization between ATP7A and cisplatin (Pt), ATP7A and DAPI, and cisplatin (Pt) and DAPI was assessed using the Similarity Feature included in the software package, based on single, focused cells. The Similarity Feature is a measure of the degree to which two input images within a masked region are linearly correlated and is a pixel by pixel comparison based on the log transformed Pearson's coefficient. The ImageStream analyses were performed in triplicate utilizing cell populations from at least 3 tumors for each treatment group.

Microarray analysis

1.0×10^5 basal type MDA-MB-231 breast cancer cells (ATCC) were treated with 36 μM cisplatin (Sigma), 10 μM TM, both drugs, or a vehicle control in triplicate in 6-well plates and harvested at 12 and 24 hours. RNA was prepared from cell pellets using the Qiagen RNeasy RNA purification kit. RNA quality was determined by 2100 Bioanalyzer analysis (Agilent Technologies) and microarray was performed in triplicate for each drug treatment group using Affymetrix gene Chip Human Gene 1.0 ST Arrays (901086). ANOVA and pathway analysis was performed by the NIDDK Genomic Core Facility. The microarray data have been submitted to the GEO database under the accession number GSE77515.

Statistical analysis

Raw intensity data were normalized by the Robust Multi-array Average (RMA) method [47] using the 'affy' package in R-Bioconductor. Normalized data were performed in R–Bioconductor using 'limma' package to identify differentially expressed genes between different treatment samples and control samples at each time point [48]. The list of differentially expressed genes at each time point of each treatment group was further filtered by the criteria of $P < 0.05$ and fold change > 1.5 to identify the set of significant differentially expressed genes in each group. Gene function enrichment was analyzed using analysis of variance (ANOVA) and Fisher's exact test. Unless otherwise stated, P values were considered significant if $P < 0.05$. Error bars represent SEM of 3 experiments unless otherwise stated. All statistical analysis was performed using R Statistical Software (version 3.1.2; R Foundation for Statistical Computing, Vienna, Austria).

ACKNOWLEDGMENTS AND FUNDING

We thank members of the Deng laboratory for critical review of the manuscript and technical advice. We are grateful for the technical assistance of Ms. Leigh Samsel in the Flow Cytometry Core Facility, National Heart Lung and Blood Institute, NIH. This work was supported, in part, by the Intramural Research Program of the NIDDK, NIH and by Chair Professor Grant (CPG) and Startup Research Grant (SRG) granted to CXD by University of Macau, Macau SAR, China.

CONFLICTS OF INTEREST

No potential conflicts of interest.

Authors' contributions

C.L.C. and C.X.D. conceived this research. C.L.C., H.W., A.H.W., G.V.O. and X.X. conducted the experiments. H.W. and W.C. processed and analyzed the microarray analysis. C.L.C., H.W., A.H.W. and C.X.D. prepared the manuscript. All authors reviewed the manuscript.

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MOLYBDENUM PRODUCTS, INC.

MOLYBDENUM AND RHENIUM CHEMICALS FOR INDUSTRY

CERTIFICATE OF ANALYSIS

PRODUCT: AMMONIUM TETRATHIOMOLYBDATE

ITEM NUMBER: 1008
LOT: 20-267
QUANTITY: 250 gms
CAS #: 15060-55-6
MW: 260.208 g/mol
EXPIRATION: 24SEP2021
FORMULA: $(NH_4)_2MoS_4$

AAA MOLYBDENUM ASSAY	PERCENT ASSAY	PERCENT SPEC
ALUMINUM (Al)	0.0001	0.0010
CALCIUM (Ca)	0.0001	0.0010
CHROMIUM (Cr)	0.0001	0.0007
COPPER (Cu)	< 0.0005	0.0010
IRON (Fe)	< 0.0003	0.0010
LEAD (Pb)	0.0001	0.0005
MAGNESIUM (Mg)	0.0001	0.0005
NICKEL (Ni)	0.0001	0.0005
SILICON (Si)	0.0001	0.0010
TIN (Sn)	0.0001	0.0010
TITANIUM (Ti)	0.0001	0.0005

This product is produced to a chemical specification, which we guaranteed. We DO NOT, express or implied, warrant the use of this product for any specific or general application.

PACKED UNDER ARGON: 24SEP2020
EXPIRATION DATE: 24SEP2021
COUNTRY OF ORIGIN: USA

CERTIFIED: Andrew D. Tulach
LAB SUPERVISOR

CUSTOMER NAME: Pharmacy Solutions

CUSTOMER PO# 01112021

AAA MOLYBDENUM INVOICE # 11589

SHIPPING DATE: 01/11/2021

1-12-21

7233 W. 116th Place
Broomfield, CO 80020

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ARL BIO PHARMA
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Certificate Of Analysis

CLIENT: Pharmacy Solutions-MI
Patrice Shook

ARL #: 465154-01
LOT #: 04232018@64
DESCRIPTION: AMMONIUM TETRATHIOMOLYBDATE 20 MG CAP
DATE RECEIVED: 04/30/2018
STORAGE: 2°C to 8°C (35.6°F to 46.4°F)
CONTAINER: One blue bottle w/10 capsules

Test	Test Method	Limits	Results	Date Tested
Ammonium Tetrathiomolybdate	ICP-MS	90.0% - 110.0%	99.0%	05/09/2018

Formulation ID: 92663
The Molybdenum Test was referenced to another facility.
The Ammonium Tetrathiomolybdate result was calculated from the molybdenum assay.

T
5-14-18

Cynthia L Pickens

05/10/2018

Cindy Pickens - Wet Chemistry Supervisor

Date Reported

Results reported above relate only to the sample that was tested.

ARL Form QUF-078-V7 09/29/2014

January 24, 2020

To Whom It May Concern:

I am a medical oncologist who treats only patients with metastatic and high risk for recurrent breast cancer and have conducted translational breast cancer research for more than 20 years. Pharmacy Solutions reached out to me regarding the pending FDA ban on tetrathiomolybdate compounding.

I unequivocally and strongly support continued compounding of tetrathiomolybdate for patient use. Based on the clinical work we have performed on IRB, and FDA approved clinical trials (under IND 71,380) and extensive correlative investigation driven by meticulous *in vitro* and *in vivo* laboratory work, copper depletion with tetrathiomolybdate (TM) clearly alters the tumor microenvironment, pulling out the infrastructure that tumors need to spread and reprogramming the metabolic microenvironment. Consequently, we strongly believe this prevents metastases. We completed accrual to and reported on a phase 2 clinical trial in 75 breast cancer patients with striking results, especially in those with triple negative breast cancer (a very difficult to treat breast cancer) and because the results are so encouraging we are in the process of developing a large randomized phase 2 clinical trial in partnership with the *Translational Breast Cancer Research Consortium* and the NCI NExT program. **TM when administered on the dose and schedule in our trial is safe, well tolerated and inexpensive**.

There is a groundswell of interest in metals in cancer and specifically copper. The 1st *Copper in Cancer Consortium* is taking place March 1-4, 2020 at Cold Spring Harbor Labs, NY in the form of a Banbury meeting which is a “think tank” meeting with thought leaders in copper biology, chemistry, signaling, metabolism and microenvironment from all over the world. The 12th Annual Copper meetings in Sorrento, Italy is an international meeting that is translational across multiple disciplines. I am invited to talk about our work in copper depletion as a therapeutic strategy in triple negative breast cancer in an Educational Session at the annual meeting of the American Association for Cancer Research (AACR) in April.

The drug development process is a long one. The development of copper depletion as a therapeutic strategy has been longer than most because it represents a completely different way to think about treating cancer. Since we haven’t cured cancer yet, most agree a different approach is needed. Development of TM has been longer than most because its development in cancer has been completely funded to date by foundations, grants, philanthropy and crowdfunding. So far, Pharma (Wilson Therapeutics and Alexion) have declined to develop it in cancer because they are focused on developing it in Wilson’s disease- a disease of 15, 000 people for year in the US. However, because they hold the intellectual property to the drug for cancer no one else can develop it for cancer commercially.

So where does this leave patients- our friends, colleagues, neighbors and family? If you believe the data (papers attached) and one’s prognosis is dismal, how great is it that there might be an option that is steeped in science and FACT to pursue that may alter the outcome? It is a bonus

that this option is oral, safe and well tolerated. Given the fact that the resources do not exist for every patient whose treating physician thinks they may benefit from TM based on the data to go on an IND sponsored clinical trial, I believe it is a patient's right to try TM to try to prevent tumor metastases. I do believe that TM is saving the lives of many of the patients who take it. Part of our job is to understand and identify who it does and does not help. Our next step is that we must assess whether our initial observations are correct and evaluate it in rigorous well designed clinical trials. If we are successful, our hope is pharma will step up and do the right thing.

In closing, I want to let you know that I get emails from TM patients all over the world every week. They ask me many different things, however unequivocally they all tell me the same one thing and that is "thank you" for doing this research. We can't let them down and you can't let them down by cutting off their drug supply.

Respectfully,

Linda Vahdat MD MBA

Current position:
Chief of Cancer Services Memorial Sloan Kettering Physicians at Norwalk Hospital
Member, Breast Medicine Service at Memorial Sloan Kettering Cancer Center
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European Medicines Agency
Pre-authorisation Evaluation of Medicines for Human Use

Document Date: London, 10 July 2008
Doc.Ref.: EMEA/COMP/82161/2008

COMMITTEE FOR ORPHAN MEDICINAL PRODUCTS

PUBLIC SUMMARY OF
POSITIVE OPINION FOR ORPHAN DESIGNATION
OF
ammonium tetrathiomolybdate
for the treatment of Wilson's disease

On 1 April 2008, orphan designation (EU/3/08/539) was granted by the European Commission to JJGConsultancy Ltd, United Kingdom, for ammonium tetrathiomolybdate for the treatment of Wilson's disease.

What is Wilson's disease?
Wilson's disease is a genetic disorder that causes excessive copper accumulation in the body, typically in the liver and brain. The liver of a person who has Wilson's disease does not release copper into the bile, as it should. Bile is a liquid produced by the liver that helps with digestion. In Wilson's disease, the copper that is normally absorbed from the food by the intestines builds up in the liver and injures liver tissue. Eventually, the damage causes the liver to release the copper directly into the bloodstream, which carries the copper throughout the body. The copper accumulated and transported by the bloodstream can then cause damage in other organs like the kidneys, brain, and eyes. If not treated, Wilson's disease can be chronically debilitating and life threatening.

What are the methods of treatment available?
Treatment of Wilson's disease generally consists of medicinal products to remove excess copper from the body and to prevent it from re-accumulating in different parts of the body by trapping copper. Several medicinal products were authorised for Wilson's disease in the Community at the time of submission of the application for orphan drug designation.
Ammonium tetrathiomolybdate might be of potential significant benefit for the treatment of Wilson's disease, because it is expected to be beneficial for the patients with Wilson's disease who suffer from neurological symptoms. This assumption will have to be confirmed at the time of marketing authorisation. This will be necessary to maintain the orphan status.

What is the estimated number of patients with Wilson's disease*?
Based on the information provided by the sponsor and previous knowledge of the Committee, Wilson's disease was considered to affect not more than 0.6 in 10,000 persons in the European Union which, at the time of designation, corresponded to about 30,000 persons.

How is this medicinal product expected to act?
Ammonium tetrathiomolybdate binds to copper and to a protein called albumin in the bloodstream. Once bound to ammonium tetrathiomolybdate and albumin, copper cannot be taken up by the organs and the damaging effects of copper are expected to decrease. Ammonium tetrathiomolybdate also acts

* Disclaimer: For the purpose of the designation, the number of patients affected by the condition is estimated and assessed based on data from the European Union (EU 27), Norway, Iceland and Lichtenstein. This represents a population of 498,000,000 (Eurostat 2006). This estimate is based on available information and calculations presented by the sponsor at the time of the application.

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in the intestine, by blocking the absorption of copper into the bloodstream, then decreasing copper uptake.

What is the stage of development of this medicinal product?
The effects of ammonium tetrathiomolybdate were evaluated in experimental models.

At the time of submission of the application for orphan designation, clinical trials in patients with Wilson's disease were ongoing.

Ammonium tetrathiomolybdate was not authorised anywhere worldwide for Wilson's disease, at the time of submission. Orphan designation of ammonium tetrathiomolybdate was granted in the United States for the treatment of Wilson's disease.

According to Regulation (EC) No 141/2000 of 16 December 1999, the Committee for Orphan Medicinal Products (COMP) adopted on 6 February 2008 a positive opinion recommending the grant of the above-mentioned designation.

Opinions on orphan medicinal products designations are based on the following cumulative criteria: (i) the seriousness of the condition, (ii) the existence or not of alternative methods of diagnosis, prevention or treatment and (iii) either the rarity of the condition (considered to affect not more than five in ten thousand persons in the Community) or the insufficient return of development investments.

Designated orphan medicinal products are still investigational products which were considered for designation on the basis of potential activity. An orphan designation is not a marketing authorisation. As a consequence, demonstration of the quality, safety and efficacy will be necessary before this product can be granted a marketing authorisation.

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Translations of the active ingredient and indication in all EU languages and Norwegian and Icelandic

Language	Active Ingredient	Indication
English	Ammonium tetrathiomolybdate	Treatment of Wilson's disease
Bulgarian	Амониев тетратиомолибдат	Лечение на болест на Уилсон
Czech	Amonium terathiomolybdat	Léčba Wilsonovy choroby
Danish	Ammonium tetrathiomolybdat	Behandling af Wilson's sygdom
Dutch	Ammonium tetrathiomolybdaat	Behandeling van de ziekte van Wilson
Estonian	Ammoonium-tetratiomolübdaat	Wilsoni haiguse ravi
Finnish	Ammoniumtetratiomolybdaatti	Wilsonin taudin hoito
French	Tétrathiomolybdate d'ammonium	Traitement de la maladie de Wilson
German	Ammonium tetrathiomolybdat	Behandlung des Morbus Wilson
Greek	Τετραθειούχο μολυβδένο αμμώνιο	Θεραπεία της νόσου του Wilson
Hungarian	Ammónium tetrathiomolybdát	Wilson-betegség kezelése
Italian	Tetratiomolibdato di ammonio	Trattamento della malattia di Wilson
Latvian	Amonija tetratiomolibdāts	Vilsona slimības ārstēšana
Lithuanian	Amonio tetratiomolibdatas	Vilsono ligos gydymas
Maltese	Ammonium tetrathiomolybdate	Kura tal-marda ta' Wilson
Polish	Tetrathiomolybdate amonowy	Leczenie choroby Wilsona
Portuguese	Tetratiomolibdato de amónio	Tratamento da doença de Wilson
Romanian	Tetratiomolibdat de amoniu	Tratamentul bolii Wilson
Slovak	Tetratiomolybdénan amónny	Liečba Wilsonovej choroby
Slovenian	Amonijev tetratiomolibdat	Zdravljenje Wilsonove bolezni
Spanish	Tetratiomolibdato de amonio	Tratamiento de la enfermedad de Wilson
Swedish	ammonium tetratiomolybdat	Behandling av Wilsons sjukdom
Norwegian	Ammoniumtetratiomolybdat	Behandling av Wilsons sykdom
Icelandic	Ammóníak tetratíómólýbdat	Meðferð við Wilsons sjúkdómi

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Ammonium Tetrathiomolybdate

MARTINDALE - The Complete Drug Reference

See also Chelators Antidotes and Antagonists

Ammonium Tetrathiomolybdate

Physical And Pharmaceutical Properties

- Synonyms:Tetratiomolibdato de amonio
- Molecular Formula:$(NH_4)_2MoS_4$
- Molecular Weight:260.3
- CAS Registry:15060-55-6
- Notes and Warnings:NOTE. The name Coprexa has been used as a trade mark for ammonium tetrathiomolybdate.

Profile

- Ammonium tetrathiomolybdate is a chelator that aids the elimination of copper from the body. It is under investigation in the treatment of Wilson's disease and pulmonary fibrosis. There is also some interest in its use in the treatment of malignant neoplasms. (Last reviewed: 2011-08-11; last modified: 2011-06-24)
- References. (Last reviewed: 2011-08-11; last modified: 2011-06-16)

- 1. Medici V, Sturniolo GC. Tetrathiomolybdate, a copper chelator for the treatment of Wilson disease, pulmonary fibrosis and other indications. IDrugs 2008; 11: 592-606. (PubMed id:18683094)
- 2. Brewer GJ. The use of copper-lowering therapy with tetrathiomolybdate in medicine. Expert Opin Invest Drugs 2009; 18: 89-97. (PubMed id:19053885)
- 3. Khan G, Merajver S. Copper chelation in cancer therapy using tetrathiomolybdate: an evolving paradigm. Expert Opin Invest Drugs 2009; 18: 541-8. (PubMed id:19335282)

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Wilson's disease.

- Ammonium tetrathiomolybdate forms a complex with protein and copper. When it is taken with food it blocks the intestinal absorption of copper, and when given between meals it combines with albumin- and caeruloplasmin-bound copper. Ammonium tetrathiomolybdate is under investigation for the initial reduction of copper levels in patients with Wilson's disease (Penicillamine); it may be particularly suitable for patients with neurological symptoms.[1] Bone marrow depression[1,2] and raised liver enzymes[1] have been reported; both have responded to temporary withdrawal or dose reduction. (Last reviewed: 2011-08-11; last modified: 2005-10-31)

- 1. Brewer GJ, et al. Treatment of Wilson disease with ammonium tetrathiomolybdate III: initial therapy in a total of 55 neurologically affected patients and follow-up with zinc therapy. Arch Neurol 2003; 60: 379-85. (PubMed id:12633149)

- 2. Harper PL, Walshe JM. Reversible pancytopenia secondary to treatment with tetrathiomolybdate. Br J Haematol 1986; 64: 851-3. (PubMed id:3801328)

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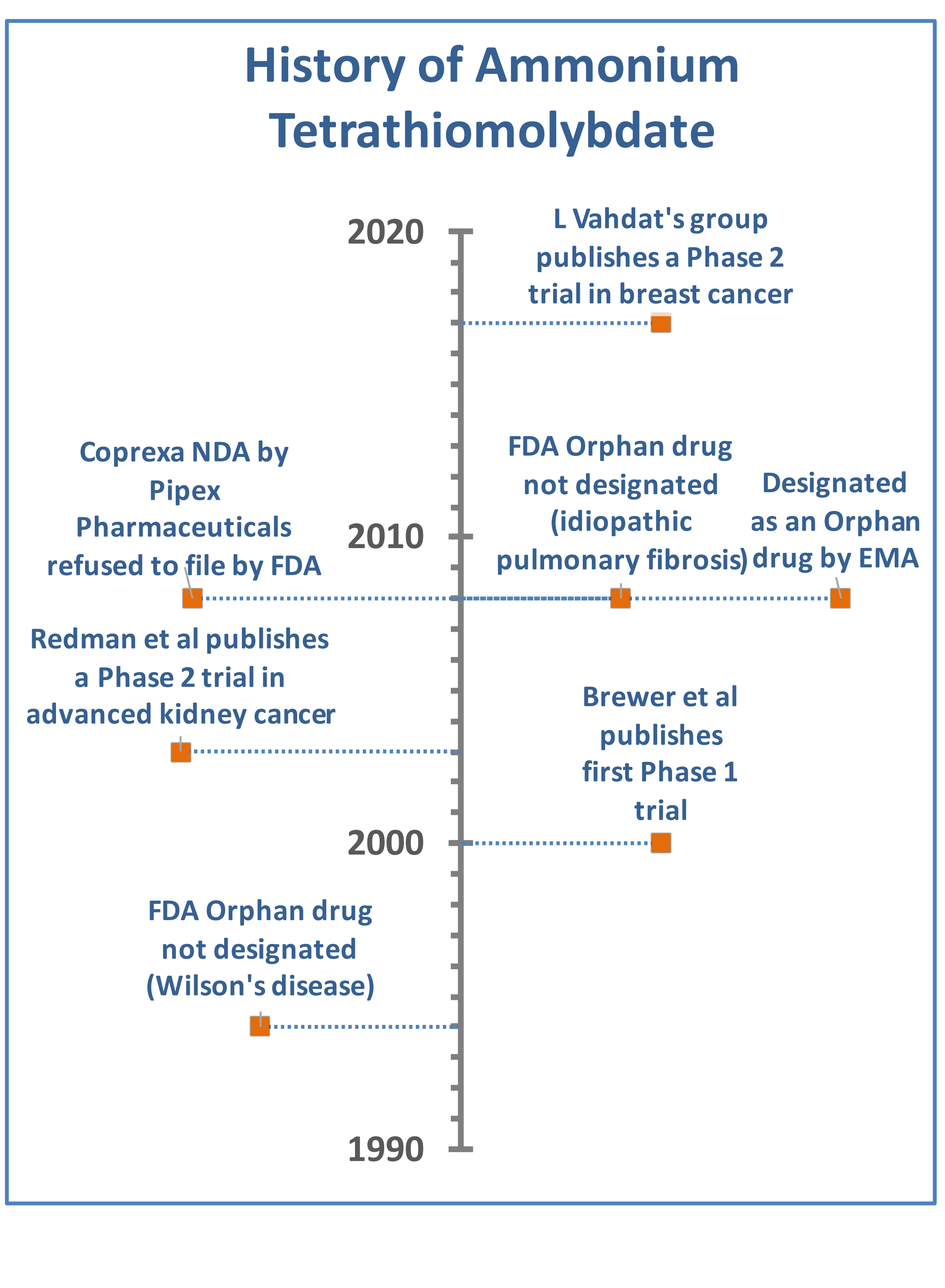
History of Ammonium Tetrathiomolybdate
2020
2010
2000
1990
L Vahdat's group publishes a Phase 2 trial in breast cancer
Coprexa NDA by Pipex Pharmaceuticals refused to file by FDA
FDA Orphan drug not designated (idiopathic pulmonary fibrosis)
Designated as an Orphan drug by EMA
Redman et al publishes a Phase 2 trial in advanced kidney cancer
Brewer et al publishes first Phase 1 trial
FDA Orphan drug not designated (Wilson's disease)

Tab 3b

FDA Evaluation of Ammonium Tetrathiomolybdate

DATE: May 2, 2022

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Charles Ganley, M.D.
Director, OSM, OND

Frances Gail Bormel, R.Ph., J.D.
Director, OCQC, OC

TO: Pharmacy Compounding Advisory Committee

SUBJECT: Evaluation of Ammonium Tetrathiomolybdate for Inclusion on the 503A Bulk Drug Substances List

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I. INTRODUCTION

Ammonium tetrathiomolybdate[1] (ATTM) was nominated for inclusion on the list of bulk drug substances for use in compounding under section 503A of the Federal Food, Drug, and Cosmetic Act (FD&C Act).[2] We evaluated ATTM for potential use in the treatment of Wilson disease and use as copper chelation therapy for the treatment of breast cancer, kidney cancer, prostate cancer, colorectal cancer, esophageal cancer, and malignant pleural mesothelioma[3,4] The proposed route of administration is oral using 20 mg to 60 mg capsules.

There are no FDA-approved drug products that contain ATTM as an active ingredient; ATTM does not have an applicable US Pharmacopeia (USP) or National Formulary (NF) drug substance monograph.

We have reviewed publicly available data on the physicochemical characteristics, safety, effectiveness, and historical use in compounding of this substance. For the reasons discussed below, we believe the evaluation criteria ***weigh against*** placing ATTM on the list of bulk drug substances that can be used to compound drug products in accordance with section 503A of the FD&C Act (503A Bulks List).

II. EVALUATION CRITERIA

A. Is the substance well-characterized, physically and chemically, such that it is appropriate for use in compounding?[5]

Figure 1. Chemical Structure of Ammonium Tetrathiomolybdate

$$(NH_4^+)_2 \left[MoS_4 \right]^{2-}$$

[1] The CAS (Chemicals Abstract Service, a division of American Chemical Society) name is (T-4_-Tetrathioxomolybdate(2-)ammonium (1:2). The Merck Index Online, https://www-rsc-org.fda.idm.oclc.org/Merck-Index/monograph/m1827/ammonium%20tetrathiomolybdate?q=authorize Accessed Jul 14, 2021.

[2] Pharmacy Solutions nominated ATTM (Document ID: FDA-2015-N-3534-0295) on February 1, 2021; the nomination can be accessed at https://www.regulations.gov/document/FDA-2015-N-3534-0295.

[3] ATTM was nominated for use in the treatment of Wilson disease and copper chelation therapy. For reasons detailed in Section II.C.2. of this evaluation memo, FDA evaluated ATTM for its use in specific types of cancer. Of note, due to structural relevance, some of the discussion in subsequent sections of this evaluation is from references that use the terminology TTM instead of ATTM. Such discussion is only to provide supportive information for the evaluation for ATTM. Any conclusion that is drawn in this review only pertains to the substance ATTM.

[4] The nomination references 13 published articles of which 10 describe the use of ATTM to treat various types of cancer.

[5] Among the conditions that must be met for a drug compounded using bulk drug substances to be eligible for the exemptions in section 503A of the FD&C Act is that the bulk drug substances are manufactured by an establishment that is registered under section 510 of the FD&C Act and that each bulk drug substance is accompanied by a valid certificate of analysis. Sections 503A(b)(1)(A)(ii) and (iii). A bulk drug substance is deemed to be adulterated if the methods used in, or the facilities or controls used for, its manufacture, processing, packing, or holding do not conform to or are not operated or administered in conformity with current good manufacturing practice. Section 501(a)(2)(B).

ATTM, also known as diammonium tetrathiomolybdate, is an inorganic salt, and is the ammonium salt of tetrathiomolybdate. It is a chelating reagent for copper. Tetrathiomolybdate (TTM) is the active moiety of ATTM, and it does not exist as an individual substance.

Databases searched for information on ATTM in preparation of this section included PubMed, SciFinder, Analytical Profiles of Drug Substances, the European Pharmacopoeia, British Pharmacopoeia, and Japanese Pharmacopoeia, and USP/NF.

1. *Stability of the active pharmaceutical ingredient (API) and likely dosage forms*

ATTM is stable at temperatures up to 150°C; at higher temperatures, it will decompose into molybdenum trisulfide (MoS_3), ammonia (NH_3) and hydrogen sulfide (H_2S). Multiple commercial resources have claimed ATTM to be stable but sensitive to oxygen.[6, 7] Moreover one study reported that ATTM decomposes via an induced internal electron transfer reaction caused by atmospheric oxygen followed by hydrolysis at room temperature (Chandrasekaran 1987). Therefore, this compound is likely to be stable only if protected from moisture and air when compounded as capsules.

2. *Probable routes of API synthesis*

ATTM can be obtained by reduction of molybdenum oxide with hydrogen sulfide and ammonium hydroxide (Srinivasan et al., 2004; McDonald et al., 1983).

$$2NH_4^+ + MoO_4 + 4\ H_2S \longrightarrow [NH_4]_2MoS_4 + 4H_2O$$

3. *Likely impurities*[8]

Likely impurities may include:

1) Residual starting materials and reaction intermediates, including ammonium molybdate.
2) Byproducts from the decomposition of ATTM, such as molybdenum trisulfide.

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[6] See, e.g., ATTM safety data sheet by Sigma Aldrich is available at https://www.sigmaaldrich.com/US/en/sds/aldrich/323446. Accessed Jan 18, 2022.

[7] See, e.g., ATTM safety data sheet by Fisher Scientific is available at https://www.fishersci.pt/store/msds?partNumber=10137133&productDescription=50GR+Ammonium+tetrathiomolybdate%2C+99.95%25%2C+%28trace+metal+basis%29&countryCode=PT&language=en. Accessed Jan 18, 2022.

[8] This evaluation contains a non-exhaustive list of potential impurities in the bulk drug substance and does not address fully the potential safety concerns associated with those impurities. The compounder should use the information about the impurities identified in the certificate of analysis accompanying the bulk drug substance to evaluate any potential safety and quality issues associated with impurities in a drug product compounded using that bulk drug substance taking into account the amount of the impurity, dose, route of administration, and chronicity of dosing.

4. *Toxicity of those likely impurities*

Impurities are unlikely to be toxic.

5. *Physicochemical characteristics pertinent to product performance, such as particle size and polymorphism*

ATTM is a dark red, crystalline solid, soluble in water. No further information on the influence of particle size or polymorphism on the drug's bioavailability were found in the literature.

6. *Any other information about the substance that may be relevant, such as whether the API is poorly characterized or difficult to characterize*

ATTM can be characterized using readily available analytical techniques, e.g., elemental analysis, infrared spectroscopy, UV-Vis spectroscopy, and X-ray crystallography.

Conclusions: Based on the available information, ATTM can be characterized using readily available analytical techniques. ATTM is likely to be stable if protected from moisture and air when compounded as capsules.

B. Are there concerns about the safety of the substance for use in compounding?

1. *Nonclinical Assessment*

The following databases were consulted in preparation of this section: PubMed, PubChem, Embase, SCOPUS Web of Science, ToxNet, GRAS Notices database, NIH dietary supplement database, LactMed, LiverTox, Google, and Google Scholar.

a. General pharmacology of the drug substance

Anti-copper agents have been studied as general inhibitors of angiogenesis due to the dependence of several angiogenic factors on the presence of copper. Among these, ATTM[9] acts to interfere with intestinal uptake of copper when administered with meals. ATTM can also remove copper from metallothionein and form insoluble copper complexes that are deposited in the liver. TTM, the active moiety of ATTM, has been studied in animal tumor models where it has been shown that TTM forms a complex with food protein and copper to prevent copper absorption. When given without food, it is absorbed into the blood and binds to albumin; the copper-albumin complex becomes unavailable for cellular uptake and becomes nontoxic (Brewer 2003).

[9] Molybdenum (Mo) is an essential trace element with a provisional recommended dietary intake, based on average reported intake, of 75-250 g/day for adults and older children. Mo functions as an electron transport agent in the molybdenum-flavoprotein enzyme, xanthine oxidase. It is also a cofactor for aldehyde oxidase, NADH-dehydrogenase, xanthine dehydrogenase, and sulfite oxidase (reviewed by the Environmental Protection Agency, EPA https://rais.ornl.gov/tox/profiles/molybdenum_f_V1.html).

b. Phamacokinetics/Toxicokinetics

ATTM in rats:
ATTM administered orally to rats (n=5/group) as TTM ion (MoS_4^{2-}) at 4 or 12 mg Mo/kg diet strongly inhibited copper absorption and decreased hepatic and renal uptake by increasing plasma retention of copper. Clinical and biochemical effects induced by orally administered MoS_4^{2-} were not seen when the dietary concentrations of copper were increased. Such treatment also inhibited the absorption and tissue retention of ^{99}Mo derived from $^{99}MoS_4^{2-}$. Intraperitoneal administration of copper ameliorated the clinical effects attributable to MoS_4^{2-} but neither inhibited ^{99}Mo absorption nor the appearance of systemic defects in copper metabolism (Mills 1981).

ATTM in dogs:
A pharmacokinetic study was conducted in adult beagle dogs (n=4/sex) where dogs received TTM (1 mg/kg) either intravenously (IV) or orally in a randomized crossover study. Serum molybdenum and copper concentrations were measured at 0 to 72 hours after administration. Under the conditions of the study, an increase in the serum copper concentrations was seen after TTM administration, suggesting that TTM mobilized tissue copper (Chan 2015).

ATTM in sheep:
The systemic distribution and retention of supplemented copper (150 mg/kg) and molybdenum in TTM-treated sheep of different breeds and copper status was investigated following subcutaneous exposure to TTM (3.4 mg/kg, subcutaneously, on three alternate days per month, for 5 months). Tissue levels of copper were measured in the following tissues: brain, liver, kidney, heart, skeletal muscle, pituitary, adrenals, testes, and ovaries. Molybdate accumulated in all organs, including brain and pituitary, in all TTM treated groups and was still present in tissues after cessation of treatment, except in liver, kidney, and skeletal muscle. Copper was detected in the cerebellum and medulla oblongata in the TTM-treated high-copper groups. Brain copper vs. molybdenum concentrations showed a strongly positive correlation in the high-copper group 7 months after TTM treatment. TTM was not excreted, but molybdenum was widely distributed and retained in many organs including brain and pituitary. In addition, TTM redistributed some displaced excess liver copper (Cu-TTM) to the brain (Haywood 1998).

c. Acute toxicity[10]

No studies were found in the literature.

[10] Acute toxicity refers to adverse effects observed following administration of a single dose of a substance, or multiple doses given within a short period (approximately 24 hours). Endpoints captured in acute toxicity studies usually include mortality and gross clinical observations. Acute toxicity data are usually superseded by data obtained from longer term toxicity studies.

d. Repeat dose toxicity[11]

A subchronic study conducted in dogs for 12 weeks with TTM did not show changes in biochemical, hematological, or histological changes. Out of 10 dogs, one dog developed immune-mediated anemia and thrombocytopenia (Langlois 2019).

Copper-associated hepatopathy (CAH) is a common cause of liver disease in dogs. To study the effects of ATTM on CAH in dogs, a study was conducted in dogs which were orally treated with TTM for 6 weeks, and hepatic biopsies were performed after the treatment course. Dogs experiencing initial decreases in hepatic copper concentrations ($[Cu]_H$) received 6 additional weeks of TTM treatment and underwent an additional biopsy. Eight of 10 dogs showed decreases in $[Cu]_H$ when compared to baseline levels (median, 1606 µg/g; range, 572-5158 µg/g) at 6 weeks (1033 µg/g, 450-2975 µg/g; $P = 0.04$) and 12 weeks (931 µg/g, 218-1677 µg/g; $P = 0.02$). Hepatic molybdenum concentrations increased >50-fold ($P < 0.001$). Changes in histologic scores and hematologic and biochemical results were variable and not significantly different from baseline. The results of this study suggest that TTM can decrease $[Cu]_H$ in some dogs with CAH (Langlois 2019).

e. Genotoxicity[12]

No studies were found in the literature.

f. Developmental and reproductive toxicity[13]

A flock of sheep 18 months of age which were accidentally fed a proprietary diet that contained a toxic amount of copper (227 mg/kg) for a 3-month period, were successfully treated with TTM. The following year, all animals developed fertility problems (lack of estrous in females and lack of libido in males), were not able to reproduce, and their general health progressively declined and most of the sheep died 2–3 years later (Haywood 2004). The last five surviving animals were euthanized. Necropsy revealed marked morbidity with depletion of the pituitary and adrenal glands, testicular atrophy, and ovarian cystic follicles. Histopathological examination revealed a non-inflammatory atrophy or degeneration of the adenohypophysis and loss of trophic cells as well as adrenocortical and testicular atrophy and ovarian degeneration. The adverse findings cited above could have resulted from an interference with the hypothalamo-adenohypophyseal axis. Whether ATTM exerts its effect centrally or directly on the pituitary is not well understood.

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[11] Repeat dose toxicity studies consist of in vivo animal studies that seek to evaluate the toxicity of the test substance by observing the changes that emerge in clinical observations, clinical chemistry, gross pathology, and histology endpoints when the test substance is repetitively administered daily for a predetermined period of time.

[12] The genotoxicity assessment battery usually consists of a gene mutagenicity assay (for single dose trials) and a variety of clastogenicity/genotoxicity assays. To support multiple dose administration in humans, additional genotoxicity testing assessment is usually conducted to detect chromosomal damage in mammalian systems.

[13] Developmental and reproductive toxicity studies are usually designed to assess the potential adverse effects in humans of both sexes and include females from various age groups that will be exposed to the proposed substance. *Developmental toxicity* or *teratogenicity* refers to adverse effects (can include embryo-fetal mortality, structural abnormalities, functional impairment, or alterations to growth) and can occur in pups either as a result of the exposure of their parents to the substance, prior to the pups' birth, or by direct exposure of the pups to the substance after birth.

Pregnant ewes (day 32 of gestation) were administered diammonium TTM doses of 0, 20, or 60 mg per day via intraruminal controlled-release devices. Ewes also received an oral dose of either 120 or 360 mg TTM twice weekly starting on day 86 of gestation. Liver and kidney samples were taken from lambs 48 hours after birth and from ewes on day 18 postpartum. Liver copper concentrations were decreased ($P < 0.05$), but kidney copper concentrations increased ($P < 0.05$) by 16-fold in ewes administered the higher dose of TTM. Liver and kidney molybdate concentrations were elevated ($P < 0.05$) 9- and 30-fold, respectively, in ewes treated with TTM. Plasma glucose concentrations were decreased ($P < 0.05$) in the highest TTM-treated ewes. Lambs of ewes given oral TTM had a fivefold increase ($P < 0.05$) in liver molybdate concentration; kidney molybdate concentration was not affected ($P > 0.05$) whereas liver copper concentration was reduced ($P < 0.05$). It appears that molybdate mobilized copper from the liver, and a copper-molybdate complex accumulated in the kidney. Some of the molybdate crossed the placenta, but only a limited amount of molybdate accumulated in fetal livers.

Weanling rats fed thiomolybdate (6 mg/kg) and copper (3 mg/kg) for 2 days and up to 3 weeks showed severe effects on the skeleton of treated rats. Findings included severe changes at the long bones' growth plates, muscle insertions and below the periosteum, growth plate cartilaginous dysplasia with subsequent interference with endochondral ossification. Other findings include subperiosteal multiplication of osteogenic cells and production of large amounts of disorganized bone, resorption of trabecular bone, and interference with fibrogenesis at ligamentous attachment to the bones (Spence 1980).

g. Carcinogenicity[14]

No studies were found in the literature.

Conclusions: ATTM, also known as diammonium tetrathiomolybdate, is an inorganic salt, and is the ammonium salt of tetrathiomolybdate. ATTM acts to chelate copper thus reducing the toxicity associated with high doses of copper. TTM, the active moiety of ATTM, has been studied in animal tumor models where it was shown to form a complex with food protein and copper to prevent copper absorption. The anti-copper activity of ATTM has been shown in rat, dog, and sheep models. Treatment with TTM may be able to reduce the incidence of copper-associated hepatopathy in dogs. ATTM may be associated with an increased incidence of immune-mediated anemia and thrombocytopenia in dogs. Exposure to TTM and copper resulted in malformations in growing bones of weanling rats. While the toxicity profile of ATTM has been described in the presence of copper, especially in reproductive studies, the toxicology profile of ATTM alone is not well described in the literature.

[14] Studies that assess cancer risk in animals are used as predictive tools to evaluate the potential for drugs to result in tumors when used by humans on a chronic basis. Carcinogenicity studies are conducted if the clinical use is expected to be continuous for a minimum of 6 months of life, or if intermittent clinical use is expected to total 6 months or more of life.

2. *Human Safety*

Databases consulted in preparation of this section include PubMed, EMBASE, ClinicalTrials.gov, FDA Adverse Event Reporting System (FAERS), and various online clinical references and professional healthcare organization websites.

a. Reported adverse reactions in FAERS

The Division of Pharmacovigilance (DPV) in the Office of Surveillance and Epidemiology conducted a search of the FAERS database for adverse event (AEs) reports from January 1, 2000 through December 25, 2017. At that time, the FAERS database retrieved a total of 16 worldwide cases with ATTM as a suspect drug. The most common reported reason for use of ATTM was malignancy (13), followed by Wilson disease (2); one case did not report reason for use. All events reported were observed in adult (> 18 years old) patients. The total daily dose of ATTM ranged from 40 mg to 280 mg. All cases[15] reported serious outcomes which included death (1), life-threatening adverse drug experiences (2), hospitalization (7), required intervention (2), and other medically significant events (5). Cases of serious outcomes were not mutually exclusive. These cases are briefly described below.

Death (n=1)
One case reported death in association with the use of ATTM after nine months of therapy. The reported reason for use was for the treatment of hepatocellular carcinoma. The patient's past medical history included hepatitis C and cirrhosis. The likely cause of death was reported as bacteremia due to *Escherichia coli* that led to hepatic and renal decompensation. DPV opined that this case was confounded by worsening of the patient's underlying disease of hepatocellular carcinoma and limited by lack of documentation of concomitant medication.

Hematologic abnormalities (n = 8):

- Anemia (1)
- Anemia and neutropenia (1)
- Neutropenia (2)
- Leukopenia (2)
- Anemia and leukopenia (2)

In these cases, the reported reasons for ATTM use were hepatocellular carcinoma (4), mesothelioma (3), or colon cancer (1). The overall time to onset of the event in relation to initiation of daily administration of ATTM ranged from 14 to 75 days. Of the 8 cases; 4 did not report concomitant medications, 3 were not taking any other medications, and 1 reported concomitant use of gabapentin, hydrochlorothiazide (HCTZ), vitamin C infusion, and verapamil.

In cases that reported anemia, treatment included blood transfusion (3) or hospitalization (1). Seven cases reported discontinuation of ATTM; 5 were re-challenged with a lower dose, and of these, 1 had persistent anemia. DPV opined that the hematologic abnormalities in the 8 cases may have been confounded by underlying disease. For the case where the concomitant

[15] "Cases" and "patients" mean the same and are used interchangeably within this evaluation.

medications were reported, the FDA-approved labels for HCTZ and gabapentin include hematologic adverse events (such as anemia and leukopenia for HCTZ). Because the concomitant medications are associated with these AEs, we cannot determine that the adverse events resulted from the use of ATTM. The duration of therapy for these medications are not documented. The time to onset of the anemia and leukopenia was 14 days after initiation of ATTM, suggesting a temporal association.

Thromboembolic/cardiac disorder (n = 3):

- Acute pulmonary embolism (1)
- Chronic pulmonary embolic disease (1)
- Palpitations, minimal ectopy on electrocardiogram (1)

In these cases, the reported reasons for ATTM use were metastatic colon cancer (1), mesothelioma (1), and unknown (1). The overall time to onset of the AE in relation to daily administration of ATTM ranged from 35 to 103 days. One case involved a patient with metastatic colon cancer who experienced an acute pulmonary embolism; however, the patient was also taking Premarin® (conjugated estrogen). Another case involved a patient who experienced chronic pulmonary embolic disease (unknown reason for use); the patient had a significant past medical history for interstitial lung disease. All the cases were likewise confounded by underlying disease states, concomitant medication or limited by insufficient documentation.

Hepatic abnormalities (n = 2):

- A patient who developed elevated (4x above baseline) aspartate transaminase (AST) and alanine transaminase (ALT) three weeks after initiating treatment with 120 mg of ATTM daily for treatment of Wilson disease. After the medication was held temporarily and then restarted at a lower dose, the transaminases returned to baseline. Concomitant medications were not reported.
- A patient had a significant increase in AST, ALT, and a total bilirubin after an increase in dose of ATTM from 120 mg to 180 mg daily for one week for treatment of Wilson disease. The aminotransferases peaked above 1000 IU/L (normal range: AST 12 to 38 IU/L and ALT 7 to 41 IU/L) and total bilirubin peaked at 196 µmol/L (normal range: 5.1 to 22 µmol/L). Also, the patient's total cholesterol and triglycerides were elevated to 642 mg/dL (normal level < 200 mg/dL) and 3047 mg/dL (normal level < 150 mg/dL), respectively. The patient had septal fibrosis of the liver prior to initiation of ATTM; baseline AST and ALT not specified. The patient was also receiving zinc sulphate which was continued throughout the course of the presentation and after ATTM was discontinued. The patient's liver function tests and lipids slowly returned to baseline after discontinuing ATTM.

DPV opined that although both cases were possibly confounded by their underlying Wilson disease, the temporal association, acute presentation of the event, and improvement after reduction in dose or discontinuation of the medication (positive de-challenge) suggests a possible association between ATTM and hepatic impairment.

Cutaneous reaction (n = 1)
This case involved a patient being treated for metastatic breast cancer with docetaxel, tetrathiomolybdate, and trientine dihydrochloride; dosing regimens and initiation dates were not documented. The patient developed ulcerations of the mucous membranes of the mouth, vagina, anus, and eyes. Papular skin lesions also spread to her torso, back, and thigh which became pustular. The patient improved after docetaxel was held. DPV opined that the cutaneous reaction was most likely attributable to docetaxel.

Fever, diarrhea, and dehydration (n = 1)
A patient reported fever, diarrhea, and dehydration that required hospitalization. The patient was being treated with fluorouracil, irinotecan, and leucovorin for metastatic colon cancer; the patient did not have neutropenia, and infection was ruled out. DPV opined that the reported AEs were most likely attributable to the chemotherapy the patient was receiving.

To update its 2018 FAERS report, DPV conducted another search from December 26, 2017, to August 10, 2021, which retrieved no additional reports. DPV concluded that the limited reports do not necessarily imply that the substance is safe or lacks toxicities, and given limitations in FAERS reporting, a definitive conclusion on the safety of compounded ATTM cannot be made.

b. Clinical Trials Assessing Safety

Adverse Events Reported in Clinical Trials
We found clinical trials in the medical literature that evaluated the safety of oral ATTM in humans. AEs in trials that reported safety outcomes are summarized below.

Safety in Wilson Disease Clinical Trials and Case series
The ClinicalTrials.gov site lists 40 ongoing or completed clinical trials in patients with Wilson disease. Five of these studies assessed tetrathiomolybdate, of which three evaluated safety. Two of these studies are complete; none has reports of results posted to the site.

A PubMed and EMBASE search on July 7, 2021, on ATTM and Wilson disease retrieved five publications from 1994 to 2006; these studies are summarized below.

An open-label study was conducted in 17 adult patients with Wilson disease treated with ATTM orally for 8 weeks followed by oral zinc acetate for maintenance (Brewer et al. 1994). Per authors, the objective of the study was to test the efficacy and toxicity of a new drug, ATTM, in the initial treatment of patients with neurologic signs and symptoms caused by Wilson disease. ATTM doses ranged from 120 mg to 240 mg in 6 daily doses. Complete blood counts (CBC) and iron status as well as hepatic and kidney function tests were performed as part of safety assessment. Based on the study report, all parameters remained stable through the 8 weeks of ATTM treatment. The authors concluded that ATTM appeared to be free of side effects when used for 8 weeks; hepatic function is preserved during ATTM therapy. No information was provided regarding AEs during the study. Patients were followed up at yearly intervals, with follow-up periods of 1 to 5 years reported.

An open-label study was conducted in 33 adult patients with Wilson disease with neurologic symptoms to test the efficacy and toxic effects of ATTM (Brewer et al. 1996). Patients received ATTM for 8 weeks, with an average daily dose of 208 mg (range 120 mg to 360 mg) given in 6 daily doses, followed by zinc acetate for long-term maintenance. Safety parameters included hepatic and kidney function tests and CBC. One patient developed a clinically significant non-hemolytic anemia; hemoglobin (Hgb) decreased from 13 g/dL to ~7.5 g/dL (normal range: 12-18 g/dL) 30 days into ATTM treatment. The dose this patient received is not specified. Anemia improved soon after cessation of ATTM (Hgb about 11.5 g/dL by Day 40) but recurred with re-initiation of ATTM; the re-initiated dose is not specified. Bone marrow examination showed depression of hematopoiesis in the red blood cell line. In addition, nine patients had an increase in liver enzymes: mean ALT increased from 46 to 123 U/L (reference value 2-35 U/L) at 5 weeks. ALT levels returned to baseline after discontinuing ATTM, but the article does not specify how long this return to baseline took. Patients were followed-up at yearly intervals up to 8 years.

An open-label study evaluated safety and efficacy of ATTM in 55 adult patients with neurologic Wilson disease (Brewer et al. 2003). This study included the 33 patients originally studied in Brewer et al. 1996 plus 22 new patients. The objective of the study was to evaluate the frequency of neurologic worsening and drug adverse effects with ATTM. The dose of ATTM administered ranged from 120 mg to 410 mg for 8 weeks followed by zinc acetate maintenance therapy; patients were followed for 3 years. Five of the 22 new patients developed bone marrow suppression during the first 3 to 6 weeks of ATTM therapy: Hgb decreased from a mean of 13.8 g/dL to a mean of 9.8 g/dL by week 6; mean white blood cell count decreased from 5800/µL to 3500 µL (normal range 4000-10,000/ µL) and platelets decreased from 112 $\times 10^3$/µL to 86 $\times 10^3$/µL (normal range 200-400 $\times 10^3$/µL) by 6 weeks. The authors noted that bone marrow suppression was most common in patients who receive ATTM doses of 200 mg or more. In addition, 3 of the 22 patients developed significant liver dysfunction with a mean ALT peak of 413 IU/L at 5 weeks; AST, lactate dehydrogenase, and alkaline phosphate were elevated as well. Per the authors, liver dysfunction has not been reported with ATTM use in populations other than patients with Wilson disease. They speculated that by removing large amounts of copper from hepatic pools, ATTM causes some hepatitis. The authors concluded that with rapid escalation of ATTM dose, bone marrow suppression or aminotransferase elevations can occur.

A randomized, double-blind, controlled, 2-arm study was conducted in 48 adult patients with neurologic presentation of Wilson disease with the objective of comparing ATTM and trientine on the frequency of neurologic worsening, AEs, and degree of neurologic recovery (Brewer et al. 2006). A total of 23 patients in the trientine group received trientine HCl 1000 mg in two divided daily doses; 25 patients in the ATTM group received 120 mg/day. Treatment continued for 8 weeks followed by maintenance with zinc acetate, and patients were followed annually for 3 years. AEs reported were transaminase elevations (4 - ATTM group, 0 - trientine group) and anemia and/or leukopenia (3 - ATTM group, 1- trientine group). There were 6 deaths reported, 2 in the ATTM group and 4 in the trientine group. The two patients in the ATTM group died during follow-up, one with neurologic deterioration and one with leukemia, presumably unrelated to ATTM. Four patients in the trientine group died during follow-up, 3 of whom had neurologic deterioration while receiving trientine therapy; in all 4 deaths, severe neurologic deterioration was listed as cause of death in addition to inanition, infection, and pulmonary

congestion. All deaths in both groups occurred 6 to 22 months into the study, not during the initial 8 weeks of active treatment.

An uncontrolled longitudinal study describes a case series of five adult patients with neurologic Wilson disease who received ATTM (De Fabregues et al. 2020). The objective was to present the hospital's experience with ATTM in the decoppering phase treatment of Wilson disease with neurological symptoms. Four patients received ATTM treatment for 8 weeks, and one patient received it for 16 weeks. The ATTM dose was 120 mg/day; maintenance with zinc acetate was initiated after completion of ATTM. Except for one patient who received ATTM as initial therapy, all patients had previously been treated with penicillamine or trientine and received ATTM as compassionate use primarily due to progression of symptoms while on or intolerance to other treatments. Assessment under the Unified Wilson's Disease Rating Scale (UWDRS), Global Assessment Scale (GAS) for Wilson disease and the Brewer-adapted Unified Huntington's Disease Rating Scale (UHDRS) for Wilson disease as well as magnetic resonance imaging (MRI) and monitoring for potential AEs were carried out in all patients before starting ATTM and 3 months after ATTM was stopped and zinc treatment was initiated. Per authors, all 5 patients had neurological clinical improvement with neuroimaging improvement (brain edema reduction) in 2 patients. One patient, without prior treatment for Wilson disease, developed anemia, leukopenia, and elevation of transaminases three weeks after starting ATTM, which resolved after stopping ATTM for 1 week and restarting it at a half dose.

In summary, published data on ATTM and Wilson disease are limited to a few small studies that are mostly open-label and uncontrolled. Despite the paucity of data, the available studies have raised considerable safety concerns that appear to be related to ATTM use, particularly potential bone marrow suppression and liver dysfunction. We are also concerned about the lack of safety data on the use of ATTM in pediatric populations and in pregnant women.

Safety in Cancer Trials

Clinical trial data using ATTM/TTM in cancer patients is limited. The following sections summarize ALL publications retrieved under search term "ammonium tetrathiomolybdate" AND "cancer" on October 27, 2021. We focused on the AEs and safety information reported in these studies.

Breast Cancer

Our search identified two studies on ATTM and breast cancer. Both publications state that ATTM is being investigated in patients with breast cancer under an Investigation New Drug (IND) application, IND #71380 (Jain et al. 2013; Chan et al. 2017).

The first study evaluated the effect of ATTM-associated copper depletion on circulating markers of angiogenesis in patients at high risk for breast cancer recurrence (Jain et al. 2013). This was an open-label, single arm, phase 2 study of 39 patients with breast cancer who were currently without evidence of disease (or no evidence of disease (NED)), including stage III and IV of any subtype if NED, or stage II triple-negative breast cancer (TNBC).[16] The study objectives included to assess the change in the number of circulating markers of angiogenesis in patients

[16] Triple-negative breast cancer is a term that has historically been applied to cancers that lack expression of the estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2.

treated with ATTM and to evaluate safety. Standard cancer treatment (i.e., chemotherapy, biologic therapy, surgery, or radiation) needed to have been completed at least 6 weeks prior to the start of the study. ATTM was administered to outpatient in two phases: a 180 mg/day induction dose in four divided doses until ceruloplasmin[17] concentration levels decreased to a target range of 5–16 mg/dL, then a reduced dose of 100 mg/day as maintenance; the dose was reduced in 20 mg increments to minimize toxicity and/or increased in 20 mg increments every 2 weeks to maintain ceruloplasmin target. The duration of the trial was 2 years. AEs reported were Grade 1/2 (mild) neutropenia in 23 (59.0%) patients and Grade 3/4 (more significant) neutropenia in 9 (23.1%) patients; one patient required hospital admission for neutropenic fever and was taken off study. Mild anemia was reported in 14 patients (35.9%); a more significant anemia was reported in one patient who later was diagnosed with B12 deficiency. No patients required growth factor support. Sulfurous eructation (sulfur burps) was common.

The second study (Chan et al. 2017) evaluated the effect of ATTM on endothelial progenitor cells (EPCs) and other biomarkers in patients with breast cancer at high risk of relapse. This was a phase 2 open-label, single-arm study in patients with stage II TNBC and stage III and stage IV NED breast cancer. ATTM was administered in two phases, induction and maintenance; the drug was administered for 2 years. Study endpoints included changes in the number of circulating markers of angiogenesis in patients treated with ATTM and survival. A total of 75 patients were enrolled; 51 completed two years. The entire cohort received standard chemotherapy either in the adjuvant or metastatic setting prior to enrolling in this study. The most common Grade 3/4 AEs were hematologic in nature, including neutropenia and thrombocytopenia. The most common AEs were sulfur eructation, neutropenia, and fatigue; however, most were grade 1 and 2. One patient who developed febrile neutropenia discontinued from the study. A patient with grade 3 anemia also discontinued from the study but the cause was later found to have been vitamin B12 deficiency. The authors concluded that ATTM is safe and well tolerated. The highest risk patients without evidence of relapse or unacceptable toxicity were given the option to participate in a sequence of 2-year extension studies; results of the extended studies are not yet published.

Kidney Cancer

A phase 2 study evaluated the antitumor activity of TTM in patients with advanced kidney cancer (Redman et al. 2003). This study enrolled 15 patients with metastatic kidney cancer who either did not respond to prior high-dose interleukin 2 (IL-2) therapy or were not eligible to receive IL-2;[18] thirteen were evaluated for response. All prior therapy including nephrectomy must have been completed at least 4 weeks before trial entry. Life expectancy needed to be at least 5 months. Patients initially received TTM 180 mg/day in divided doses, subsequently adjusted to target ceruloplasmin level of 5–15 mg/dL (reference range 16-60 mg/dL, Lang 2004) or based on toxicity. Study duration was 6 months. One patient discontinued therapy secondary to toxicity (fatigue). Grade 1–2 fatigue was reported by almost all of the patients, as was the occurrence of sulfurous eructation after taking TTM. Other AEs reported that did not require a dose reduction were nausea (4 patients), diarrhea (3 patients), occasional dizzy episodes without

[17] Ceruloplasmin is the major copper-carrying protein in the blood, accounting for approximately 90 percent of the circulating copper. Thus, it is used as part of the evaluation of copper status.

[18] High-dose bolus IL-2 was reportedly the only FDA approved systemic treatment for metastatic kidney cancer when this study was conducted (Redman 2003). With the advent of newer agents, IL-2 is used less frequently.

blood pressure changes (4 patients), and macular rash on trunk (2 patients). Eleven patients had dose reductions in TTM. The most frequent cause for a dose reduction of TTM was grade 3 or 4 granulocytopenia. Four patients had one dose reduction; two patients had two dose level reductions; and one patient each, had three and four dose level reductions. After stopping TTM, the granulocytopenia decreased to grade 1 or 2 by 5 days. There were no episodes of febrile neutropenia. Granulocytopenia was not seen before 12 weeks on study. One patient had a single dose level reduction for anemia. According to the authors, TTM was relatively well tolerated, but in our opinion, the study corroborates prior findings that ATTM may cause significant bone marrow suppression requiring 3- and 4-level dose reduction in some patients.

Prostate Cancer
A phase 2 study evaluated the antitumor activity of TTM in patients with hormone-refractory prostate cancer (HRPC) (Henry et al. 2006). Nineteen patients with adenocarcinoma of the prostate with progression following hormonal therapy with minimal disease were started on TTM therapy at a dose of 180 mg a day in divided doses to target ceruloplasmin level of 5–15 mg/dL; doses were adjusted as needed to maintain ceruloplasmin level. All patients were evaluated for AEs, and again hematological abnormalities were the most common. Three patients experienced neutropenia and four experienced lymphopenia, but the patients who developed lymphopenia had been reportedly lymphopenic prior to initiation of TTM therapy. One of the patients with neutropenia developed musculoskeletal pain; no febrile neutropenia was reported. The author did not include a causality assessment for two other nonhematologic AEs, hematuria and unstable angina, which occurred nine weeks after TTM therapy.

Malignant Pleural Mesothelioma (MPM)
A phase 2 study evaluated the effect of copper depletion on progression and survival in 30 patients who had undergone cytoreductive surgery for MPM (Pass et al. 2008). No other treatment, either cytotoxic chemotherapy, molecularly targeted therapy, or radiation therapy, was allowed while the patients were taking TTM. Four to six weeks after cytoreduction, patients were started on an induction dose of TTM of 180 mg/day in divided doses to achieve a ceruloplasmin level of 5-15 mg/dL, after which the dose was reduced to 60 mg/day. TTM was discontinued in any patient in whom MPM recurred, and patients were given the option to receive first-line mesothelioma treatment. Fatigue was a commonly seen AE reported by 90% of the patients; dizziness unrelated to blood pressure or posture was also common (53%). A total of Sixteen (16) patients (53%) needed TTM held, or dose reduced because of bone marrow dysfunction, i.e., granulocytopenia (40%) with or without anemia (13%) or thrombocytopenia (3%). The granulocytopenia reversed within 1 week of holding the TTM in all cases, and the patients were then restarted on the drug at a reduced dose. There were no episodes of febrile neutropenia. Seven patients (4 extrapleural pneumonectomy, 3 pleurectomy) required blood transfusion for anemia combined with fatigue. The authors concluded that TTM is well tolerated, but we opine that while some of the AEs may be confounded by the underlying malignancy, there are significant AEs that cannot be ignored and warrant further evaluation to better understand the risks associated with TTM therapy in cancer patients.

Colorectal Cancer
A pilot study was conducted in 24 patients with primary colon or rectal cancer with distant metastasis with the objective of determining the tolerability and toxicity of TTM in combination chemotherapy (Gartner et al. 2009). Treatment protocol consisted of irinotecan, 5-fluorouracil, and leucovorin (IFL) plus 180 mg/day of oral TTM in divided doses, with dose adjustments per ceruloplasmin level or toxicity. The most common AEs were anemia (25%) and neutropenia (12.5%); common non-hematologic AEs included diarrhea (16.7%), nausea/vomiting (4.2%), and 'others' (20.8%), which included a case of deep venous thrombosis (DVT) prior to documented copper deficiency. It is not known whether DVT may be related to ATTM-induced inhibition of vascular endothelial growth factor (VEGF). The authors do not provide a causality assessment, but on DVT, they state that, "…there were no other serious adverse events attributable to VEGF inhibition …" suggesting a potential link that deserves further consideration.

Esophageal Cancer
A phase 2 study evaluated whether TTM prevents or delays progression of residual micrometastatic disease after maximal cytoreduction of esophageal cancer with chemoradiation and surgical resection (Schneider et al. 2013). In this study, patients with resectable, locally advanced esophageal cancer received cisplatin, paclitaxel, and radiotherapy for 3 weeks followed by esophagectomy. Daily oral TTM 20 mg was started 4 weeks post-op and continued for 2 years to maintain the ceruloplasmin level between 5 and 15 mg/dL. Forty-eight patients received TTM. Disease recurrence was the main reason for discontinuing TTM (13 of 48 patients); prolonged neutropenia caused TTM cessation in one patient. Other AEs included nausea, dizziness, and diarrhea, which was consistent with AEs reported in other cancer trials of TTM.

The ClinicalTrials.gov site lists a clinical trial with results posted titled *Chemoradiation and Tetrathiomolybdate (TM) in Patients with Esophageal Carcinoma* (NCT00176800) which reported serious adverse events (SAE) in 40% of the patients, including febrile neutropenia, thrombotic microangiopathy, myocardial infarct, arrythmia, hypotension, supraventricular tachycardia, and colitis, as well as gastrointestinal issues such as nausea and diarrhea.[19]
In general, the safety evaluation of ATTM in this study was largely confounded by the underlying disease and/or concomitant cancer treatment. It is not clear whether this study has been published.

Clinical Trials in Various Types of Cancer
A phase 1 study conducted in 18 patients with various metastatic types of cancers treated with TTM primarily sought to obtain information on dose and dose response but also evaluated some safety parameters (Brewer et al. 2000). Briefly, patients with breast, colon, lung, melanoma, pancreas, prostate, angiosarcoma, chondrosarcoma, nasopharyngeal, hemangioendothelioma, and renal cancer who had demonstrable progression of their cancer in the previous three months after standard treatments or progressive disease after declining conventional therapy were enrolled. They were not undergoing active cancer treatment during the study. Patients received one of

[19] NIH U.S. National Library of Medicine (clinicaltrials.gov), search term "ammonium tetrathiomolybdate" https://clinicaltrials.gov/ct2/results?cond=&term=ammonium+tetrathiomolybdate&cntry=&state=&city=&dist=, Accessed Oct 14, 2021.

three dose regimens consisting of a fixed 20 mg TTM oral dose three times daily (TID) with meals plus an escalating (levels I, II, and III) in-between meals dose given TID for a total of six doses/day. Total TTM daily doses were 90 mg, 105 mg, or 120 mg/day, and treatment duration was 90 days to 17 months. Anemia was reported in four patients, two of whom had been reportedly treated with cytotoxic chemotherapy, and two of whom had evidence of extensive bone marrow involvement with their disease at the time of entry into the trial. Some patients required a blood transfusion, but the study does not specify how many patients. The authors attributed the anemia to causes other than treatment except in one patient where they felt it was "very likely that the copper deficiency caused by TTM produced the anemia." They also reported that several patients experienced transient, occasional sulfur-smelling burping, within 30 minutes of TTM ingestion. They noted no additional AEs of any type with long-term maintenance of mild clinical copper deficiency over 8–15 months.

c. Pharmacokinetic Data

We did not find pharmacokinetic data on ATTM in humans. A publication by Maiti and Moura (2021) explains that TTM (the active moiety of ATTM) does not circulate in the free form in vivo and has the ability to form a stable tripartite complex with any protein, copper, and itself. The authors further state there are two possible mechanisms of TTM with copper in vivo: (a) with food and (b) without (away from) food. The publication provides Figure 2 below which illustrates probable mechanism of TTM with and without food in vivo.

Figure 2. Probable Mechanism of the TTM Drug With and Without Food in Vivo

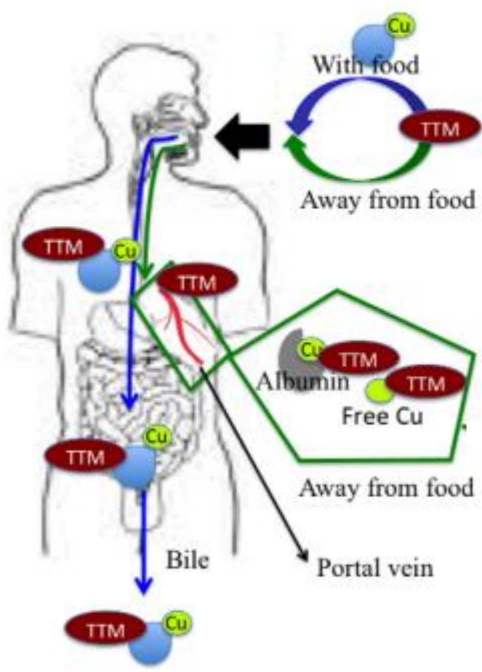

Figure was adapted from Maiti and Moura 2021

When TTM is given with food, it immediately binds with food containing copper, forming a stable tripartite complex, which binds directly with the bile without being absorbed in the gastrointestinal track and is excreted in the stool. When TTM is given without food ("away from food" in Figure 2, it forms a stable tripartite complex with albumin-bound copper as well as free copper in the blood, which is slowly metabolized and finally excreted into the bile (Brewer et al. 1991; Pan et al. 2002).

d. Other Safety Information

Case Report:
PubMed was searched for potential cases of ATTM misuse and abuse (Lang et al. 2004). The Department of Clinical Biochemistry at the John Radcliffe Hospital, Oxford, UK reported a case of iatrogenic copper deficiency in 2004 involving a 56-year-old woman with a 7-year history of metastatic cancer who presented with severe copper deficiency following self-treatment with TTM 120 mg/day; the publication did not specify length of treatment. The patient developed severe neutropenia as her serum copper concentration fell from 19.8 mmol/L to 3.3 mmol/L (normal range 11-20 mmol/L) and ceruloplasmin concentration fell from 35 mg/dL to 4 mg/dL (normal range 16-60 mg/dL, Lang et al. 2004).

ATTM and Copper Deficiency:
According to the American Association for the Study of Liver Diseases[20] (AASLD), potential AEs of ATTM include bone marrow suppression, hepatotoxicity, and overly aggressive copper removal, which causes neurological dysfunction.

Copper is an essential trace element, necessary for the activity of many key enzymes including superoxide dismutase, lysyl oxidase, dopamine β-hydroxylase, cytochrome oxidase, and ceruloplasmin. Acquired copper deficiency has been well documented in humans (Danks 1988). The most common causes are malabsorption and zinc ingestion as well as over-treatment of Wilson disease with zinc and chelators; treatment with TTM has been associated with copper deficiency (Lang et al. 2004). The most common neurologic manifestation of acquired copper deficiency is myelopathy or myeloneuropathy. Rare neurologic symptoms include myopathy (along with myelopathy) (Kumar and Low 2004), myelo-optico-neuropathy with hyposmia and hypogeusia (Spinazzi 2007), and cognitive impairment (Nations 2008). The hematologic hallmark of copper deficiency is anemia and leukopenia. Thrombocytopenia and pancytopenia are relatively rare.

[20] AASLD is a membership organization of scientists and health care professionals that generates practice guidelines for providers and educational materials for patients on prevention and management of liver disease.
The organization also facilitates programs and initiatives aimed at improving liver health and quality patient care: https://www.aasld.org/sites/default/files/2019-06/Wilson-Disease2009.pdf , Accessed Jul 29, 2021.

e. Availability of alternative approved therapies that may be as safe or safer

There are currently FDA-approved therapies for Wilson disease and the various cancers discussed in this evaluation.

FDA-Approved Therapies for Wilson Disease

Drug therapy in the treatment of Wilson disease primarily consists of copper chelation, to bind systemic excess copper, and inhibitors of copper intestinal absorption. There are currently four FDA-approved drug products indicated for the treatment of Wilson disease.

- Penicillamine oral capsule (Cuprimine®, NDA 019853[21] is a chelating agent indicated for the treatment of Wilson disease and for the treatment of cystinuria and patients with severe, active rheumatoid arthritis who have failed to respond to an adequate trial of conventional therapy. It is labeled in pediatric patients with cystinuria. There are no controlled studies on the use of penicillamine in pregnant women, but it may be used in pregnancy at a reduced dose.
- Penicillamine oral tablet (Depen®, NDA 019854)[22] indicated in the treatment of Wilson disease, cystinuria, and in patients with severe, active rheumatoid arthritis.
- Trientine hydrochloride oral capsule (Syprine®, NDA 019194)[23], another chelating compound used for removal of excess copper from the body. It is indicated in the treatment of Wilson disease patients who are intolerant to penicillamine. It is labeled for use in adults and pediatric patients. There are no adequate well-controlled studies in pregnant women. Used in pregnancy only if the potential benefit justifies the potential risk to the fetus.
- Zinc acetate (Galzin, NDA 020458) inhibits copper absorption; it is indicated for maintenance treatment of patients with Wilson disease who have been initially treated with a chelating agent.[24] It is also used during pregnancy.

In summary, FDA-approved therapies that have been evaluated for safety and effectiveness are available for the treatment of Wilson disease. These are considered to be safe for use under the conditions specified in their FDA-approved labels, which include warnings and precautions for safe use of the product including in pregnant and pediatric patients.

FDA-Approved Therapies for Cancer

Cancer treatment depends on the type of cancer and its stage. The National Cancer Institute provides extensive listings of currently approved drug products for the treatment of different

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[21] Cuprimine® (penicillamine) Capsule, approved December 4, 1970; current labeling approved October 26, 2004 https://www.accessdata.fda.gov/drugsatfda_docs/label/2004/19853s012,014lbl.pdf.

[22] Depen® (penicillamine) Tablet, approved November 8, 1978 https://dailymed.nlm.nih.gov/dailymed/getFile.cfm?setid=38f8ae60-b354-11de-8a39-0800200c9a66&type=pdf

[23] Syprine® (trientine hydrochloride) Capsule, approved November 8, 1985 https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=c34f77a7-996b-4470-b5df-d946a7fe5dbe, Accessed Jul 29, 2021.

[24] Galzin (zinc acetate) Capsule, approved January 28, 1997 https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=a0c72bff-20f3-4241-b966-34a95178d1a3, Accessed Nov 15, 2021.

types of cancers, including breast, colorectal, esophageal, kidney, MSM, and prostate cancer.[25] Some patients with cancer will have only one type of treatment but most patients will need a combination of treatments, such as surgery with chemotherapy and/or radiation therapy. Other treatment modalities available include hormone therapy, immunotherapy, and stem cell transplant, among other modalities. There are no FDA-approved products intended for use as copper-chelating agents in the treatment of breast, colorectal, esophageal, kidney, MSM, and prostate cancers.

Conclusion: Based on available clinical information, safety concerns associated with the use of ATTM in clinical trials for the treatment of Wilson disease and chelation therapy in various types of cancer include hepatoxicity and bone marrow suppression (i.e., anemia, leukopenia, and thrombocytopenia), which are potentially serious. We are also concerned about the AEs of significant copper removal associated with the use of ATTM, its long-term effects, and the lack of safety data associated with its use in pregnant women and children. Nonclinical studies have shown that ATTM produces epiphyseal damage in growing bone. There are currently available FDA-approved therapies for the treatment of Wilson disease and for the types of cancer addressed by the nomination that meet established criteria for safety and effectiveness and are labeled accordingly to inform their safe use.

C. Are there concerns about whether a substance is effective for a particular use?

The following databases were consulted in the preparation of this section: PubMed, EMBASE, ClinicalTrials.gov, and various online clinical references and professional healthcare organization websites. In addition to a comprehensive review of pertinent information from these databases, this section provides a brief overview of Wilson Disease and copper chelation in cancer treatment, pertinent regulatory history, and a discussion of the proposed uses of ATTM.

1. *Background*

Brief overview of copper metabolism and Wilson Disease

Free copper is extremely toxic and can produce irreversible cellular damage. Dietary copper intake is approximately 1 to 2 mg per day (Ma 2000). Excess copper is predominantly excreted into the bile, where it ends up as fecal copper. The transport of copper within hepatocytes is regulated by *ATP7B*[26] where it mediates the incorporation of copper into ceruloplasmin (Cater et al. 2006; Nagano et al. 1998; Pfeiffer 2007). Ceruloplasmin is then secreted into the bloodstream. Copper in ceruloplasmin accounts for approximately 90 percent of the circulating copper (See Appendix 1 of this evaluation for additional information).

Wilson disease is a rare genetic disorder with an autosomal recessive pattern of inheritance caused by mutations that lead to impaired function of the intracellular copper transporter *ATP7B*, leading to copper excess in tissue despite low circulating levels of ceruloplasmin. This buildup can lead to damage of the liver, brain, and eyes. Signs and symptoms of Wilson disease include

[25] See NIH National Cancer Institute website for detail listing of approved cancer treatments https://www.cancer.gov/about-cancer/treatment/drugs/cancer-type, Accessed Nov15, 2021.
[26] ATP7B is an acronym for ATPase activity, 7 distinct domain, and B class for second P-type ATPase copper binding pump.

chronic liver disease, central nervous system abnormalities, and psychiatric (mental health-related) disturbances.[27] The majority of patients with Wilson disease are diagnosed between the ages of 5 and 35 years (mean age of 13 years) (Lin et al. 2014). Wilson disease is progressive and may be fatal if left untreated. Treatment is aimed at reducing the amount of copper that has accumulated in the body and maintaining normal copper levels thereafter.

The AASLD (AASLD 2008) recommends the following pharmacological treatment for Wilson disease:

- Initial treatment for symptomatic patients should include a chelating agent (D-penicillamine or trientine) (See Appendix 2 of this evaluation). Trientine may be better tolerated.
- Patients should avoid intake of foods and water with high concentrations of copper, especially during the first year of treatment.
- Treatment of pre-symptomatic patients or those on maintenance therapy can be accomplished with a chelating agent or with zinc. Trientine may be better tolerated.
- Patients with acute liver failure due to Wilson disease should be referred for and treated with liver transplantation immediately. Patients with decompensated cirrhosis unresponsive to chelation treatment should be evaluated promptly for liver transplantation.
- Treatment for Wilson disease should be continued during pregnancy, but dosage reduction is advisable for D-penicillamine and trientine.
- Treatment is lifelong and should not be discontinued unless a liver transplant has been performed.

There are no FDA-approved drug products that contain ATTM as an active ingredient. ATTM has been studied under investigational new drug applications (INDs) (Jain 2013 and Chan 2017). Pipex Pharmaceuticals submitted new drug application (NDA) for Coprexa (oral tetrathiomolybdate) for the treatment of initially presenting neurologic Wilson Disease. In 2008, Pipex announced that the NDA was issued a Refuse to File (RTF) letter, which cited, among other deficiencies, issues concerning the adequacy of the clinical evidence of safety and efficacy of Coprexa and a request to conduct an additional short term reproductive drug safety study in animals.[28] No further public information is available on the current status of this application.

2. *Reports of trials, clinical evidence, and anecdotal reports of effectiveness, or lack of effectiveness, of the bulk drug substance*

Clinical trials on efficacy in Wilson disease

Five studies, including four clinical trials and one case-series, with efficacy outcomes of ATTM in Wilson disease were identified through a PubMed and EMBASE search on July 7, 2021. Three of the four clinical trials were uncontrolled, open-label studies evaluating the effectiveness of ATTM as initial treatment in neurologically presenting Wilson disease. The number of

[27] Wilson disease. NIH Genetic Rare Diseases Information Center (GARD) available at https://rarediseases.info.nih.gov/diseases/7893/wilson-disease. Accessed Nov 3, 2021.

[28] Pipex Pharmaceuticals Update on Coprexa (oral tetrathiomolybdate) New Drug Application https://www.drugs.com/nda/coprexa_080129.html. Accessed Nov 22, 2021.

patients included in the analyses of successive studies was cumulative. In each of these studies, ATTM was administered orally 6 times a day (3 with meals; 3 without meals) for 8 weeks followed by long-term maintenance with oral zinc acetate. The primary outcome measure in these studies was neurologic function assessed by quantitative neurologic and speech examinations both during ATTM therapy and at yearly follow-up. Neurologic assessment consisted of subjective scoring of the degree of tremor, chorea, dysarthria, walking, fine motor coordination, facial expression, resting dystonia, and rigidity; speech assessment consisted of subjective scoring of the severity of the dysarthria in spontaneous speech, expository speech, diadochokinetic rates, and verbal and nonverbal agility scores. In addition, quantitative scoring of MRI scans of the brain were obtained at baseline and at yearly follow-ups. Assessments were performed by the same evaluator who was blinded to treatment status but was aware that patients on ATTM therapy were undergoing frequent repeat assessments. These studies are individually summarized below.

An open-label study was conducted on 17 adult patients with neurologic Wilson disease treated with ATTM orally for 8-weeks followed by oral zinc acetate for maintenance (Brewer et al. 1994). Per authors, the objective was to test the efficacy and toxicity of a new drug, ATTM, in the initial treatment of patients with neurologic signs and symptoms caused by Wilson disease including dysarthria (most common), tremor, incoordination, drooling, mood swing, insomnia, memory loss, headache, fatigue, and depression. Total ATTM daily dose was 120 mg to 240 mg (mean 168 mg/day), and doses were escalated until the criterion for control of nonceruloplasmin plasma copper was reached. Patients were treated for 8 weeks, followed by zinc acetate maintenance. Patient's neurologic status was evaluated by a quantitative neurologic examination, a quantitative speech pathology examination, and quantitative scoring MRI scans of the brain. For efficacy analysis, worsening on any of these parameters was considered to be due to ATTM instead of underlying worsening of the disease. The study reported that during the 8 weeks of ATTM administration, neurologic function was protected, which seems to contrast neurologic deterioration reported in up to 50% of patients during initial treatment with penicillamine (Brewer 1987; Walshe 1993). The study also reports improvement on brain MRI scans at 1 year with no patients showing significant deterioration. The authors concluded that ATTM appears to be an "ideal" drug for the initial treatment of patients with Wilson disease who present with neurological symptoms but recognized the need for controlled studies comparing ATTM to penicillamine.

Another open-label study was conducted in 33 adult patients with Wilson disease with neurologic symptoms to test the efficacy and toxic effects of ATTM (Brewer et al. 1996). This study included further follow up data on the original 17 patients published by Brewer et al., in 1994. Only patients who received ATTM as *initial* therapy were studied. Patients received ATTM for 8 weeks at a mean daily dose of 208 mg (range 120 mg to 960 mg) followed by maintenance with zinc acetate. Efficacy outcome measures were preservation of neurological function and long-term recovery. The neurologic examinations quantitated tremor, chorea, dysarthria, walking, facial expression (masked faces), fine motor coordination, resting dystonia, and rigidity. The same neurologist scored each neurologic examination. Because of some fluctuation in scores unrelated to progressive worsening, an increase in score of 5 was set as indicating meaningful deterioration. The same speech pathologist (as in the previous study) scored each speech pathology examination. Because of some fluctuations in scores unrelated to

progressive worsening, an increase in score of 2 was set as indicating meaningful deterioration. The MRI scans of the brain were quantitatively scored by the reported system above.
Patients were generally seen 1 year later for reexamination, and annually thereafter for 1 to 8 years. Following their neurologic criteria, only 1 of the 33 patients showed deterioration in neurologic function during the 8 weeks of ATTM administration. The authors concluded that neurologic recovery was good to excellent in most patients during the 1 to 6 years follow up. However, this study is limited by the small size and possible observer bias as it is not controlled and not blinded.

Another open-label study evaluated safety and efficacy of ATTM in 55 adult patients with neurologic Wilson disease (Brewer et al. 2003). This study includes the 33 patients originally studied in Brewer 1996 plus 22 new patients. The objective of the study was to evaluate the frequency of neurologic worsening and drug adverse effects with ATTM. The daily dose of ATTM administered ranged from 120 mg to 410 mg for 8 weeks followed by zinc acetate maintenance therapy; patients were followed for 3 years. Neurologic function was assessed by scored neurologic and speech tests. Overall, two patients showed neurological deterioration, one within the 8-week period, another at the one year follow up. Subsequent neurologic scores improved in both patients by Year 3. The authors concluded that ATTM showed excellent efficacy in patients with Wilson disease who present with neurologic manifestation. However, we opine that this study has limitations regarding size and study design.

The three studies showed no significant change in mean neurology, speech, and MRI scores in most patients during the 8 weeks of ATTM treatment; neurologic deterioration was documented in only two patients, which the authors contrast with up to 50% deterioration with initial treatment with penicillamine. However, neurology data was available for fewer than 50% of the patients enrolled, for both the initial 8 weeks and subsequent yearly follow-up. Overall, the interpretation of these studies is limited without a comparator.

A randomized, double-blind, controlled, 2-arm study was conducted in 48 adult patients with the neurologic presentation of Wilson disease to compare ATTM and trientine for the frequency of neurologic worsening, AEs, and degree of neurologic recovery (Brewer et al. 2006). A total of 23 patients received trientine HCl 1000 mg; 25 patients received ATTM 20 mg/day. Treatment continued for 8 weeks followed by maintenance with zinc acetate; the patients were followed annually for 3 years. The main outcome measure was neurologic function assessed by semiquantitative neurologic and speech examinations. Most but not all patients were newly diagnosed; a few patients who had been receiving long-term treatment with penicillamine stopped therapy for more than a year and then developed new neurologic symptoms. During the 8-week treatment, the quantitative neurologic test and a quantitative speech test as described in earlier studies were performed weekly. The neurologists and speech pathologist were blinded. According to the study results, 6 of 23 patients in the trientine group vs. 1 of 25 patients in the ATTM had neurological deterioration ($P<0.05$) during initial treatment. The authors concluded that ATTM is a better choice than trientine for preserving neurologic function in patients who present with neurologic disease. This is the only controlled study of ATTM in Wilson disease. The prevention of worsening neurologic symptoms over three years after 8 weeks of treatment may not have any clinical relevance to a chronic neurologic disease such as Wilson disease.

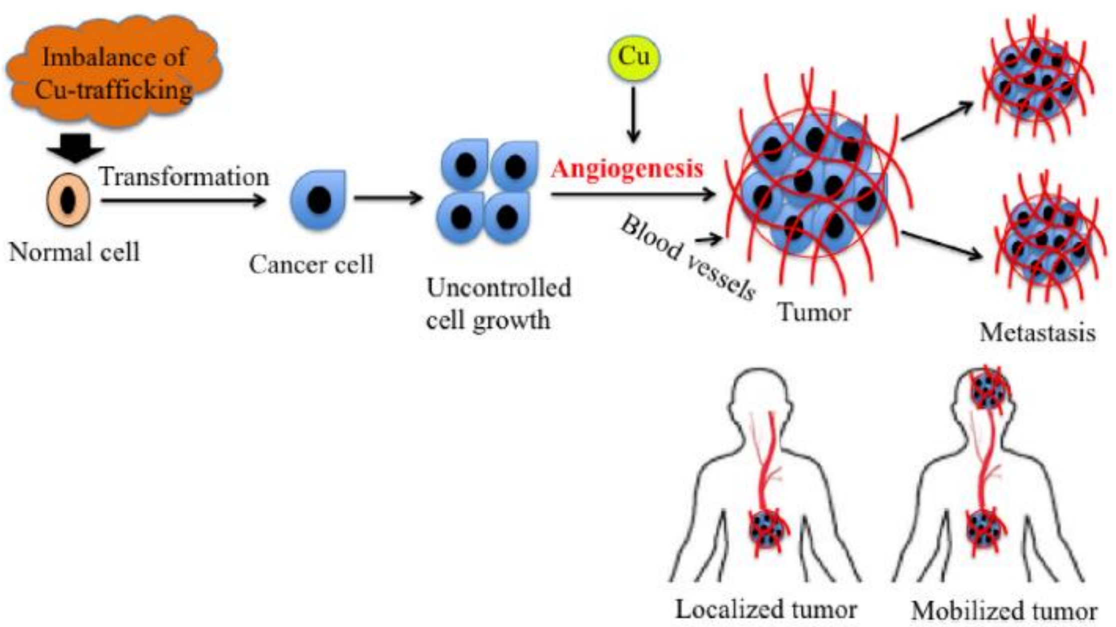
An uncontrolled, longitudinal study described a case series of five adult patients with neurologic Wilson disease who received ATTM (De Fabregues et al. 2020). The study objective was to present the hospital's experience with ATTM in the decoppering phase treatment of Wilson disease with neurological symptoms. Four patients received ATTM treatment for 8 weeks, and one patient received it for 16 weeks given neurological improvement on ATTM treatment. ATTM was dosed as 120 mg/day (20 mg TID with meals and 20 mg TID between meals); maintenance with zinc acetate was initiated after completion of ATTM. Except for one patient who received ATTM as initial therapy, all patients had previously been treated with penicillamine or trientine and received ATTM as compassionate use primarily due to progression of symptoms while on or intolerance to other treatments. Assessment under the UWDRS, Global GAS for Wilson disease and the Brewer-adapted UHDRS for Wilson disease as well as MRI and monitoring for potential AEs were carried out in all patients before starting ATTM and 3 months later when ATTM was stopped, and zinc treatment was initiated. According to the authors, all five patients had neurological clinical improvement with neuroimaging improvement (brain edema reduction) in 2 patients, and they concluded that, "ATTM could be a good treatment for the initial treatment of Wilson disease with neurological symptoms due to its high efficacy, with a lower rate of neurological deterioration than the drugs currently available, despite the potential adverse events." However, we opine that these findings should be confirmed in a large randomized clinical trial to establish a better benefit-risk balance.

Copper Chelation for the Treatment of Various Types of Cancer

ATTM in Cancer Treatment

Cancer is a multifactorial disease that involves abnormal cell growth along with invasion, dissemination, and metastasis (Blockhuys et al. 2017). It has been hypothesized that the progression of cancer cells was dependent on copper (Ishida et al. 2010), and it has been shown that the copper level in a cancer cell is markedly higher (up to 2–3-fold) compared to a healthy normal cell (Kuo et al. 2002). Angiogenesis, the formation of new capillary branches from existing blood vessels resulting in the growth and spread of tumors or cancer cells (Urso 2015; Folkman 1995; Carmeliet 2003), is tightly controlled by a net balance between angiogenic stimulating factors and inhibitors. In contrast, an imbalanced expression between activator and inhibitor leads to the progression of a tumor at the primary and metastatic sites (See Figure 3) (Denoyer et al. 2015; Urso, 2015; Iruela-Arispe 1997; Hanahan 1996).

Figure 3. Inter-Relation Between Cancer, Angiogenesis, and Copper (Maiti and Moura 2021)



The second nominated use for ATTM, i.e., copper chelation therapy,[29] as such is not a disease or condition. However, the publications submitted to support the proposed use were studies in patients with various types of cancer; among these are studies that describe clinical outcomes or potential clinical benefit in patients with breast and kidney cancer. In addition, we found publications on the use of ATTM in patients with esophageal cancer, colorectal cancer, prostate cancer, and MPM that describe clinical outcome or potential clinical benefit. Therefore, we evaluated ATTM use in patients with these specific types of cancers.

Clinical trials using ATTM/TTM in cancer patients are scant. The following sections summarize ALL publications retrieved under search term "ammonium tetrathiomolybdate" AND "cancer" on October 27, 2021. The focus of the studies presented is on efficacy outcomes. Nonclinical, in vitro studies as referenced in the nomination are not relevant to our clinical evaluation of efficacy; therefore, they are not discussed. The following studies are the same studies described in the safety section of this evaluation (See Section II.B.2.b).

Clinical Trials in Various Types of Cancer

Breast Cancer

Our search identified two studies on ATTM and breast cancer. The first study evaluated the effect of ATTM-associated copper depletion on circulating markers of angiogenesis in patients at high risk for breast cancer recurrence (Jain et al. 2013). This was an open-label single arm, phase 2 study of 39 patients with breast cancer who were currently without NED, including stage

[29] Pharmacy Solutions, ATTM nomination (Document ID: FDA-2015-N-3534-0295) (February 1, 2021), available at https://www.regulations.gov/document/FDA-2015-N-3534-0295.

III and IV of any subtype of NED (no evidence of disease), or stage II TNBC (triple-negative breast cancer). Standard cancer treatment (i.e., chemotherapy, biologic therapy, surgery, or radiation) needed to have been completed at least 6 weeks prior to the start of the study; only concurrent hormonal therapy was permitted. The primary endpoint was change in bone marrow-derived endothelial progenitor cells (EPCs), considered critical for metastatic progression; secondary objectives included relapse free survival (RFS), number of hematopoietic progenitor cells, and levels of plasma angiogenic factors and cytokines. ATTM was administered to outpatients in two phases: a 180 mg daily induction dose in four divided doses until ceruloplasmin levels decreased to a target range of 5–16 mg/dL. Ceruloplasmin levels were tested every 2 weeks for the first 4 weeks, then weekly until target ceruloplasmin was reached. When ceruloplasmin was within target, patients were switched to maintenance: 100 mg daily in divided doses, reduced in 20 mg increments to minimize toxicity and/or increased in 20 mg increments every 2 weeks to maintain ceruloplasmin target. The duration of the trial was 2 years. The study reports successful copper depletion in 75% of patients and significant reduction in EPCs/mL by 27 in copper-depleted patients (P=0.04). Overall RFS at 10 months was 85% (95% CI 74.6%-96.8%). %); six of 39 patients (15%) relapsed during the first 12 months on ATTM, but 27 (70%) remained relapse-free through the course of the study. The remaining six patients discontinued the study due to toxicity (3), patient's decision (2), or lost to follow-up (1). The authors concluded that ATTM may promote tumor dormancy and ultimately prevent relapse but acknowledged that a large, randomized, multicenter trial with more triple negative and stage 4 NED would be necessary.

The second study (Chan et al. 2017) evaluated the effect of ATTM on EPCs and other biomarkers in patients with stage II TNBC or stage III and stage IV NED as well as the effect on lung metastasis in preclinical models. Like the study above, this was a phase 2, open-label, single-arm study. ATTM was administered in two phases, induction and maintenance, for 2 years; 28 days of TTM administration comprised one cycle. The primary endpoint was change in vascular endothelial growth factor receptor 2-positive endothelial progenitor cells (VEGFR2+ EPCs); secondary endpoints included event-free survival (EFS) and overall survival (OS). A total of 75 patients were enrolled; 51 completed two years. The majority of patients were at high risk of relapse, including 55% of patients with stage III disease and 40% of patients with stage IV NED; 48% had TNBC. The entire cohort received standard chemotherapy either in the adjuvant or metastatic setting prior to enrolling in this study; only concurrent hormonal therapy was permitted. The investigators reported copper to target level in 80% of patients within 4 cycles. Lowering ceruloplasmin levels resulted in a significant decrease in EPC levels. At a median follow-up of 6.3 years, the EFS for all 75 patients was 72% (90% for all stage II/III patients with TNBC). The OS was 84%. The authors concluded that ATTM affects copper-dependent components of the tumor microenvironment; whether this effect would translate to an increase in survival rate remains to be determined. We did not identify any additional clinical studies on ATTM in breast cancer.

These studies do not provide evidence that ATTM contributes to a clinical response because the trials were single arm and patients continued to receive other cancer therapy.

Kidney Cancer

A phase 2 study was published in 2003 evaluating the antitumor activity of TTM in patients with advanced kidney cancer (Redman et al. 2003). This study enrolled 15 patients with metastatic kidney cancer who either did not respond to prior high-dose IL-2 therapy or were not eligible to receive IL-2; thirteen were evaluated for response. All prior therapy, including nephrectomy, must have been completed at least 4 weeks before trial entry. Life expectancy needed to be at least 5 months. Patients initially received TTM 180 mg/day in divided doses, subsequently adjusted to target ceruloplasmin level of 5–15 mg/dL or based on toxicity. Study duration was 6 months. The authors collected information on changes in tumor vascularity by dynamic contrast-enhanced MRI to evaluate disease progression and changes in the levels of several angiogenic factors (i.e., VEGF, basic fibroblast growth factor (bFGF), IL-6 and IL-8) to assess the impact of copper depletion on these potential proangiogenic factors. All the patients reached copper deficiency as predefined for this study. The median time to reach a ceruloplasmin level ≤15 mg/dL was 5 weeks, with a range of 1–15 weeks. In this study, copper depletion was associated with reduced levels of the four proangiogenic factors ($P \leq 0.0003$); however, this effect did not persist at a later study time point. Five of the 13 patients had evidence of progressive disease before 12 weeks (median, 7.5 weeks) while they were copper deficient. Of the remaining eight patients, four had progressive disease by 12 weeks. Overall, the 6-month PFS rate was 31%, and the median time to progression was 13 weeks (range, 3–45 weeks). Four patients had stable disease and continued TTM therapy beyond the 6-month cutoff (median duration, 34.5 weeks). All of them ultimately progressed. The authors concluded that serum levels of IL-6, IL-8, VEGF, and bFGF may correlate with copper depletion but not with disease stability and that TTM alone showed no efficacy in patients with advanced kidney cancer in this small cohort. This study did not support the effectiveness of ATTM in the treatment of kidney cancer.

Prostate Cancer

This phase 2 study evaluated the antitumor activity of TTM in patients with HRPC (Henry et al. 2006). The primary objective was to evaluate the efficacy of TTM in delaying disease progression; secondary objectives included assessing TTM's effect on several angiogenic factors, i.e., VEGF, bFGF, IL-6 and IL-8. The final objective was to assess prostate-specific antigen (PSA) as an indicator of response to antiangiogenic therapy. Nineteen patients with adenocarcinoma of the prostate with progression following hormonal therapy with minimal disease were started on TTM therapy at a dose of 180 mg a day in divided doses to target a ceruloplasmin level of 5 – 15 mg/dL. Doses were adjusted as needed to maintain the target ceruloplasmin level. The study reported that 17 of 19 patients became copper deficient per pre-specified ceruloplasmin targets. The median duration on study was 13.7 weeks, yet ranged from 8.1 to 33.9 weeks. PSA levels increased significantly between baseline and time of progression ($P < 0.001$); however, no difference was noted in the levels of any of the proangiogenic factors, VEGF, bFGF, IL-6, and IL-8, within the first 3 months. Fourteen patients had disease progression. The study was terminated after enrollment of only 19 patients because more than 11 patients had progressed, a criterion for closing the study based on prespecified statistical considerations. The authors concluded that copper depletion with TTM did not delay disease progression in patients with asymptomatic metastatic HRPC. This study did not support the effectiveness of ATTM in hormone-refractory prostate cancer.

Malignant Pleural Mesothelioma (MPM)

One publication is available evaluating the effect of copper depletion on progression and survival after cytoreductive surgery for MPM (Pass et al. 2008). In this phase 2 study, no other treatment, including cytotoxic chemotherapy, molecularly targeted therapy, or radiation therapy, was allowed while the patients were taking TTM. Thirty patients were eligible for postoperative copper-reduction therapy, which was compared to stage I through III historical controls treated with cytoreductive surgery for mesothelioma. Four to six weeks after cytoreduction, patients were started on an induction dose of TTM of 180 mg/day in divided doses to achieve ceruloplasmin 5-15 mg/dL, after which the dose was reduced to 60 mg/day. Computerized tomography (CT) scans were performed within 1 week of starting TTM and then every 4 months for disease monitoring. In this study, target ceruloplasmin was reached at a median of 4.9±0.3 weeks (range, 2 to 9 weeks) associated with a significant reduction in serum VEGF levels. Twenty-seven patients were maintained on TTM until disease progressed (range 2 to 30 months); in three patients without recurrence, TTM was discontinued between 51 and 61 months. The time to progression for all stage I or stage II patients in the study was 20 months compared to 10 months in the non-TTM historical controls (P=0.046). No difference in time to progression for the stage III patients, a median of 7 months, was observed. Median survival was 41 months for stage I or II and 15 months for stage III compared to 25 months for stage I and II patients in historical controls. The authors concluded that TTM has antiangiogenic effects in MPM after surgical resection, but they cautioned about the potential for bias because this study was not a randomized trial; they recommended validating this trial in a larger randomized study.

Colorectal Cancer

A pilot study evaluating TTM in combination with chemotherapeutic agents for metastatic colorectal cancer was published in 2009 (Gartner et al. 2009). The study enrolled 24 patients with primary colon or rectal cancer with distant metastasis. Treatment regimen consisted of irinotecan, 5-fluorouracil, and leucovorin (IFL) on days 1, 5, 15, and 22 of a 6-week cycle plus 180 mg/day of oral TTM in divided doses, with dose adjustments per ceruloplasmin level or toxicity as described in the studies above. Primary endpoints included tolerability and toxicity of TTM in combination with IFL, and secondary endpoints included survival time and serum levels of angiogenic factors (VEGF, bFGF, IL-8, and IL-6) as well as serum levels of carcinoembryonic antigen (CEA). Twenty-two of 24 patients (91.7%) achieved target levels of ceruloplasmin within 3 months of initiation of study treatment, with a majority of patients reaching target ceruloplasmin levels within 1 month. The study failed to find any significant correlation between baseline serum cytokine levels and time to disease progression (TTP) for any of the cytokines measured. The overall response rate was 25% (95% CI 9.8–46.7). Ten patients had at least a 50% reduction in baseline CEA level during study treatment. Six patients with stable disease and partial response discontinued chemotherapy following at least 6 months of IFL and continued TTM alone, as specified in the study protocol. Tumor progression was seen in each of these patients within the next 5 months. The authors suggested continuing both treatments, TTM and chemotherapy, rather than TTM alone to control tumor progression in further studies of TTM. Overall, it is not possible to draw any meaningful conclusion on efficacy from this study given the small number of patients and the exploratory nature of the study.

Esophageal Cancer
A phase 2 study evaluated whether TTM prevents or delays progression of residual micro metastatic disease after surgical resection and chemoradiation for esophageal cancer (Schneider et al. 2013). In this study, patients with resectable, locally advanced esophageal cancer received cisplatin (days 1 and 22), paclitaxel (days 1, 8, 15, and 22), and radiotherapy for 3 weeks followed by esophagectomy. Daily oral TTM 20 mg was started 4 weeks post-op and continued for 2 years to maintain the ceruloplasmin level between 5 and 15 mg/dL. The primary objective was 3-year recurrence-free survival, and secondary endpoints were 3-year OS rate and treatment toxicity. A total of 69 patients were enrolled; 47 received adjuvant TTM, 14 of which completed 2 years of therapy. All 69 patients were included in the survival analysis, median follow up was 55 months (4.5 years). Twenty-seven patients had disease recurrence, 25 patients were alive without disease, 1 was alive with disease, and 43 had died. The three-year recurrence-free survival rate was 44 % (95 % confidence interval [CI], 32–55 %) and the three-year OS rate was 45 % (95 % CI 33–56 %). The study did not compare survival rates between the patients who receive TTM and the ones who did not in this cohort, but the authors compared their results to a group of 69 historical controls treated with a similar protocol minus TTM. Disease-free survival (DFS) probabilities were 65 % for the study group vs. 55% for the historical controls at 1 year; 49 % vs. 39% at 2 years; and 44% vs. 32% at 3 years. Similarly, OS probabilities were 74 % for the study group vs. 75% for the historical controls at 1 year; 57 % vs. 50% at 2 years; and 45 % vs. 34% at 3 years. These differences did not reach statistical significance but suggest the addition of TTM offered a slight survival advantage in this small study. The authors concluded that DFS and OS are promising when compared to historical controls treated at their institution with a similar regimen that did not include TTM. There was no association between decreased level of ceruloplasmin with recurrence free survival or OS. This study did not support the effectiveness of ATTM in esophageal cancer.

3. *Whether the product compounded with this bulk drug substance is intended to be used in a serious or life-threatening disease*

ATTM is being evaluated for the treatment of Wilson Disease and as a chelating agent for the treatment of various cancers. These are both serious and life-threatening conditions for which there are available FDA-approved therapies. Given the insufficient evidence to support the effectiveness of oral ATTM to treat Wilson disease and the types of cancer addressed by the nomination, we are concerned that ATTM may be used in some cases instead of the FDA-approved drug products intended to treat these conditions, which have been evaluated for safety and effectiveness for their labeled indications.

4. *Whether there are any alternative approved therapies that may be as effective or more effective*

See Section II.B.2.e.

Conclusions: We conclude there is insufficient information to support the effectiveness of oral ATTM for the treatment of Wilson disease and as chelation therapy for the treatment of breast, colorectal, esophageal, kidney, MPM, and prostate cancer. There are currently FDA approved

therapies available with established efficacy for the treatment of Wilson disease and the types of cancer addressed by the nomination.

D. Has the substance been used historically in compounding?

This section describes the historical and current use of ATTM as an oral formulation. Databases searched for information on ATTM regarding Section II.D of this evaluation included PubMed, EMBASE, National Organization for Rare Disorders (NORD), Natural Medicines, ClinicalTrials.gov, Google/Google Scholar, UptoDate, Micromedex, United States Pharmacopeia – National Formulary, European Pharmacopoeia, British Pharmacopoeia, and Japanese Pharmacopoeia.

1. Length of time the substance has been used in pharmacy compounding

The nominator did not provide historical use data. ATTM, the ammonium salt form of TTM, has been used orally to treat Wilson disease and various types of cancer for many years.

According to Walshe 1999, ATTM was first used to treat patients with Wilson disease in 1984. Brewer et al. 1991 later described the use of compounded ATTM to treat patients with Wilson disease who presented with acute neurological symptoms and became clinically worse when treated with penicillamine. The authors noted that ATTM was purchased “in a form suitable for human administration” and was placed in capsules that contained 10, 20, 30, or 40 mg.

In 2000, Brewer et al. reported the first human phase I clinical trial of an anti-copper approach to antiangiogenesis therapy based on the oral use of TTM in 18 patients with metastatic solid tumors. According to the authors, TTM “was purchased in bulk lots suitable for human administration.” Research pharmacists placed the appropriate dose of TTM in gelatin capsules at the time the prescription was written. Three dose levels of oral TTM (90, 105, and 120 mg/day) were administered in six divided doses with and in-between meals.

Overall, there is insufficient evidence in the literature to determine the exact length of time ATTM has been used in pharmacy compounding; however, it appears that the substance has been used in pharmacy compounding from 1984 to at least April 2021 based on the last verification and update posted to ClinicalTrials.gov for NCT00195091, a “Phase II Study of Tetrathiomolybdate (TM) in Patients with Breast Cancer.” Chan et al. 2017 and Jain et al. 2013 describe this trial and how research pharmacists compound the bulk drug substance per protocol into oral capsules. The estimated study completion date is June 2022.

2. The medical condition(s) it has been used to treat

ATTM is predominantly used to treat Wilson disease by complexing excess copper (Bandmann et al. 2015). In addition, numerous clinical trials evaluated ATTM as a new treatment for various forms of cancer, including breast cancer (Jain et al. 2013; Chan et al. 2017), MPM (Pass et al. 2008), prostate cancer (NCT00150995; Henry et al. 2006), esophageal carcinoma (Schneider et al. 2013), hepatocellular carcinoma or liver cancer (NCT00006332), colorectal cancer (Gartner et al. 2009), advanced kidney cancer (Redman et al. 2003), and non-small cell

lung cancer (NCT00560495). Furthermore, the substance has been studied in clinical trials as a treatment for idiopathic pulmonary fibrosis (Medici and Sturniolo 2008; NCT00189176), primary biliary cirrhosis (Askari 2010), and psoriasis vulgaris (NCT00113542).

Of the studies and articles listed above, several detailed the use of compounded ATTM drug product to treat various medical conditions while others did not. As previously noted, Brewer et al. 1991 described the use of compounded ATTM 10, 20, 30, or 40 mg capsules to treat patients with Wilson disease. In a phase II study that enrolled female patients with breast cancer at high risk for recurrence (NCT00195091), "clinical grade tetrathiomolybdate (produced by [Good Manufacturing Practice] GMP) was purchased in bulk" and dispensed by research pharmacists in gelatin (oral) capsules (Chan et al. 2017; Jain et al. 2013). In a phase II study in patients with MPM, TTM was purchased "in bulk lots suitable for human administration and "placed in 20-mg gelatin (oral) capsules for patient use" (Pass et al. 2008). Lastly, in a trial in patients with primary biliary cirrhosis, "tetrathiomolybdate, manufactured using good manufacturing practices," was purchased in bulk lots and aliquoted into 100-g bottles (Askari et al. 2010).

It is unknown whether drug products compounded using ATTM as a bulk drug substance were used to treat the remaining medical conditions above (i.e., prostate, esophageal, liver, colorectal, advanced kidney, and non-small cell lung cancers; idiopathic pulmonary fibrosis and psoriasis vulgaris).

3. How widespread has its use been?

Overall, there is evidence of how widespread the use of compounded ATTM has been both within and outside of the United States. According to outsourcing facility product reports submitted to FDA,[30] one outsourcing facility reported making an ATTM capsule in 2017. Results from an internet search for compounded drug products containing ATTM revealed four compounding pharmacies within the United States compounding ATTM.[31,32,33,34] One of the four compounding pharmacies specified dosage form, the intended route of administration, and strength (e.g., "40 mg oral capsules") of the drug product. An Australian compounding pharmacy advertised compounding ATTM 2.5, 5, and 10 mg capsules.[35] In addition, the International Journal of Pharmaceutical Compounding (IJPC) has published compounding

[30] The Drug Quality and Security Act, signed into law on November 27, 2013, created a new section 503B in the Federal Food, Drug, and Cosmetic Act. Under section 503B, a compounder can become an outsourcing facility. Outsourcing facilities are required to provide FDA with a list of drugs they compounded during the previous six-month period upon initial registration and in June and December each year. This retrospective information does not identify drugs that outsourcing facilities intend to produce in the future.
[31] Available at https://www.kauaipharmacy.com/compounding/cancer .
[32] Available at https://tcfam.com/treatments/repurposed-medications/ammonium-tetrathiomolybdate/
[33] Available at https://imcwc.com/wp-content/uploads/2019/08/Unique-Product-Dosing-Chart.pdf.
[34] Available at https://www.fda.gov/inspections-compliance-enforcement-and-criminal-investigations/warning-letters/pharmaceutical-care-solutions-dba-pharmacy-solutions-610201-08022021.
[35] Available at https://www.customcompounding.com.au/wp-content/uploads/2016/04/MASTER-PRICE-LIST-2018-V5.pdf.

formulations for ATTM 20 mg and 50 mg oral capsules.[36,37] Currently, there is no compounded drug product monograph for any ATTM dosage form in the United States Pharmacopeia-National Formulary (USP-NF).

4. Recognition of the substance in other countries or foreign pharmacopeias

ATTM remains an experimental therapy, and it is not commercially available in countries outside the United States. Nonetheless, the European Commission gave ATTM orphan designation for the treatment of Wilson disease on April 1, 2008. In addition, European clinical practice guidelines (EASL 2012) recommend the use of chelators such as ATTM as the initial treatment of symptomatic patients with Wilson disease.

We found no evidence of compendial monographs in countries outside of the United States.

Conclusion: There is evidence of the historical and current use of ATTM in compounding as an oral formulation for the treatment of Wilson disease or as copper chelation therapy for treatment of cancer both within the United States and outside of the United States.

III. RECOMMENDATION

We have balanced the criteria described in section II above to evaluate ammonium tetrathiomolybdate for inclusion on the 503A Bulks List. After considering the information currently available, a balancing of the criteria ***weighs against*** ammonium tetrathiomolybdate being placed on that list based on the following:

1. Based on the available information, ATTM can be characterized using readily available analytical techniques. ATTM is likely to be stable if protected from moisture and air when compounded as capsules.

2. Based on available clinical information, safety concerns associated with the use of ATTM include hepatoxicity and bone marrow suppression (i.e., anemia, leukopenia, and thrombocytopenia), which are potentially serious. We are also concerned about the adverse effects of significant copper removal associated with the use of ATTM, its long-term effects, and the lack of safety data associated with its use in pregnant women and children. Nonclinical studies have shown that ATTM produces epiphyseal damage in growing bones. There are currently available FDA-approved therapies for the treatment of Wilson disease and for the types of cancer addressed by the nomination that meet established criteria for safety and effectiveness and are labeled accordingly to inform safe use of the product.

3. We conclude there is insufficient information to support the effectiveness of oral ATTM for the treatment of Wilson disease and as chelation therapy for the treatment of breast,

[36] Available at CompoundingToday.com | Formulation: Ammonium Tetrathiomolybdate 20 mg Oral Capsules (subscription required).
[37] Available at CompoundingToday.com | Formulation: Ammonium Tetrathiomolybdate 50 mg Oral Capsules (subscription required).

colorectal, esophageal, kidney, MPM, and prostate cancer. There are currently FDA-approved therapies available with established efficacy for the treatment of Wilson disease and for the types of cancer addressed by the nomination.

4. ATTM has been used in pharmacy compounding since 1984 in oral dosage forms. There is evidence of the historical and current use of ATTM in compounding as an oral formulation for the treatment of Wilson disease or as copper chelation therapy for treatment of cancer both within the United States and outside of the United States.

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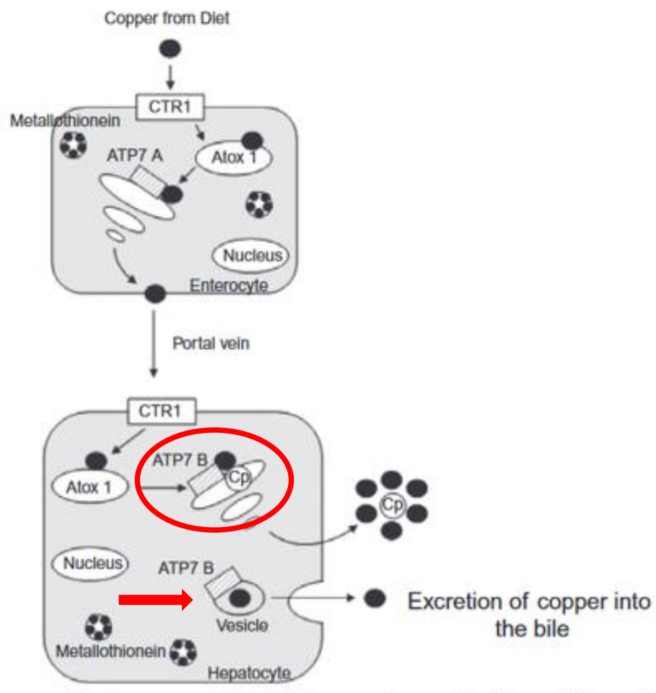
APPENDIX 1: COPPER METABOLISM AND WILSON DISEASE

Copper Metabolism and Wilson Disease

Free copper is extremely toxic and can produce irreversible cellular damage. Dietary copper intake is approximately 1 to 2 mg per day (Ma 2000). Copper is absorbed in the stomach and duodenum, binds mainly to circulating albumin, and is taken up by various tissues (Cox 1995, Brewer 1992, Lorincz 2010). The daily requirement for copper is approximately 0.75 mg. Excess copper is predominantly excreted into the bile, where it ends up as fecal copper.
The transport of copper within hepatocytes is regulated by *ATP7B*, which normally resides in the trans-Golgi network in hepatocytes (liver cells) where it mediates the incorporation of six copper molecules into apoceruloplasmin (the ceruloplasmin peptide without copper) forming ceruloplasmin (Cater 2006, Nagano1998, Pfeiffer 2007). Ceruloplasmin is then secreted into the bloodstream. Copper in ceruloplasmin accounts for approximately 90 percent of the circulating copper. Under high copper conditions, ATP7B is also redistributed to cytoplasmic vesicles where it transports excess copper across the hepatocyte apical membrane into the bile canaliculus for subsequent biliary excretion into the bile (Figure 4).

Figure 4. Copper Metabolism (Adapted Woimant 2014)



CTR: copper transporter; ATOX 1: copper specific chaperone protein; ATP7: copper-transporting P-type ATPases; Cp: ceruloplasmin (Apoceruloplasmin, without copper, holoceruloplasmin with copper)

Wilson disease, also known as hepatolenticular degeneration, is a rare genetic disorder with an autosomal recessive pattern of inheritance caused by mutations that lead to impaired function of the intracellular copper transporter *ATP7B*. This mutation leads to decreased incorporation of copper into apoceruloplasmin and decreased transport of copper from the liver into bile, leading

to copper excess in tissue despite low circulating levels of ceruloplasmin (the major form of circulating copper). This buildup can lead to damage of the liver, brain, and eyes. Signs and symptoms of Wilson disease include chronic liver disease, central nervous system abnormalities, and psychiatric (mental health-related) disturbances.[38] The majority of patients with Wilson disease are diagnosed between the ages of 5 and 35 years (mean age of 13 years) (Lin 2014). While children are more likely to present with hepatic manifestations and rarely with neurologic symptoms, adolescents and adult patients present more often with neurologic manifestations. The mean age at presentation for patients with neurologic symptoms ranges between 15 and 21 years (Lorincz 2010; Walshe 1989; Oder 1991; Ferenci 2007). Wilson disease is progressive and may be fatal if left untreated. There is currently no cure for Wilson disease. Treatment is aimed at reducing the amount of copper that has accumulated in the body and maintaining normal copper levels thereafter.

[38] Wilson disease. NIH Genetic Rare Diseases Information Center (GARD) available at https://rarediseases.info.nih.gov/diseases/7893/wilson-disease. Accessed Nov 3, 2021.

APPENDIX 2: AMERICAN ASSOCIATION FOR THE STUDY OF LIVER DISEASE (AASLD) PHARMACOLOGIC THERAPY FOR WILSON DISEASE

AASLD Pharmacologic Therapy for Wilson Disease

Table 1 summarizes pharmacological therapy for Wilson disease as detailed in the AASLD website accessed on October 15, 2021, on its *Practice Guideline Diagnosis and Treatment of Wilson Disease: An Update* (Hepatology, 2008).[39]

Table 1. Pharmacological Therapy for Wilson Disease

Drug	Mode of Action	Neurological Deterioration	Side Effects	Comments
D-Penicillamine	General chelator induces cupruria	10%-20% during initial phase of treatment	• Fever, rash, proteinuria, lupus-like reaction • Aplastic anemia • Leukopenia • Thrombocytopenia • Nephrotic syndrome • Degenerative changes in skin • Elastosis perforans serpingosa • Serous retinitis • Hepatotoxicity	Reduce dose for surgery to promote wound-healing and during pregnancy Maximum dose 20 mg/kg/day; reduce by 25% when clinically stable
Trientine	General chelator induces cupruria	10%-15% during initial phase of treatment	• Gastritis • Aplastic anemia rare • Sideroblastic anemia	Reduce dose for surgery to promote wound-healing and during pregnancy Maximum dose 20 mg/kg/day; reduce by 25% when clinically stable
Zinc	Metallothionein inducer, blocks intestinal absorption of copper	Can occur during initial phase of treatment	• Gastritis; biochemical pancreatitis • Zinc accumulation • Possible changes in immune function	No dosage reduction for surgery or pregnancy Usual dose in adults: 50 mg elemental Zn three times daily; *minimum* dose in adults: 50 mg elemental Zn twice daily
Tetrathiomolybdate	Chelator, blocks copper absorption	Reports of rare neurologic deterioration during initial treatment	• Anemia; neutropenia • Hepatotoxicity	Experimental in the United States and Canada

As shown in Table 1, AASLD current practice guidelines includes tetrathiomolybdate as a potential pharmacological therapy for Wilson disease, but it is listed as experimental in the United States and not commercially available. This practice guideline also states ATTM is a very strong decoppering agent which works by two mechanisms: interfering with intestinal uptake of copper (if administered with meals) and binding copper from plasma (when taken between meals). It further states that recent data indicate its utility because it does not cause neurological deterioration. It also mentions its utility as an antiangiogenic agent due to its extensive decoppering effect.

[39] AASLD Practice Guidelines and Guidances: Wilson Disease, published June 2008 https://www.aasld.org/sites/default/files/2019-06/Wilson-Disease2009.pdf, Accessed Jul 20, 2021.

Tab 4

Ferric Subsulfate

Tab 4a

Ferric Subsulfate Nominations

Division of Dockets Management (HFA-305)
Food and Drug Administration
Department of Health and Human Services
5630 Fishers Lane
Rm. 1061
Rockville, MD 20852

Re: Docket FDA-2013-N-1525

> "List of Bulk Drug Substances That May Be Used in Pharmacy Compounding; Bulk Drug Substances That May Be Used To Compound Drug Products in Accordance With Section 503A of the Federal Food, Drug, and Cosmetic Act"

Dear Sir or Madam,

Fagron appreciates the opportunity to address the FDA's request for nominations of bulk drug substances that may be used to compound drug products that are neither the subject of a United States Pharmacopeia (USP) or National Formulary (NF) monograph nor components of FDA-approved drugs.

We hereby nominate the bulk drug substances in the attached spreadsheets for FDA's consideration as bulk drug substances that may be used in pharmacy compounding under Section 503A.

None of these items appear on an FDA-published list of drugs that present demonstrable difficulties for compounding. In addition, none are a component of a drug product that has been withdrawn or removed from the market because the drug or components of the drug have been found to be unsafe or not effective.

We include references in support of this nomination for your consideration.

Thank you for your consideration. If Fagron can answer any questions, please contact me (j.letwat@fagron.com; 847-207-6100).

Respectfully submitted,

Julie Letwat, JD, MPH
Vice-President, Regulatory and Government Affairs

Re: Docket FDA-2013-N-1525

Substances submitted (see corresponding .xlxs file)

7-Keto Dehydroepiandrosterone
Acetyl-D-Glucosamine
Aloe Vera 200:1 Freeze Dried
Astragalus Extract 10:1
Beta Glucan (1,3/1,4 –D)
Boswellia Serrata Extract
Bromelain
Cantharidin
Cetyl Myristoleate Oil
Cetyl Myristoleate 20% Powder
Chrysin
Citrulline
Dehydroepiandrosterone
Deoxy-D-Glucose (2)
Diindolylmethane
Domperidone
EGCg
Ferric Subsulfate
Glycolic Acid
Glycosaminoglycans
Hydroxocobalamin Hydrochloride
Kojic Acid
Methylcobalamin
Nicotinamide Adenine Dinucleotide
Nicotinamide Adenine Dinucleotide Disodium Reduced (NADH)
Ornithine Hydrochloride
Phosphatidyl Serine
Pregnenolone
Pyridoxal 5-Phosphate Monohydrate
Pyruvic Acid
Quercetin
Quinacrine Hydrochloride
Ribose (D)
Silver Protein Mild
Squaric Acid Di-N-Butyl Ester
Thymol Iodide
Tranilast
Trichloroacetic Acid
Ubiquinol 30% Powder

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What is the name of the nominated ingredient?	**Ferrric Subsulfate**
Is the ingredient an active ingredient that meets the definition of "bulk drug substance" in § 207.3(a)(4)?	Yes, Ferric Subsulfate is an active ingredient as defined in 207.3(a)(4) because when added to a pharmacologic dosage form it produces a pharmacological effect. Update in Cochrane Database Syst Rev. 2010;(6):CD00142124. Monsel's solution: a kinder, gentler hemostatic. Jetmore AB, Heryer JW, Conner WE. Dis Colon Rectum. 1993 Sep;36(9):866-7. No abstract available.
Is the ingredient listed in any of the three sections of the Orange Book?	The nominated substance was searched for in all three sections of the Orange Book located at http://www.accessdata.fda.gov/ scripts/cder/ob/docs/queryai.cfm. The nominated substance does not appear in any section searches of the Orange Book.
Were any monographs for the ingredient found in the USP or NF monographs?	The nominated substance was searched for at http://www.uspnf.com. The nominated substance is not the subject of a USP or NF monograph.
What is the chemical name of the substance?	Iron(+3) cation dihydroxide pentasulfate
What is the common name of the substance?	Monsel's Salt; Ferric Sulfate Basic
Does the substance have a UNII Code?	39R4TAN1VT
What is the chemical grade of the substance?	No grade
What is the strength, quality, stability, and purity of the ingredient?	Description: Brown powder Solubility: Conforms Identification <Ferric Salts 191>: Conforms Assay Ratio: >= 24%
How is the ingredient supplied?	Powder
Is the substance recognized in foreign pharmacopeias or registered in other countries?	No

Has information been submitted about the substance to the USP for consideration of monograph development?	No
What dosage form(s) will be compounded using the bulk drug substance?	Topical powder, topical solution
What strength(s) will be compounded from the nominated substance?	21% - 10% (for topical powder)
What are the anticipated route(s) of administration of the compounded drug product(s)?	Topical powder, topical solution
Are there safety and efficacy data on compounded drugs using the nominated substance?	Follen Mitchell, Michelle, et al. "A randomized clinical trial of cryotherapy, laser vaporization, and loop electrosurgical excision for treatment of squamous intraepithelial lesions of the cervix." Obstetrics & Gynecology 92.5 (1998): 737-744. http://www.sciencedirect.com/science/article/pii/S0029784498002464. Cochrane Database Syst Rev. 2000;(2):CD001421. Interventions for preventing blood loss during the treatment of cervical intraepithelial neoplasia.Martin-Hirsch PL, Kitchener H. Update in Cochrane Database Syst Rev. 2010;(6):CD001421. 24. Monsel's solution: a kinder, gentler hemostatic.Jetmore AB, Heryer JW, Conner WE. Dis Colon Rectum. 1993 Sep;36(9):866-7. No abstract available.
Has the bulk drug substance been used previously to compound drug product(s)?	Yes, to compound Monsel's Solution. Astirngent and hemostaic: Described by Monsel in the 1850s. ~21%w/v Fe; sp. Gr. ~1.6. Solution for topical use-do not administer orally Commercially available (1-2 yr) Rev Hist Pharm (Paris) 2009 Jul;57(362):193-200. [Léon Monsel and his solution].[Article in French] Brun G. Monsel is overall well known by his publications about coagulation power of ferric sub-sulfate from which he described three preparation ways. Current publications are exclusively foreigner. They point out advantages and disadvantages of Monsel's solution. It is used to day as hemostatic agent in minor surgical procedures such as biopsies in genecology, dermatology, proctology and O.R.L. PMID: 20027795. [PubMed - indexed for MEDLINE]
What is the proposed use for the drug product(s) to be compounded with the nominated substance?	Astingent and hemostatic

What is the reason for use of a compounded drug product rather than an FDA-approved product?	benzalkonium chloride and povidone. There are an estimated 10% of the population can be allergic to Benzalkonium Chloride. The current population is approximately 318,700,524. (http://www.census.gov/popclock/) This would give 31,870,052.6 individuals in the US population with a potential allergy to Benzalkonium Chloride. The current FDA approved medications contain this preservative. A preservative free option would be needed for this portion of the population.
Is there any other relevant information?	All relevant information was expressed in the above questions

September 30, 2014

Division of Dockets Management (HFA-305)
Food and Drug Administration
Department of Health and Human Services
5630 Fishers Lane, Room 1061
Rockville, Maryland 20852

[Docket No. FDA-2013-N-1525]

Re: FDA-2013-N-1525; List of Bulk Drug Substances That May Be Used in Pharmacy Compounding in Accordance with Section 503A

Dear Sir or Madam:

Thank you for the opportunity to submit our comments on FDA's request for a list of bulk drug substances that may be used in pharmacy compounding as defined within Section 503A of the Federal Food, Drug and Cosmetic Act. As FDA receives these lists from the public, the medical and pharmacy practice communities, the International Academy of Compounding Pharmacists (IACP) appreciates the opportunity to identify and share drug substances which are commonly used in the preparation of medications but which have neither an official USP (United States Pharmacopeia) monograph nor appear to be a component of an FDA approved drug product.

IACP is an association representing more than 3,600 pharmacists, technicians, academicians students, and members of the compounding community who focus on the specialty practice of pharmacy compounding. Compounding pharmacists work directly with prescribers including physicians, nurse practitioners and veterinarians to create customized medication solutions for patients and animals whose health care needs cannot be met by manufactured medications.

Working in tandem with the IACP Foundation, a 501(c)(3) non-profit organization dedicated to enhancing the knowledge and understanding of pharmacy compounding research and education, our Academy is submitting the accompanying compilation of 1,215 bulk drug substances which are currently used by compounding pharmacies but which either do not have a specific USP monograph or are not a component of an FDA approved prescription drug product.

These drug substances were identified through polling of our membership as well as a review of the currently available scientific and medical literature related to compounding.

INTERNATIONAL ACADEMY OF COMPOUNDING PHARMACISTS

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Although the information requested in FDA-2013-N-1525 for each submitted drug substance is quite extensive, there are many instances where the data or supporting research documentation does not currently exist. IACP has provided as much detail as possible given the number of medications we identified, the depth of the information requested by the agency, and the very short timeline to compile and submit this data.

ISSUE: The Issuance of This Proposed Rule is Premature

IACP is concerned that the FDA has disregarded previously submitted bulk drug substances, including those submitted by our Academy on February 25, 2014, and created an series of clear obstructions for the consideration of those products without complying with the requirements set down by Congress. Specifically, the agency has requested information on the dosage forms, strengths, and uses of compounded preparations which are pure speculation because of the unique nature of compounded preparations for individual patient prescriptions. Additionally, the agency has developed its criteria list without consultation or input from Pharmacy Compounding Advisory Committee. Congress created this Advisory Committee in the original and reaffirmed language of section 503A to assure that experts in the pharmacy and medical community would have practitioner input into the implementation of the agency's activities surrounding compounding.

As outlined in FDCA 503A, Congress instructed the agency to convene an Advisory Committee ***prior*** to the implementation and issuance of regulations including the creation of the bulk ingredient list.

> (2) Advisory committee on compounding.--Before issuing regulations to implement subsection (a)(6), the Secretary shall convene and consult an advisory committee on compounding. The advisory committee shall include representatives from the National Association of Boards of Pharmacy, the United States Pharmacopeia, pharmacists with current experience and expertise in compounding, physicians with background and knowledge in compounding, and patient and public health advocacy organizations.

Despite a call for nominations to a Pharmacy Compounding Advisory Committee (PCAC) which were due to the agency in March 2014, no appointments have been made nor has the PCAC been formed to do the work dictated by Congress. Additionally, the agency provides no justification in the publication of criteria within FDA-2013-N-1525 which justifies whether this requested information meets the needs of the PCAC.

In summary, IACP believes that the absence of the PCAC in guiding the agency in determining what information is necessary for an adequate review of a bulk ingredient should in no way preclude the Committee's review of any submitted drug, regardless of FDA's statement in the published revised call for nominations that:

> General or boilerplate statements regarding the need for compounded drug products or the benefits of compounding generally will not be considered sufficient to address this issue.

IACP requests that the Pharmacy Compounding Advisory Committee review each of the 1,215 drug substances we have submitted for use by 503A traditional compounders and we stand ready to assist the agency and the Committee with additional information should such be requested.

Thank you for the opportunity to submit our comments and IACP looks forward to working with the FDA in the future on this very important issue.

Sincerely,

David G. Miller, R.Ph.
Executive Vice President & CEO

Bulk Drug Substances for Consideration by the FDA's Pharmacy Compounding Advisory Committee

Submitted by the International Academy of Compounding Pharmacists

General Background on Bulk Drug Substance

Ingredient Name	Ferric subsulfate
Chemical/Common Name	Ferric Sulfate, Basic
Identifying Codes	1310-45-8
Chemical Grade	Provided by FDA Registered Supplier/COA
Description of Strength, Quality, Stability, and Purity	Provided by FDA Registered Supplier/COA
How Supplied	Varies based upon compounding requirement
Recognition in Formularies *(including foreign recognition)*	Not Listed in USP/NF for this specific salt/form

Information on Compounded Bulk Drug Preparation

Dosage Form	Varies based upon compounding requirement/prescription
Strength	Varies based upon compounding requirement/prescription
Route of Administration	Varies based upon compounding requirement/prescription
Bibliography *(where available)*	Federal Register 1999
Past and Proposed Use	The very nature of a compounded preparation for an individual patient prescription as provided for within FDCA 503A means that the purpose for which it is prescribed is determined by the health professional authorized to issue that prescription. FDA's request for this information is an insurmountable hurdle that has not been requested by the PCAC.

Tab 4b

FDA Evaluation of Ferric Subsulfate

DATE: May 2, 2022

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TO: Pharmacy Compounding Advisory Committee

SUBJECT: Evaluation of Ferric Subsulfate for Inclusion on the 503A Bulk Drug Substances List

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I. INTRODUCTION

Ferric subsulfate solid or powder was nominated for inclusion on the list of bulk drug substances for use in compounding under section 503A of the Federal Food, Drug, and Cosmetic Act (FD&C Act). Ferric subsulfate was proposed for use as an astringent and hemostatic agent during minor surgical procedures.[1, 2] The proposed route of administration was for topical use in the following dosage forms, as a solution and as a powder (10-21%).

There is no ferric subsulfate drug substance monograph in the United States Pharmacopoeia (USP), National Formulary (NF), British Pharmacopoeia (BP), or European Pharmacopoeia (EP). Although there is no "applicable" USP or NF drug substance monograph for ferric subsulfate for the purposes of section 503A(b)(1)(A)(i)(I),[3] there is a USP drug product monograph for ferric subsulfate solution or Monsel's Solution**.**

Fagron submitted literature for the nominated substance ferric subsulfate,[4] where the substance was referred to as "Monsel's." There is a lack of clarity in the nomination and literature submitted by Fagron regarding whether the nominator intended for FDA to consider ferric subsulfate and Monsel's (solution, paste) to refer to a bulk drug substance and a drug product compounded from that substance, respectively, or whether the nominator intended to mean that they are the same products and the names are used interchangeably; FDA interprets the nomination to be for the bulk drug substance ferric subsulfate solid or powder, and data submitted by Fagron on "Monsel's" will be considered for the overall assessment and recommendation with respect to the use proposed by the nominators.

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[1] Ferric subsulfate was nominated by Fagron and the International Academy of Compounding Pharmacists (IACP). Based on information submitted by Fagron, we construe its nomination to be for the bulk drug substance ferric subsulfate supplied in a solid or powder form. Fagron's nomination says the "ingredient [is] supplied" as a "powder." Fagron provided supporting literature reference to one Cochrane review, one prospective randomized study, one opinion article, and one abstract with the citation PMID: 20027795. The literature reference with PMID 20027795 included an abstract that was in the English language, but the full text of the article was in French. We did not consider this foreign language article because it was in French, and the nominator did not submit a verified English translation. Accordingly, the nominator did not submit sufficient information for the Agency to evaluate whether the substance is appropriate for use in compounded drug products for all minor surgical procedures. See 21 CFR 10.20(c)(2) ("If a part of the material submitted is in a foreign language, it must be accompanied by an English translation verified to be complete and accurate, together with the name, address, and a brief statement of the qualifications of the person making the translation. A translation of literature or other material in a foreign language is to be accompanied by copies of the original publication.")

[2] Fagron proposed the topical route of administration and dosage formulation as a solution and as a powder (10-21%). IACP did not nominate ferric subsulfate with sufficient information for FDA to evaluate because the nomination did not identify specific dosage forms, strengths, routes of administration, or propose reason(s) for using compounded drug products containing ferric subsulfate, so we did not consider this nomination.

[3] For purposes of section 503A(b)(1)(A)(i)(I) of the FD&C Act, FDA interprets the term "an applicable United States Pharmacopeia (USP) or National Formulary (NF) monograph" to refer to official drug substance monographs. See Final Rule, List of Bulk Drug Substances That Can Be Used To Compound Drug Products in Accordance With Section 503A of the Federal Food, Drug, and Cosmetic Act, 84 FR 4696, 4705 (March 21, 2019).

[4] We note that ferric sulfate (Chemical Abstract Service number (CAS# 10028-22-5), is not the same bulk drug substance as ferric subsulfate (CAS# 1310-45-8). Ferric sulfate has a different molecular formula than ferric subsulfate, based on the USP drug product monograph for ferric subsulfate solution and the USP drug substance monograph for ferric sulfate. See (Bandi 2017) (describing different clinical uses).

We evaluated publicly available data on the physicochemical characteristics, safety, effectiveness, and historical use in compounding of ferric subsulfate. For the reasons discussed below, we believe the evaluation criteria ***weigh against*** placing ferric subsulfate solid or powder on the list of bulk drug substances that can be used to compound drug products in accordance with section 503A of the FD&C Act (503A Bulks List).

II. EVALUATION CRITERIA

A. Is the substance well-characterized, physically and chemically, such that it is appropriate for use in compounding?[5]

Databases such as PubMed, SciFinder, Google, the EP, and USP/NF were searched for information on ferric subsulfate, and very limited responsive information was found about ferric subsulfate solid or powder. The information below summarizes what FDA found in these databases.

Ferric subsulfate is also called iron subsulfate, Monsel's salt, iron hydroxide sulfate ($Fe_4(OH)_2(SO_4)_5$), or basic ferric sulfate, and its CAS number is 1310-45-8.[6] A USP drug product monograph is available for ferric subsulfate solution which is also called Monsel's solution. The monograph states that ferric subsulfate solution contains, in each 100 mL, basic ferric sulfate equivalent to not less than 20 g and not more than 22 g of iron (Fe).

1. Stability of the API and likely dosage forms

Ferric subsulfate solid or powder is available directly through several vendors. It is described as a yellow rhombic crystalline salt that is water soluble and is used in industrial waste processing as a coagulant and as a pigment in pickling baths for steel and aluminum.[7] The material safety data sheet states that the compound is sensitive to light and that exposure to light accelerates decomposition, but it is stable when it is stored at room temperature in light-resistant containers, protected from light, and kept in a container tightly closed in a dry and well-ventilated place.[8]

The USP drug product monograph for ferric subsulfate solution indicates that the product should be stored in tight, light-resistant containers at temperatures above 22°C. Crystallization may occur if the solution is exposed to temperatures below 22°C, but warming will redissolve the crystals.

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[5] Among the conditions that must be met for a drug compounded using bulk drug substances to be eligible for the exemptions in section 503A of the FD&C Act is that the bulk drug substances are manufactured by an establishment that is registered under section 510 of the FD&C Act and that each bulk drug substance is accompanied by a valid certificate of analysis. Sections 503A(b)(1)(A)(ii) and (iii). A bulk drug substance is deemed to be adulterated if the methods used in, or the facilities or controls used for, its manufacture, processing, packing, or holding do not conform to or are not operated or administered in conformity with current good manufacturing practice. Section 501(a)(2)(B).

[6] One nominator used the term "ferric sulfate basic" as a common name for ferric subsulfate. We consider this to mean the same as "basic ferric sulfate."

[7] https://www.spectrumchemical.com/ferric-subsulfate-powder-purified-f1042.

[8] https://www.spectrumchemical.com/media/sds/F1042_AGHS.pdf.

2. *Probable routes of API synthesis*

Information found in the databases listed above details how to make ferric subsulfate solution. It is prepared by oxidation of ferrous sulfate with nitric acid in the presence of sulfuric acid as depicted by the scheme below:

$$12\ FeSO_4 + 4\ HNO_3 + 3\ H_2SO_4 \rightarrow 3\ Fe_4(OH)_2(SO_4)_5 + 4\ NO + 2H_2O$$

The preparation procedure for the ferric subsulfate solution is provided in the USP drug product monograph and starts with ferrous sulfate. Other than what is included above, no additional information has been found on ferric subsulfate in the databases searched, and no reference has been found for synthesis of the solid or powder form of ferric subsulfate. The solid or powder may presumably be made by crystallization of ferric subsulfate from its solution. This assumption is based on the labeling information in the USP drug product monograph for ferric subsulfate solution which indicates that crystallization may occur if the ferric subsulfate solution is exposed to temperatures below 22°C. However, no detail has been found to support this assumption. It is unclear how ferric subsulfate solid or powder is isolated and purified.

3. *Likely impurities*[9]

Based on the above method of synthesis, likely impurities include elemental impurities, ferrous salt, and nitrate or by-product from oxidation reagents. The USP drug product monograph for ferric subsulfate solution includes a test for the limit of nitrate. It's unclear whether any other ferrous or ferric salt complex is formed in ferric subsulfate.

4. *Toxicity of those likely impurities*

Toxicity of elemental impurities is outlined in ICH Q3D. The toxicity of other iron complexes, if formed, is unknown.

5. *Physicochemical characteristics pertinent to product performance, such as particle size and polymorphism*

No references have been found about the physicochemical characteristics including particle size and polymorphism for ferric subsulfate solid or powder. It is unclear how ferric subsulfate is distinguished from ferric sulfate or ferric sulfate hydrate during characterization. Particle size and polymorphs are not expected to be critical for aqueous formulation of ferric subsulfate because ferric subsulfate is water soluble.

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[9] This evaluation contains a non-exhaustive list of potential impurities in the bulk drug substance and does not address fully the potential safety concerns associated with those impurities. The compounder should use the information about the impurities identified in the certificate of analysis accompanying the bulk drug substance to evaluate any potential safety and quality issues associated with impurities in a drug product compounded using that bulk drug substance taking into account the amount of the impurity, dose, route of administration, and chronicity of dosing.

6. *Any other information about the substance that may be relevant, such as whether the API is poorly characterized or difficult to characterize*

As pointed out above, it is unclear how ferric subsulfate solid or powder is distinguished from ferric sulfate or ferric sulfate hydrate during characterization.

Conclusions: Very limited information was found on ferric subsulfate solid or powder in public databases. It is unclear how ferric subsulfate solid or powder is manufactured, isolated, purified or characterized. Although industrial grade ferric subsulfate solid or powder is available, there is insufficient data available to support adding ferric subsulfate solid or powder to the 503A Bulks List because of a lack of information regarding the manufacturing, quality, and characterization of the commercially available ferric subsulfate solid or powder. The USP drug product monograph for ferric subsulfate solution describes a procedure that starts with ferrous sulfate to prepare ferric subsulfate solution. Ferric subsulfate solution is chemically and physically well-characterized when the USP drug product monograph is followed, but this solution is made starting from ferrous sulfate rather than ferric subsulfate solid or powder, which we construe to be the subject of the nomination. Ferric subsulfate solid or powder is not well-characterized, physically and chemically, because there is not sufficient information known regarding how the ferric subsulfate solid or powder is manufactured, isolated, purified, or characterized. Because ferric subsulfate solid or powder is not well characterized chemically and physically, we do not have assurance that its properties and toxicities, when used in compounding, would be the same as the properties and toxicities reported in the literature and considered by the Agency.

B. Are there concerns about the safety of the substance for use in compounding?

The following sections consider available information about the safety of certain drug products containing ferric subsulfate. These products are typically described in the literature as "Monsel's solution" or "Monsel's paste." We were unable to determine from the literature whether these products were produced from ferric subsulfate solid or powder or whether these products were produced following the USP drug product monograph for ferric subsulfate solution.

1. *Nonclinical assessment*

The following databases were consulted in the preparation of this section: PubMed, National Toxicology Program (NTP) website, Embase, Web of Science, ToxNet, NIH dietary supplement label database, Google, GRAS notice inventory, and Drugs@FDA.

a. General pharmacology of the drug substance

Monsel's solution or paste is an unapproved drug that contains ferric subsulfate (CAS # 1310-45-8). Different sources define ferric subsulfate as an astringent, hemostatic, or styptic agent. The definitions of these terms are discussed below.

The hemostatic activity of Monsel's solution appears to be caused by the denaturation and agglutination of proteins such as fibrinogen by ferric ions, which is augmented by the low pH and subsulfate group (Miller 2015). The hemostatic agent's acidic and oxidizing properties result in phagocytic and histiocytic proliferation with tissue damage (Wood 1981; Armstrong

1986; Miller 2015). An astringent is a substance (or agent) that causes the contraction of skin cells and other body tissues through its acidic nature. In the literature, the use of ferric subsulfate is also referred to as a styptic, an antihemorrhagic agent that assists with hemostasis (Gilbert 1989). Because of the astringent and hemostatic mechanism of action of ferric subsulfate, it is referred to in the Monsel's literature as a caustic agent.[10]

Nonclinical studies included a study in Yucatan miniature hairless pigs where the hemostatic effect of 30% aluminum chloride and ferric subsulfate (Monsel's solution) on the rate of wound healing was investigated. Wound healing was examined in occluded and nonoccluded full thickness 3 mm punch biopsy wounds. Wounds were treated with either 30% aluminum chloride solution or ferric subsulfate solution. The wounds in the control groups were allowed to clot with minimal pressure from a gauze pad (Sawchuck 1986). Delay in re-epithelialization was noted histologically in wounds treated either with aluminum chloride or ferric subsulfate when compared to controls. The authors proposed that this delay in re-epithelialization maybe the result of tissue necrosis caused by these hemostatic agents, resulting in visibly slightly larger scars in treated pigs.

Another animal model is the rat tail bleeding model which was used to test the hemostatic efficacy of Monsel's solution. The three types of styptics that were tested for hemostatic efficacy included a powder form and a gel form containing ferric subsulfate and chitosan, and a solution containing ferric subsulfate and lidocaine. Each of the agents were applied with a minimal pressure to the wounded area. In the control group, equal pressure was applied to the wound without any styptic applied (Byun et al. 2018). In the transected rat tails, hemostasis was improved with the three styptics compared to the control group.

b. Pharmacokinetics/Toxicokinetics

No data were found in the literature.

c. Acute Toxicity[11]

No data were found in the literature.

d. Repeat dose toxicity[12]

No data were found in the literature.

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[10] Topical caustic agents, like ferric subsulfate, coagulate proteins leading to thrombus formation and hemostasis (ACOG 2020; See additional discussion in Section II.C.3 of this evaluation memo on Alternative therapies).

[11] Acute toxicity refers to adverse effects observed following administration of a single dose of a substance, or multiple doses given within a short period (approximately 24 hours). Endpoints captured in acute toxicity studies usually include mortality and gross clinical observations. Acute toxicity data are usually superseded by data obtained from longer term toxicity studies.

[12] Repeat-dose toxicity studies consist of in vivo animal studies that seek to evaluate the toxicity of the test substance by observing the changes that emerge in clinical observations, clinical chemistry, gross pathology, and histology endpoints when the test substance is repetitively administered daily for a predetermined period of time.

e. Genotoxicity[13]

No data were found in the literature.

f. Developmental and reproductive toxicity[14]

Despite its extensive use in localized cervical and vaginal bleeding (VB) in gynecological procedures, no nonclinical articles were found in the literature that describe Monsel's solution's effect on reproduction, fertility or development.

g. Carcinogenicity[15]

No carcinogenicity studies have been conducted with Monsel's solution.

Conclusions: Nonclinical safety data for ferric subsulfate solution was limited to pharmacology studies that investigated the mechanism of action of this substance as an astringent and a hemostatic agent. No data were found in the literature that describe the acute toxicity, repeat dose toxicity, reproductive toxicity, genetic toxicology, or carcinogenicity aspects of Monsel's solution.

2. Human Safety

The following databases were consulted in the preparation of this section: PubMed, EMBASE, Cochrane Database of Systematic Reviews, FDA Adverse Event Reporting System (FAERS), the Center for Food Safety and Nutrition (CFSAN) Adverse Event Reporting System (CAERS), and ClinicalTrials.gov.

a. Reported adverse reactions (FAERS, CAERS)

<u>FAERS</u>

The Office of Surveillance and Epidemiology conducted a search of the FAERS database for reports of adverse events (AEs) through August 25, 2021. Fifteen cases of potential drug-event associations were reported, with most cases involving application site reactions (e.g., inflammation, pain, irritation, chemical burn, dysuria). While these AEs were expected due to

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[13] The genotoxicity assessment battery usually consists of a gene mutagenicity assay (for single dose trials) and a variety of clastogenicity/genotoxicity assays. To support multiple dose administration in humans, additional genotoxicity testing assessment is usually conducted to detect chromosomal damage in mammalian systems.

[14] Developmental and reproductive toxicity studies are usually designed to assess the potential adverse effects in humans of both sexes and include females from various age groups that will be exposed to the proposed substance. *Developmental toxicity* or *teratogenicity* refers to adverse effects (can include embryo-fetal mortality, structural abnormalities, functional impairment, or alterations to growth) and can occur in pups either as a result of the exposure of their parents to the substance, prior to the pups' birth, or by direct exposure of the pups to the substance after birth.

[15] Studies that assess cancer risk in animals are used as predictive tools to evaluate the potential for drugs to result in tumors when used by humans on a chronic basis. Carcinogenicity studies are conducted if the clinical use is expected to be continuous for a minimum of 6 months of life, or if intermittent clinical use is expected to total 6 months or more of life.

the inflammatory potential of ferric subsulfate and are consistent with its known mechanism of action as discussed in the literature, many cases lacked sufficient detail to determine the causality of the drug-event association (i.e., whether the AEs were related to the unapproved drug products). The AEs also primarily consisted of acute reactions to exposure rather than exposure data for longer-term effects. The majority of the cases identified the suspect product as “Monsel’s solution” or “paste.” It is likely that the identified Monsel’s solution or paste in the FAERS database referred to the unapproved drug containing ferric subsulfate.

Of the 15 FAERS cases reported through August 2021, there were 10 serious adverse events (SAEs), and three cases involved ferric subsulfate from compounded products that resulted in hospitalizations (Case IDs 15334124, 15395442, 17999873). There were no deaths reported. Description of the three cases with the compounded products is listed below:

- Case ID 15334124 (U.S.; 2018). A 31-year-old female reported that Monsel’s solution was applied to her urethra after intrauterine device (IUD) removal. She reported her “vagina completely closed and was crusted over and peeling off.” She also had swelling in her urethra, dysuria, and skin pigmentation. She referred to the AE as a chemical burn and required hospitalization.
- Case ID 15395442 (U.S.; 2018). A 33-year-old female reported Monsel’s solution was applied to her cervix prior to IUD placement. She had a burning sensation and then 2 hours later she had intense pain. She went back to the physician the same day for an evaluation and her vagina was swollen shut, which prompted the physician to remove the IUD. The following day, the patient reported that she had small amounts of tissue and blood coming out of her vagina. On the fourth day, a 6- by 3-inch tissue came out and she had cramping, burning, and pain. The patient went to the emergency room and a tissue biopsy was sent, but the results were not reported. She was told by her physician that three other women had a similar application site burn due to Monsel’s solution.
- Case ID 17999873 (U.S.; 2020). A 29-year-old female reported that Monsel’s paste was applied to her cervix to control bleeding. She immediately had a burning sensation. Because the burning persisted throughout the night, the patient went to hospital the next morning. The patient stated that unspecified creams and medications prescribed did not help.

<u>CAERS</u>
The CFSAN collects reports of AEs involving food, cosmetics, and dietary supplements in the CAERS. A search of CAERS was conducted for AEs associated with ferric subsulfate on October 5, 2021, based on the terms “ferric subsulfate” and “Monsel’s solution.” No cases related to the ingredient were found.

b. Clinical trials assessing safety

<u>Randomized controlled trials</u>
No published clinical trials were found that were conducted to specifically assess the safety of ferric subsulfate drug products in humans.

The nominator provided a reference to Mitchell et al. (1998), a prospective, randomized controlled trial where ferric subsulfate solution (Monsel’s solution) was used in two (laser, loop

electrical excision procedure [LEEP] with ball electrode) of the three surgical gynecological procedures. The objective of the trial was to compare cryotherapy, laser vaporization, and LEEP (also referred to as loop diathermy) for the treatment of squamous intraepithelial lesions. It is not possible to assess whether ferric subsulfate solution (Monsel's solution) alone or the surgical procedures contributed to the postoperative complications (bleeding, pain, infection) seen in this study.

We identified at least two randomized, controlled trials that investigated the efficacy of ferric subsulfate drug products used for gynecological procedures (i.e., conization, cryotherapy, laser vaporization, LEEP, ball electrode), and provided safety information on ferric subsulfate.

Hilal et al. (2016) reported a randomized, controlled trial assessing the efficacy and safety of Monsel's solution (20% ferric subsulfate) after colposcopy guided cervical biopsies. In this trial, 75 women were randomized to the Monsel's group compared to the 70 women in the control, "wait and see" group who did not receive any hemostatic agent or other procedures following colposcopic examination for cervical abnormalities.[16] From the Monsel's group, 5/75 (7%) women had an uncomfortable discharge up to 1-3 days after colposcopic examination, 1/75 woman reported severe bleeding for >5 days but did not require further treatment, and 1/75 woman had severe VB 3 hours after colposcopy, requiring inpatient care. The hospitalized patient received a vaginal packing without ferric subsulfate for 24 hours. The article reported that although AEs of discharge were not observed in the control group, the occurrence of severe complications that required treatment, including bleeding was not different between the groups (7/75 [9%] in the Monsel's solution group vs. 2/70 [3%] in the control group). The study authors noted that prolonged discharge may be a specific side effect of Monsel solution: "Because of the coagulation of blood, it can appear as 'tissue.' Thus, patients should be made aware of these side effects when undergoing the application of Monsel['s] solution."

In the Kietpeerakool (2007) trial, women undergoing LEEP were randomized to Monsel's solution (N=140) or the control group (povidone-iodine solution) (N=145) to study the occurrence of postoperative bleeding after LEEP.[17] The investigational product was applied to the cervical bed using a cotton-tip applicator. The most common AE reported was vaginal irritation, which was mild in severity, and more common in the Monsel's (ferric subsulfate) group, compared to the control group. Overall, postoperative infection was observed in 4.56% of women and was successfully treated with oral antibiotics: infection was reported in 6 (4.29%) women in Monsel's group, and in 7 (4.83%) women in control group. As shown in Table 1, the postoperative complications of severe bleeding and infection were not different among the groups who underwent LEEP, 8.57% in Monsel's group vs. 11.72% in the control, respectively. The article concluded that the severity of AEs from the application of Monsel's solution

[16] In the Hilal (2016) study, authors designated group 1 as "Monsel's group," where women received local hemostasis using cotton swabs with an extra-large head drenched in Monsel's solution (20% ferric subsulfate), which were gently pressed on the bleeding areas of the cervix until no more bleeding was visible.
[17] In the "Introduction" section of the Kietpeerakool (2007) article, authors refer to "Monsel's solution" as ferric subsulfate. The concentration of ferric subsulfate was not reported. The active ingredient was applied topically with a cotton swab, and then spread on the craters only.

appeared related to the amount of product used, and various amounts (or repeated dosages) of ferric subsulfate could be minimized.

Table 1: Postoperative complications after LEEP (Kietpeerakool et al. 2007)

Postoperative complications		
Complications	Monsel's group ($n = 140$)	Control group ($n = 145$)
Absent	128 (91.42)	128 (88.28)
Severe bleeding		
Early bleeding	0 (0)	1 (0.69)
Delayed bleeding	6 (4.29)	9 (6.20)
Infection	6 (4.29)	7 (4.83)

Case reports and anecdotal cases assessing safety

In cases with topical application of ferric subsulfate, histopathological evidence showed skin pigmentation (Wood and Severin 1980), tissue destruction (necrosis) acutely (2-5 days) and chronically (2-13 weeks) (Davis 1984; Spitzer and Chernys 1996), and delayed skin reepithelialization (>2 weeks) (Armstrong et al. 1986).

Pathological artifact from ferric subsulfate application and diagnostic challenges

A misdiagnosis of cervical cancer was made due to the pathological changes (hemorrhagic diathesis and artifactual changes similar to that of squamous cells) following the application of ferric subsulfate three days after the treatment of a bleeding vaginal cyst (Rahman 2020). Fortunately, the patient did not have to get a hysterectomy for the misdiagnosis of cervical cancer because the misdiagnosis was quickly cleared.

Similar to the previous case, atypical histiocytic reactions to ferric subsulfate in the skin have been confused with malignant melanoma (Wood and Severin 1980), have obscured interpretation of depth of melanoma invasion (Olmstead et al. 1980), and have impaired the interpretation of cone biopsy specimens when biopsies were performed less than three weeks since the time of ferric subsulfate application (Spitzer and Chernys 1996).

In all these cases, the authors emphasized avoiding cervical smears on patients with recent treatments of ferric subsulfate on the cervix in order to avoid confusion on future diagnosis(es). Furthermore, the authors suggested that in cases where an urgent smear or biopsy is needed within a few weeks after the application of ferric subsulfate, pathologists need information of prior ferric subsulfate use to avoid misinterpretation of the cytologic or histologic findings.

Other safety information

Monsel's solution may not be appropriate treatment option for hemostasis during in vitro fertilization (IVF) because it may inadvertently affect pregnancy outcomes. In an abstract published in the American Society for Reproductive Medicine, Cook (2013) reported that 52 patients were identified for application of Monsel's solution (ferric subsulfate) to control bleeding during oocyte retrieval from IVF cycles followed by fresh, day 5 embryo transfers. 33 patients underwent a fresh embryo transfer at the time of oocyte retrieval. Pregnancy

outcomes were reduced during IVF for the Monsel's group when fresh embryo transfer was performed compared to 419 control patients, who were undergoing IVF in the same period and did not receive Monsel's solution (15.2% vs. 33.6%, p=0.03, respectively). All pregnancies in the Monsel's solution group were documented to receive small (1 swab) or medium (2-3 swabs) dosage administration; none received the larger (greater than 3 swabs) dosage of Monsel's solution.

SAE of peritoneal perforation and mortality
A 46-year-old otherwise healthy woman who had cervical cone biopsy for carcinoma in situ resulted in iatrogenic colpotomy that was a complication of the excisional biopsy when a large surgical excision was made for management of the carcinoma that resulted in persistent bleeding (Shuhaiber et al. 2005). To control bleeding, pads soaked with Monsel's solution were applied after unsuccessful hemostasis with suture and other surgical interventions. The patient died, and the autopsy report showed diffuse internal perforation with histologic evidence of diffuse bowel necrosis, deposits of black/dark gray material around blood vessels, fibrin thrombosis with endothelialization, and extensive iron deposition in the vessel wall. The authors concluded that Monsel's solution can lead to muscle necrosis when it accidently leaks into the peritoneal cavity after topical application.

The study authors in the referenced publications in the American Journal of Obstetrics and Gynecology recommended that "Uterine perforation must be excluded before the use of a Monsel's pack because a leak into the peritoneal cavity could lead to areas of bowel damage and necrosis" (Disu et al. 2007, Miller et al. 2015). The peritoneal cavity must not be exposed to Monsel's solution, and that the consequences of spillage may be catastrophic (Witter 2015).

c. Pharmacokinetic (PK) data

There is no PK data on the topical administration of ferric subsulfate, but we found one article that reported the ability of ferric subsulfate to achieve hemostasis. Armstrong et al. (1986) concluded that ferric subsulfate (Monsel's solution) initially coats the collagen fibers of the skin in the biopsy site and then seeps into the dermis to accumulate along the dermis and the subcutaneous fat (or skeletal muscle). The ferrugination (iron deposition) of the deeper undamaged collagen fibers may also occur because the concentration of the ferric subsulfate (Monsel's solution) may be higher as it continues to seep down and accumulate (in fascial layer, cartilage, blood vessels, etc.).

d. Availability of alternative approved therapies that may be as safe or safer

See Section II.C.3.

Conclusions: Ferric subsulfate is a hemostatic agent that has acidic and oxidizing properties that results in a caustic mechanism. Ferric subsulfate applied topically for achieving hemostasis during minor gynecological surgical procedures that include cervical biopsies has been tolerated with minor AEs consisting of application site reactions (e.g., inflammation, pain, irritation, chemical burn, dysuria) and possible postoperative discharge for up to 1-3 days, delayed wound healing, and vaginal irritation. Potential AEs following the topical use of ferric subsulfate

include increased inflammation and erythema, dermal fibrosis, dyspigmentation from deposition of iron particles in the dermis, delays in epithelialization, and tissue necrosis. Dyspigmentation may distort the pathology on re-excision and lead to depth calculation error of melanoma. Histologic changes in the tissue may persist up to three weeks.

Although scientific publications in Obstetrics & Gynecology generally recognize ferric subsulfate as an appropriate hemostatic agent for small amounts of bleeding when applied on the cervical and vaginal epithelium following cervical biopsies and excisional procedures, ferric subsulfate solution (Monsel's solution) should not be used intra-abdominally because a leak into the peritoneal cavity could lead to areas of bowel damage and necrosis based on the SAE of peritoneal perforation and mortality.

C. Are there concerns about whether a substance is effective for a particular use?

The following databases were consulted in the preparation of this section: PubMed, EMBASE, Cochrane Database of Systematic Reviews, ClinicalTrials.gov, and various online clinical references and websites.

The following sections consider available information about the effectiveness of certain drug products containing ferric subsulfate. These products are typically described in the literature as "Monsel's solution" or "Monsel's paste." We are unable to determine from the literature whether these products were produced from ferric subsulfate solid or powder or whether these products were produced following the USP drug product monograph for ferric subsulfate solution.

1. *Reports of trials, clinical evidence, and anecdotal reports of effectiveness, or lack of effectiveness, of ferric subsulfate as hemostatic agent to reduce bleeding in minor surgical procedures.*

   a. Use of ferric subsulfate during cervical biopsies

Cervical precancerous lesions, often referred to as cervical intraepithelial neoplasia (CIN), are characterized by atypical squamous changes in the transformation zone of the cervix. CIN are frequently diagnosed and treated among women in reproductive and postmenopausal ages using cervical biopsies (punch biopsy, cone biopsies, endocervical curettage) with surgical procedures including cold knife conizations, laser conization, or LEEP with electrocautery.[18] LEEP is a standard surgical procedure for the treatment of high-grade squamous intraepithelial lesions (CIN II and III) (Kyrgiou 2006). However, these surgical procedures and treatment are not without risk. The two most common short-term complications after LEEP are bleeding (intraoperative and postoperative) and infection. Lopes et al. (1994) reported that 70% of women undergoing LEEP experienced some degree of VB with a mean duration of 9 days. The surgical procedures can also result in long-term complications: persistent bleeding, discharge, unsatisfactory colposcopy and cervical stenosis, dysmenorrhea, amenorrhea.

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[18] Types of cervical biopsies. Johns Hopkins Medicine. Access at https://www.hopkinsmedicine.org/health/treatment-tests-and-therapies/cervical-biopsy.

Intraoperative bleeding is generally controlled using standard surgical techniques (sutures, clips, or electrocautery) with the adjunct use of topical hemostatic agents. Based on a literature review, the American College of Obstetricians and Gynecologists (ACOG) Committee on Gynecologic Practice made the following conclusions and recommendations in their 2020 publication[19] regarding the use of topical hemostatic agents, such as ferric subsulfate 20% (Monsel's solution):

- Data on the use of topical hemostatic agents in gynecologic and obstetric surgery are limited and, therefore, recommendations largely are based on these agents in nongynecologic and nonobstetric surgeries.
- Topical hemostatic agents most commonly are used in situations where the use of electrocautery or sutures for hemostatic control of surgical bleeding is not ideal or safe.
- Topical hemostatic agents should not be used for routine prophylaxis of postoperative bleeding because these agents may increase the risk of infection, adhesion formation, and other complications.

ACOG made their recommendations regarding the use of topical agents that are generally in three broad categories: 1) caustic, 2) physical (require intact human coagulation cascade), and 3) biologic. The ACOG report states: "Topical caustic agents include aluminum chloride, ferric subsulfate 20% (Monsel's solution), silver nitrate, and zinc chloride paste.[20] They coagulate proteins leading to tissue necrosis and eschar formation, enhancing thrombus formation and hemostasis. Although used in the cervix and vagina, they are not for intrabdominal use."

The nomination from Fagron proposed the need to compound ferric subsulfate for hemostasis using citations to support its coagulation effects following cervical biopsies from one randomized, controlled trial (Mitchell 1998) and a Cochrane review (Martin-Hirsch and Bryant 2013).

The Cochrane review identified 12 randomized, controlled trials to assess the effectiveness of interventions for preventing blood loss (short term and long term) during the treatment of CIN (i.e., knife or laser cone cervical biopsy, LEEP with electrocautery, laser ablation). Of the 12 studies, the Cochrane review identified three studies (Gilbert 1989) (Lipscomb 2006) (Doyle 1992) that assessed effectiveness of ferric subsulfate in hemostasis (i.e., blood loss, hemorrhage). For purposes of our evaluation, the clinical study design and study details are summarized in Appendix 1. The three studies as summarized below.

In a randomized, controlled trial (Gilbert 1989), 200 women underwent cold-knife conization, where 100 women were randomized to the treatment group "pack" with Monsel's solution (ferric subsulfate) compared to 100 women in the standard suture technique group. Packing was defined as inserting a 3-in-wide gauze roll dipped in Monsel's solution into the cervical cone bed with firm pressure. The application of ferric subsulfate was reserved for cone bed rather than vagina. Packs were removed after 12 hours. The study showed that packing with ferric

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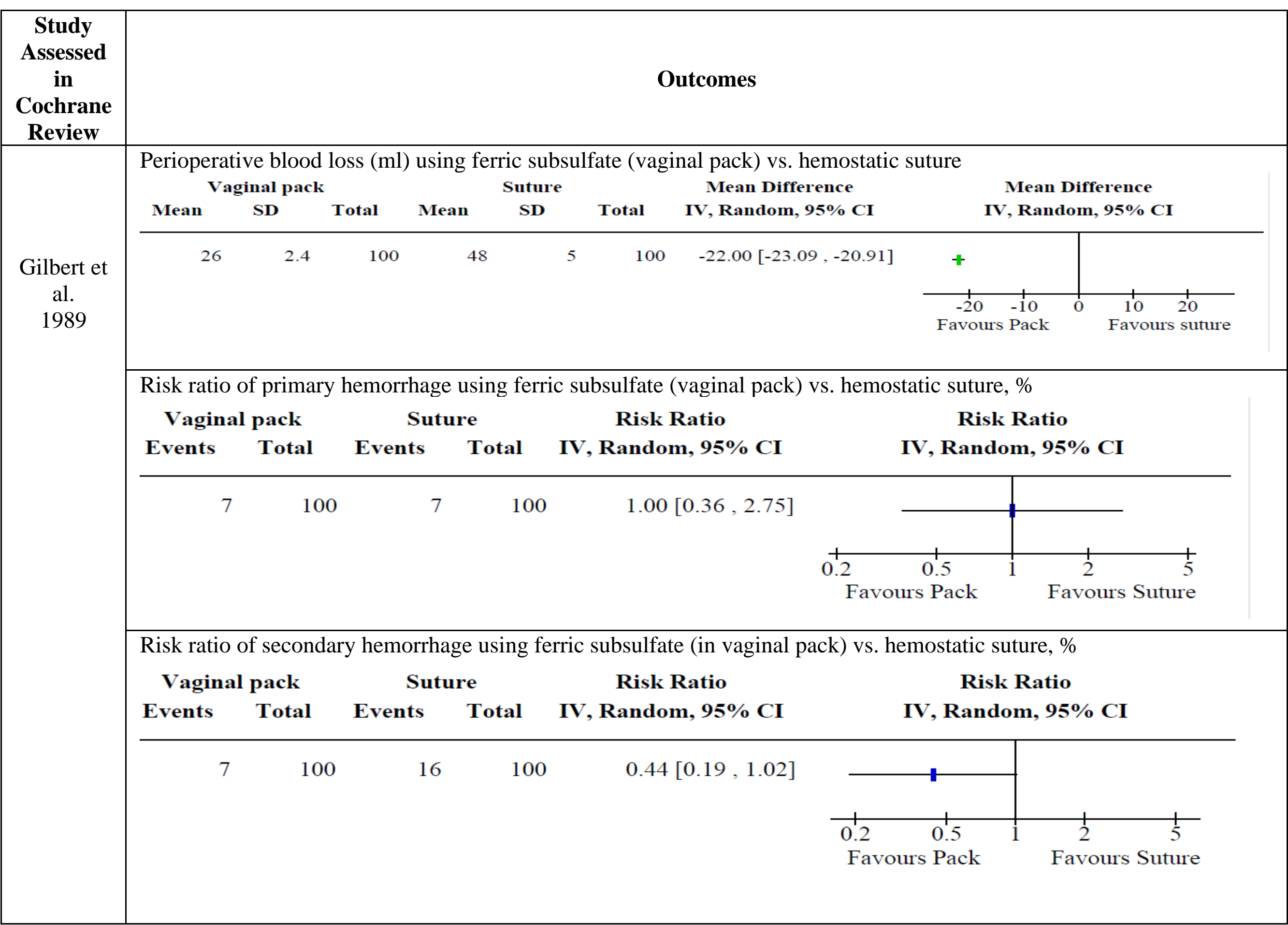
[19] ACOG Committee Opinion 2020 publication is available at https://www.acog.org/clinical/clinical-guidance/committee-opinion/articles/2020/10/topical-hemostatic-agents-at-time-of-obstetric-and-gynecologic-surgery.

[20] See also 21 CFR 310.545(a)(18)(ii) (finding that there are inadequate data to establish general recognition of the safety and effectiveness of silver nitrate and zinc chloride as over-the-counter astringent drug products).

subsulfate after cold-knife conization reduced morbidity by reducing perioperative blood loss (mL) (Mean Difference = -22.00, 95% confidence interval [CI]: -23.09 to -20.91) compared to hemostatic sutures (Figure 1). The results for primary and secondary hemorrhages were not different among the two groups. However, long-term outcomes differed between the two groups. Follow-up after 4 months showed more patients in the suture group (N=81) had menstrual symptoms and required some form of treatment for these symptoms compared to the Monsel's pack group (N=89). Also, colposcopic examination of the transitional zone of the cervix was not well visualized among the suture group (N=100) at 4-months follow-up and 12-months follow-up compared to the pack group (N=100). The article concluded that use of vaginal pack (with ferric subsulfate) may be effective in controlling hemorrhage following cone biopsy, and is an easier, quicker technique with lower rate of postoperative complications and more satisfactory colposcopic follow-up without recourse in comparison to the use of sutures. However, this study does not provide data to support efficacy of ferric subsulfate since there was not an adequate control arm; it is not possible to estimate the contribution of ferric subsulfate solution towards hemostasis as opposed to the pressure exerted on the wound by the gauze pack.

Figure 1: Analyses of outcomes in Gilbert et al. A prospective randomized trial comparing a suture versus non-suture technique (Figure adapted from Martin-Hirsch and Bryant 2013)



Study Assessed in Cochrane Review	Outcomes
Gilbert et al. 1989	See outcome tables below

Perioperative blood loss (ml) using ferric subsulfate (vaginal pack) vs. hemostatic suture

Vaginal pack Mean	Vaginal pack SD	Vaginal pack Total	Suture Mean	Suture SD	Suture Total	Mean Difference IV, Random, 95% CI
26	2.4	100	48	5	100	-22.00 [-23.09 , -20.91]

Risk ratio of primary hemorrhage using ferric subsulfate (vaginal pack) vs. hemostatic suture, %

Vaginal pack Events	Vaginal pack Total	Suture Events	Suture Total	Risk Ratio IV, Random, 95% CI
7	100	7	100	1.00 [0.36 , 2.75]

Risk ratio of secondary hemorrhage using ferric subsulfate (in vaginal pack) vs. hemostatic suture, %

Vaginal pack Events	Vaginal pack Total	Suture Events	Suture Total	Risk Ratio IV, Random, 95% CI
7	100	16	100	0.44 [0.19 , 1.02]

Lipscomb et al. (2006) attempted to compare ferric subsulfate (N=47) to fulguration[21] with ball electrode (electrocautery) (N=53) for hemostasis following LEEP in a randomized, controlled study. The study designated the treatment arm using the term Monsel's paste and concentration was not reported. Time to hemostasis was longer at 131.3 ± 144.6 seconds in the ball electrode group vs. 90.3 ± 116.4 seconds in the Monsel's group (p=0.02). Although estimated blood loss was reported higher in ball electrode group than Monsel's group (16.9 vs. 13.6 mL), overall, there was no significant difference in blood loss between the two groups (Mean Difference = 4.82 mL, 95%: CI, -3.45 to 13.09). Figure 2 shows the trend in favor of Monsel's paste for reduction in blood loss in all patients enrolled in the study, including 6 patients who required alternate means of hemostasis and were not included in the main analysis. Of note, the Visual Analogue Scale (VAS) pain scale and the hemostasis time measured were not distributed normally and were analyzed with nonparametric statistics. The article concluded that the difference between the groups was on average 41-89 seconds for full hemostasis, depending on whether the patient's condition required additional methods of hemostasis was included. Among the Monsel's group, pain was reported less and the time to complete hemostasis was shorter, compared to the group with ball electrode for hemostasis. However, based on the data, both fulguration with ball electrode and Monsel's paste appear to be equally effective for hemostasis after LEEP.

Figure 2: Analyses of outcomes in Lipscomb et al. A trial that compares Monsel's paste with ball electrode for hemostasis after loop electrosurgical excision procedure (Figure adapted from Martin-Hirsch and Bryant 2013)

Study Assessed in Cochrane Review	Outcomes							
	Blood loss (mL) in ball electrode vs. Monsel's use for hemostasis after LEEP in all enrolled patients							
	Ball electrode			Monsel's solution			Mean Difference	Mean Difference
	Mean	SD	Total	Mean	SD	Total	IV, Random, 95% CI	IV, Random, 95% CI
Lipscomb et al. 2006	20.2	26.2	53	15.38	15.1	47	4.82 [-3.45 , 13.09]	

A randomized, controlled trial (Doyle 1992) of 125 healthy women undergoing loop diathermy (commonly referred to as LEEP) for cervical biopsies, were randomized to either the Monsel's solution group or the control group. Monsel's solution was prophylactically applied by a cotton

[21] World Health Organization. Colposcopy and treatment of cervical intraepithelial neoplasia: a beginners' manual. There are three types of coagulation: desiccation, in which the active electrode touches the tissue; fulguration, in which the active electrode does not touch the tissue but 'sprays' multiple sparks between itself and the tissue; and puncture coagulation, in which an electrode, usually a needle, is inserted into the center of a lesion. Coagulation using the fulguration setting and a 3- to 5-mm ball electrode is the type of coagulation that is normally referred to in this manual (one exception is the use of a needle electrode to fulgurate a stubborn area of bleeding). The fulguration setting uses a higher peak-to-peak voltage waveform than the other coagulation settings, coagulating tissue with less current and, therefore, less potential harm to adjacent tissue. Accessed at https://screening.iarc.fr/colpochap.php?chap=13.php&lang=1.

bud to the cervical wound. Although the study did not designate if ferric subsulfate was the same substance as Monsel's solution and there is no information specifically referencing ferric subsulfate, the assumption is that the Monsel's solution studied was "ferric subsulfate." The article did not specify how much Monsel's solution was applied and its concentration. All patients were instructed to record their blood loss for 2-weeks using a sanitary pad chart adapted from Higham et. al. (1990). There was no significant reduction in postoperative bleeding measured as the percentage of women bleeding at day 14 (64% in the Monsel's group vs. 73% in the control group) and the log of mean total blood loss (3.942 ± 1.3 in the Monsel's group vs. 4.14 ± 1.2 in the control group). With limitations of the study design (such as variable sizes of biopsy excisions), there is insufficient data to make a conclusion on the efficacy of ferric subsulfate as an effective hemostatic agent following LEEP.

Fagron provided the Mitchell et al. (1998) study in support of the efficacy of ferric subsulfate. In this randomized, controlled study with a mean duration of 16 months follow up, 390 women with biopsy-proven squamous intraepithelial lesions were randomized to one of three minor surgical procedures for ablation:[22] cryotherapy (N=139), laser vaporization (N=121), or LEEP (N=130). Of note, to achieve hemostasis ferric subsulfate solution was applied postoperatively only to the patients in the groups randomized following the laser vaporization procedure and to the patients randomized to the LEEP procedure group when the lesion bed was coagulated with ball electrode, and then ferric subsulfate solution was placed in the bed. Ferric subsulfate was not used postoperatively for hemostasis in the patients undergoing cryotherapy. The concentration of ferric subsulfate was not reported. The authors used the term ferric subsulfate solution and noted it was Monsel's solution in the "Methods" section. The primary objective was to compare the rate of recurrent squamous intraepithelial lesion disease and complications among the three surgical procedures. The study was not designed to specifically determine the effects of ferric subsulfate in controlling bleeding. Results showed similar success rates of non-recurrent squamous intraepithelial lesions among all three procedures. Incidence of complications, including bleeding (postoperative <24 or >24 hours), were not different among the three surgical procedures. We did not find this study to be supportive in determining the effectiveness of ferric subsulfate for hemostasis because the study was limited in its methodological design and measurement of adequate controls. Thus, it is not possible to estimate the contribution of ferric subsulfate solution towards hemostasis as opposed to the effects of type of surgical procedures.

We identified two additional publications evaluating the effects of ferric subsulfate drug products on hemostasis, predominantly in premenopausal and postmenopausal women undergoing minor surgical procedures on the cervix.

In a randomized, placebo-controlled trial (Hilal 2016), the application of Monsel's solution (ferric subsulfate) group (N=75) was compared to the control, "wait and see" group (N=70) who did not receive any hemostatic agent or other procedures which could lead to hemostasis (e.g.,

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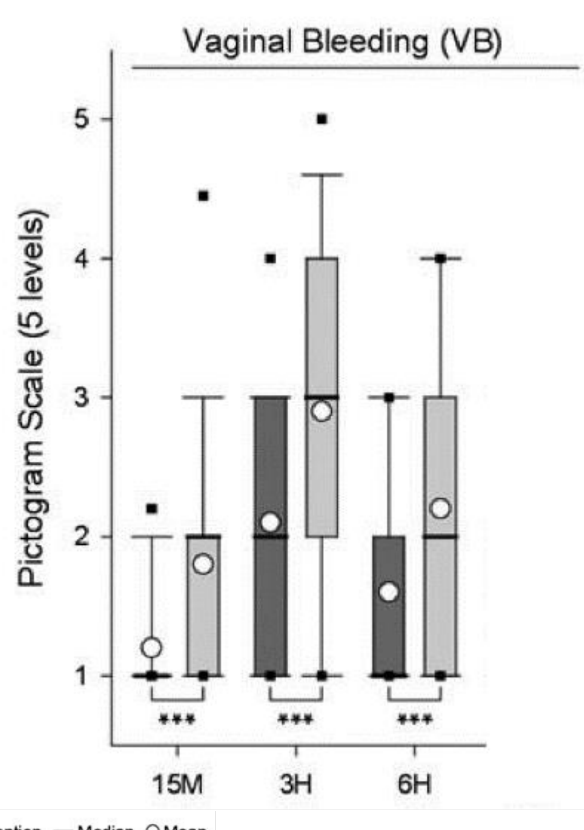
[22] Ablation of the transformation zone is used in the management of patients with biopsy-proven squamous intraepithelial lesions of the cervix who have negative findings on endocervical curettage, a satisfactory colposcopy examination, and congruent Papanicolaou smear and biopsy results. Several techniques for ablation are available: cryotherapy, laser ablation, and loop excision of the transformation zone, also called the LEEP (Mitchell et al. 1998).

pressure on the biopsy site with a cotton swab) following colposcopic examination for cervical abnormalities and endocervical curettage. The authors referred to the investigational product Monsel's solution as 20% ferric subsulfate. Monsel's solution was applied using cotton swabs. The primary endpoint was VB after 15 minutes measured by scoring a sanitary pad with a modified 5-level pictogram as an objective measure (Appendix 2). The primary outcome was assessed by a study nurse blinded to the study group allocation. The mean VB score for the active treatment and control groups was derived from the 5-level pictogram that was used to measure the amount of VB. The Monsel's solution group experienced less VB, mean score 1.2 ± 0.6 vs. 1.8 ± 1.0 in control group ($p<0.001$) (Figure 3). Of note, supplemental results showed that duration of the surgical procedure and biopsy volume independently did not significantly affect the outcome of VB at 15 minutes.[23] Additional secondary outcomes were evaluated. All women were issued a questionnaire and three sanitary pads to score the secondary end points VB after 3- and 6-, and 24-hours (measured by the same 5-level pictogram). After each measuring of VB, women used a new sanitary pad. VB measurements on the 5-level pictogram showed that after 3 and after 6 hours were also in favor of ferric subsulfate resulting in less bleeding (mean score 2.1 ± 1.1 in Monsel's solution group vs. 2.9 ± 1.2 control group, $p<0.001$; and 1.6 ± 0.7 in Monsel's solution group vs. 2.2 ± 1.0 control group, $p<0.001$, respectively). Using the same questionnaire, women additionally scored the subjective estimation of overall VB after 24 hours, estimation of overall pain after 24 hours, and overall satisfaction after 24 hours (all measured by an 11-level VAS [100 mm]). However, at 24 hours, the VB score was equivalent in both groups and Monsel's solution did not affect overall pain or satisfaction of women undergoing endocervical curettage. Considering the reduction in VB for 6 hours after cervical biopsy in the ferric subsulfate treated group, the data in this article support the efficacy of ferric subsulfate as a hemostatic agent in the reduction of VB in the short-term.

[23] See Table in Supplemental Digital Content 3 at https://cdn-links.lww.com/permalink/lgt/a/lgt_2016_06_08_hilal_2016-0079_sdc3.pdf. Accessed Nov 5, 2021.

Figure 3: Amount of vaginal bleeding (VB) compared between patients treated with Monsel's solution (dark gray) versus no intervention (light gray) after cervix sampling and measured at different time points after the procedure (15 minutes and 3- and 6- hours) using a 5-level pictogram scale (Modified from Hilal 2016)



Lower values correspond to lower amount bleeding. Box plots: thick lines indicate medians, boundaries, and whiskers, and filled squares indicate the 25th/10th/5th, and the 75th/90th/95th percentiles, respectively. Open circles represent the means. Levels of significance are indicated (Mann-Whitney U test): ***p≤0.001.

In the Kietpeerakool (2007) controlled trial, women undergoing LEEP with ball electrode were randomized to Monsel's solution (N=140) or the control group (povidone-iodine solution) (N=145) to study the occurrence of postoperative bleeding after LEEP. The investigational product was applied to the cervical bed using a cotton-tip applicator. In the "Introduction" section of their article, authors refer to Monsel's solution as ferric subsulfate, but concentration was not reported. A follow-up visit was scheduled for 2-weeks following the LEEP, and all patients were also telephoned by a gynecologic nurse, blinded to the study, at 24 hours and 4-weeks to inquire about any possible complications. The occurrence of any degree of postoperative VB was observed in 260 (93.3%) of the patients. Immediate application of Monsel's solution after LEEP significantly reduced the duration of the uncomplicated VB (defined as postoperative VB that did not require any treatment), mean 2.73 ± 3.25 days in Monsel's vs. 5.06 ± 4.35 days in control; p<0.001. Occurrence of persistent VB was defined as postoperative VB of at least 2-weeks duration and did not require any treatment (Table 2). Persistent VB was noted to be clinically significant by 3.13% (95% CI, 0.86 to 7.81) and 10.94% (95% CI, 6.11 to 17.67) of women in the Monsel's and control groups, respectively. This article

supports the efficacy of ferric subsulfate in reduction of bleeding following LEEP (procedure with ball electrode).

Table 2: Study outcomes after LEEP[†] (Kietpeerakool et al. 2007)

Symptoms[‡] Variable	Monsel's group ($n = 128$)	Control group ($n = 128$)	Difference (95% CI)	*P*-value
Uncomplicated vaginal bleeding (days)	2.73 ± 3.25	5.06 ± 4.35	2.33 (1.43–3.23)	<0.001
Persistent vaginal bleeding	4 (3.13)	14 (10.94)	0.26 (0.07–0.89)	0.014

†Values are given as mean ± SD or number (percentage) unless stated otherwise. ‡Excluding menstrual bleeding and women who had any complications. CI, confidence interval.

Conclusion: We identified at least two randomized controlled trials and a 2013 Cochrane review that support the short-term efficacy of topical ferric subsulfate as a hemostatic agent in women undergoing cervical biopsies. Clinical data from these studies showed bleeding outcomes (duration, severity of blood loss) reduced after the application of ferric subsulfate compared to adequate controls (no hemostatic agent). The size of the biopsy and duration of the procedures did not affect the outcomes. These studies provide randomized, controlled data that demonstrate effectiveness for use on cervical biopsies and excisions, in addition to safety outcome data.

FDA considered the references provided by Fagron in assessing the efficacy of topical ferric subsulfate as a hemostatic and astringent agent among women undergoing minor surgical procedures on the cervix. However, three of the studies did not have adequate controls or it was not possible to estimate the contribution of ferric subsulfate solution towards hemostasis as opposed to other factors (pressure exerted on the wound by the gauze pack, size of the biopsy excision, types of surgery procedures). One study in the Cochrane review showed both fulguration with ball electrode and Monsel's paste to be equally effective for hemostasis after LEEP.

b. Use of ferric subsulfate in minor surgical procedures for excision of hemorrhoid

Fagron provided a reference to an opinion article (Jetmore 1993) from surgeons who described the properties of ferric subsulfate (styptic and hemostasis) and its effectiveness in treating open anorectal wounds after excision of a thrombosed hemorrhoids based on their experience. The authors stated, "unlike electrocautery, silver nitrate, and suture, [Monsel's solution] is usually painless…and application of the solution over the oozing wound surface to be quicker and more efficient than the focal burning needed with a silver nitrate stick…all Monsel's solution-treated hemorrhoid thrombectomy wounds in our experience have healed well." However, the report does not provide any clinical data on the efficacy of ferric subsulfate and patient demographics.

We did not find any additional information on ferric subsulfate use in hemorrhoid surgeries.

Conclusion: There is insufficient information concerning effectiveness to support use of ferric subsulfate for hemostasis following the excision of hemorrhoids.

2. *Whether the product compounded with this bulk drug substance is intended to be used in a serious or life-threatening disease*

Ferric subsulfate has been used in minor surgical procedures as discussed above. Ferric subsulfate is not intended to be used first line in a serious or life-threatening disease.

3. *Whether there are any alternative approved therapies that may be as effective or more effective*

Multiple therapies have been FDA-approved, including drugs , biologics, and devices, for the treatment of hemostasis (ACOG 2020). See Table 3 below for details on physical, biological, and combination of topical hemostatic agents.

Table 3: Topical hemostatic agents recommended in gynecology and obstetrics (Modified from ACOG Committee on Gynecologic recommendations, 2020)

Agent (Brand Name) Reference	**Form**
Physical agents (require intact coagulation cascade)	
Oxidized regenerated cellulose (e.g., Surgicel [24])	woven mesh or powder
Microfibrillar collagen (e.g., Avitene [25])	sponge or powder
Microporous polysaccharide hemospheres (e.g., Arista [26])	Powder
Gelatin matrix (e.g., Gelform [27])	sponge or powder
Biologic agents	
Thrombin [28]	**Varies (see below)**
Bovine (e.g., Thrombin-JMI)	powder, reconstituted with sterile saline
Pooled human plasma (e.g., Evithrom)	frozen liquid
Recombinant (e.g., Recothrom)	powder, reconstituted with sterile saline
Topical tranexamic acid [29]	liquid
Combination agents	
Thrombin+gelatin granules (flowable) (e.g., Floseal [30], Surgiflo [31])	Liquid
Fibrin sealant [32] (e.g., Tisseel, [33] Evicel [34])	frozen liquid (spray)

Conclusions: There is evidence of effectiveness for the use of ferric subsulfate as a topical hemostatic agent based on data from randomized controlled trials to reduce bleeding following minor gynecological surgical procedures that include cervical biopsies. However, we did not find sufficient clinical information that supported the effectiveness of ferric subsulfate for properties of hemostasis or astringence in minor surgical procedures for excision of hemorrhoids.

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[24] See medical device premarket approval (PMA) for Surgicel™ at https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpma/pma.cfm?id=N12159S087/. (PMA N12159). PMA is the FDA process of scientific and regulatory review to evaluate the safety and effectiveness of Class III medical devices. Class III devices are those that support or sustain human life, are of substantial importance in preventing impairment of human health, or which present a potential, unreasonable risk of illness or injury.

[25] See medical device PMA for Avitene Microfibrillar Collagen Hemostat at https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpma/pma.cfm?id=N17600S035 (PMA N17600).

[26] See medical device PMA for Arista™ AH Absorbable Hemostatic Particles Device at https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpma/pma.cfm?id=P050038S038. (PMA P050038).

[27] See medical device PMA for Gelfoam (Absorbable Gelatin) Sterile Sponge at https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpma/pma.cfm?id=N18286S039 (PMA N18286).

[28] See Thrombin, Bovine, Evithrom, Recotherom from Purple book at https://purplebooksearch.fda.gov/advanced-search/. Redirected to DailyMed for biologic product labels.

[29] See tranexamic acid at https://www.accessdata.fda.gov/scripts/cder/daf/ (e.g., NDA 019281).

[30] See medical device PMA for Floseal Hemostatic Matrix at https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpma/pma.cfm?id=P990009S065/. (PMA P990009).

[31] See medical device PMA for Surgiflo Hemostatic Matrix Kit with Thrombin at https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpma/pma.cfm?id=P990004S044/. (PMA P990004).

[32] See Fibrin Sealant (human) topical labeling at https://www.fda.gov/media/108694/download. Accessed Aug 13, 2021.

[33] See Tisseel topical labeling at https://www.fda.gov/media/71674/download. Accessed Aug 10, 2021.

[34] See Evicel topical labeling at https://www.fda.gov/media/81499/download. Accessed Aug 10, 2021.

D. Has the substance been used historically as a drug in compounding?

Databases searched for information on ferric subsulfate in regard to Section II.D. of this consultation included PubMed, Natural Medicines database, The International Journal of Pharmaceutical Compounding, Japanese Pharmacopoeia, and Google.

1. Length of time the substance has been used in pharmacy compounding

We do not have information on the extent to which the products described in this section are compounded starting from ferric subsulfate solid or powder. The nominators did not provide historical use data.

Ferric subsulfate solution was developed in the late 1850s by Leon Monsel, a French military pharmacist, for hemostasis in minor disruptions of the epithelium (Epstein and Maibach 1964). Monsel reported his success with the styptic and directions for its formulation in 1856. The formulation (now called Monsel's solution) eventually reached the American literature with a publication in the *American Journal of Pharmacology* in 1859. In 1863, a publication in the *Pharmacopoeia of the United States* described a "liquor ferri subsulphatis."[35] In the middle of the 19th century, a pharmacologist named Proctor developed a less acidic Monsel's solution and the term ferric subsulfate, which is still in use today (Garrett et al. 2002).

Ferric subsulfate was not mentioned again in the medical literature until 1964, when Epstein and Maibach published a short paper describing the history, chemistry and mechanism of action of the solution (Epstein and Maibach 1964). The NF in 1955 required Monsel's solution to contain between 20% and 22% aqueous ferric ion and provided that it has been used as a topical hemostatic agent for minor surgical procedures.[36]

Although ferric subsulfate has been studied and used since the beginning of the 19th century, there is insufficient information available to determine how long it has been used specifically in pharmacy compounding.

2. The medical condition(s) it has been used to treat

According to internet searches, ferric subsulfate has been used in gynecology and surgical offices/clinics as a hemostatic agent for minor surgical procedures (Capacho and Alcobia 2014) due to its rapid hemostatic qualities. Results from a Google search using the term "ferric subsulfate compounding pharmacy" indicate that ferric subsulfate is commonly compounded as a topical solution and gel for hemostasis.[37,38]

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[35] Liquor Ferri Subsulphatis. Pharmacopoeia of the United States, 4th Decennial Revision. Philadelphia: JB Lippincott; 1863:220.

[36] National Formulary, 10th ed, Philadelphia : J. B. Lippincott Co., 1955, p. 242.

[37] See McNulty, John P., Muller, George. 2014. Compounded drugs of value in outpatient hospice and palliative care practice. IJPC, 18(3)190-200. Available at: https://pubmed.ncbi.nlm.nih.gov/25306765/. Accessed Apr 14, 2022.

[38] Available at: https://www.koiscenter.com/wp-content/uploads/2017/06/Bealls-Compounding-Pharmacy.pdf. Accessed Apr 14, 2022.

3. How widespread its use has been

Compounded products containing ferric subsulfate remain in wide use today, especially in gynecological and surgical offices/clinics due to its rapid hemostatic qualities (Miller et al. 2015). Ferric subsulfate is the active ingredient in certain unapproved prescription[39] and non-prescription[40] products marketed in the United States (U.S.) for human and animal use for the indication of hemostasis.[41,42,43] In addition, ferric subsulfate is commercially available as a solution or a gel for topical administration, such as Monsel's solution, on the internet through vendor sites, such as Amazon.com[44] and eBay.com.[45]

The International Journal of Pharmaceutical Compounding has published compounding formulations for ferric subsulfate solution NF (Monsel's Solution) (Human and Veterinary).[46] The formulation is made from ferrous sulfate, sulfuric acid 95-96%, nitric acid and purified water USP.

There is currently one USP-NF drug product monograph for ferric subsulfate solution 20% to 22% solution.[47]

4. Recognition of the substance in other countries or foreign pharmacopeias

A search of the BP 2020, EP (10.8) and the Japanese Pharmacopoeia (17th Edition) did not show any monograph listings for ferric subsulfate.

Conclusions: Available information suggests ferric subsulfate products have been used as hemostatic agents from the middle of the 19th century; however, it is not clear how long these products have been compounded, and we do not have information on the extent to which these products are compounded starting from ferric subsulfate solid or powder. There is a USP-NF drug product monograph for ferric subsulfate solution, and there are several ferric subsulfate

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[39] Some products bear national drug codes (NDCs). See National Drug Code Directory at http://www.accessdata.fda.gov/scripts/cder/ndc/index.cfm; search "ferric subsulfate" as Nonproprietary Name.
[40] FDA has determined that there are inadequate data to establish general recognition of the safety and effectiveness of ferric subsulfate (Monsel's solution) as an OTC astringent drug product. See 21 CFR 310.545(a)(18)(ii).
[41] AstriGyn [package insert]. Trumbull, CT: Cooper Surgical Inc; March 2004. The AstrinGyn summary of product characteristics (prescriber information) and labeling can be found at: AstrinGyn (fda.gov). Accessed Aug 5, 2021.
[42] GYNEX [package insert]. Gynex Corporation; March 2020. Summary of product characteristics (prescriber information) and labeling can be found at: https://www.accessdata.fda.gov/spl/data/cf32cbad-332c-2546-e053-2a95a90a5b30/cf32cbad-332c-2546-e053-2a95a90a5b30.xml. Accessed Aug 5, 2021.
[43] Wallach [package insert]. Cooper Surgical Inc; October 1992. The summary of product characteristics (prescriber information) and labeling can be found at: Ferric Subsulfate Aqueous (fda.gov). Accessed Aug 5, 2021.
[44] See Monsel's solution at https://www.amazon.com/monsels-solution/s?k=monsels+solution. Accessed Nov 9, 2021.
[45] See Monsel's solution at https://www.ebay.com/sch/i.html?_from=R40&_trksid=p2334524.m570&_nkw=monsel&_sacat=0&LH_TitleDesc=0&_odkw=ferric+subsulfate&_osacat=0. Accessed Nov 9, 2021.
[46] Available at: https://compoundingtoday.com/Formulation/FormulaInfo.cfm?ID=1566 (subscription required).
[47] Available at: https://online.uspnf.com/uspnf/document/1_GUID-10EB6EA3-AD43-461A-B169-F22A80243905_1_en-US?source=Quick%20Search&highlight=ferric%20subsulfate%20solution (subscription required).

drug products marketed for human and animal use bearing national drug codes. Based on literature and internet searches, ferric subsulfate is mostly compounded as a topical solution and gel to stop bleeding after minor surgical procedures in the U.S.

III. RECOMMENDATION

We have balanced the criteria described in section II above to evaluate ferric subsulfate for the 503A Bulks List. After considering the information currently available, a balancing of the criteria ***weigh against*** ferric subsulfate solid or powder being placed on that list based on the following:

1. Very limited information was found on ferric subsulfate solid or powder in public databases. It is unclear how ferric subsulfate solid or powder is manufactured, isolated, purified or characterized. Although, industrial grade ferric subsulfate solid or powder is available, there is insufficient data available to support adding ferric subsulfate solid or powder to the 503A Bulks List because of a lack of information regarding the manufacturing, quality, and characterization of the commercially available ferric subsulfate solid or powder. The USP drug product monograph for ferric subsulfate solution describes a procedure that starts with ferrous sulfate to prepare ferric subsulfate solution. Ferric subsulfate solution is chemically and physically well-characterized when the USP drug product monograph is followed, but this solution is made starting from ferrous sulfate rather than ferric subsulfate solid or powder, which we construe to be the subject of the nomination. Ferric subsulfate solid or powder is not well-characterized, physically and chemically, because there is not sufficient information known regarding how the ferric subsulfate solid or powder is manufactured, isolated, purified, or characterized. Because ferric subsulfate solid or powder is not well characterized chemically and physically, we do not have assurance that its properties and toxicities, when used in compounding, would be the same as the properties and toxicities reported in the literature and considered by the Agency.

2. The most frequently reported AEs with topical application of ferric subsulfate (Monsel's solution/paste) consisted of application site reactions such as pain, inflammation, irritation, chemical burn, dysuria, and wound site discharge. Experts in Gynecology and Obstetrics recommend exclusion of uterine and peritoneal perforations prior to the topical application of ferric subsulfate. Although topical caustic agents are used on the cervix or vagina, an ACOG Committee on Gynecologic Practice publication stated that topical caustic agents like ferric subsulfate are not for intraabdominal use. Exposure of the peritoneal cavity to ferric subsulfate may result in serious AEs. Anecdotal reports recommended the importance of informing pathologists of ferric subsulfate use in order to avoid misdiagnosis, since the application of ferric subsulfate has been frequently associated with pathologic artifacts. The clinical and nonclinical long-term effects of ferric subsulfate on skin and future fertility are unknown.

3. The available evidence suggests that topical use of ferric subsulfate (Monsel's solution/paste) may be effective as a hemostatic agent following minor gynecologic surgical procedures that include cervical biopsies. There is insufficient information

concerning effectiveness to support use of ferric subsulfate for hemostasis following the excision of hemorrhoids.

4. Ferric subsulfate products, e.g. Monsel's solution, have been used as a hemostatic agent from the middle of the 19th century; however, it is not clear whether or how long such products have been compounded starting from ferric subsulfate solid or powder. There is a USP-NF drug product monograph for ferric subsulfate solution, and there are several ferric subsulfate drug products marketed for human and animal use bearing NDCs. Based on literature and internet searches, ferric subsulfate is mostly compounded as a topical solution and gel to stop bleeding after minor surgical procedures in the U.S.

Based on this information we have considered, a balancing of the four evaluation criteria ***weighs against*** ferric subsulfate solid or powder being added to the 503A Bulks List primarily because of the lack of information on the physical and chemical characterization of ferric subsulfate solid or powder.

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APPENDIX 1: SUMMARY TABLE OF RANDOMIZED CONTROLLED TRIALS EVALUATING FERRIC SUBSULFATE IN THE COCHRANE REVIEW

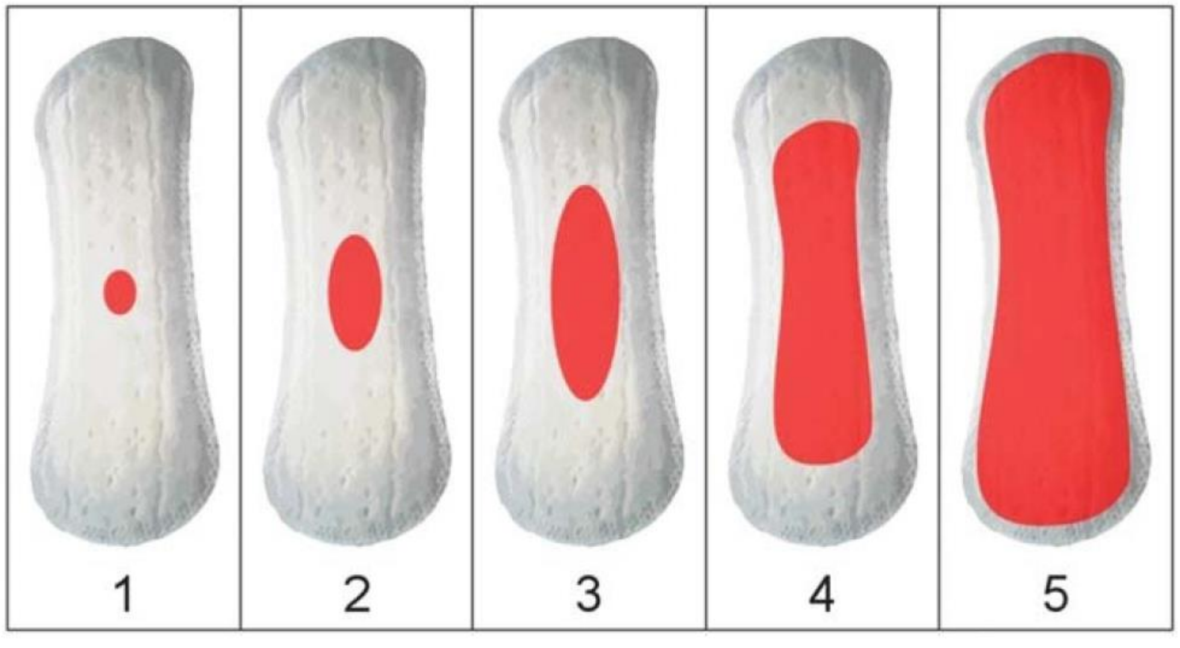
Table 4. Summary of randomized controlled trials evaluating ferric subsulfate in the Cochrane Review (Martin-Hirsch and Bryant 2013; Gilbert 1989; Lipscomb 2005; Doyle 1992)

Referenced study trial **Trial design**	**Treatment (*Dosing*) groups** **and** **Number of subjects**	**Types of procedures being described**	**Effectiveness outcomes**	**AEs - Safety discussion/Tolerability (if any)**
Lipscomb. Gary, et al. 2005 A trial that compares Monsel's paste with ball electrode for hemostasis after loop electrosurgical excision procedure. 2006 Single (patient) blinded randomized controlled study	Monsel's paste (N=47) vs. Ball electrode (N=53)	Healthy women post LEEP surgery for cervix	1. Time to hemostasis shorter with Monsel's paste (50% to 100%), vs. Ball electrode; only 41 to 89 seconds different. 2. Pain was reported less in Monsel's paste 2.2 ± 3.9 vs. 11.1 ± 13.8 (on VAS scale). 3. Estimated blood loss, although higher in the fulguration group, was not significant between groups (16.9 vs 13.6mL; P = .45).	The VAS scores and hemostasis time were not distributed normally and were analyzed with nonparametric statistics. Small amount of time difference in hemostasis may not be clinically significant to many physicians. Estimating accurate procedural blood loss rather than no true difference in blood loss. In the analysis, (n=6) patients were excluded because they required alternate means of hemostasis (2 from Monsel's paste, 4 from fulguration).
Gilbert L. et al. 1989 Hemostasis and cold knife cone biopsy: a prospective randomized trial comparing a suture versus nonsuture technique.	3-inch-wide gauze role dipped in Monsel's solution inserted into vagina for 12 hours (N=100); authors defined Monsel's solution as	Knife cut cone biopsy for abnormal cervical cytology	1. Patients receiving the pack dipped in Monsel solution associated with less intraoperative blood loss. 2. The incidence of reactionary hemorrhage (defined as bleeding in the first 24 hours that required further therapy) was identical in both groups. 3. Secondary hemorrhage (defined as bleeding occurring after the 24 hours of sufficient quantity to require readmission to	At the 2-week review, there were no differences between the suture and pack groups and the incidence of positive urine cultures (6% vs 4%) or in the mean hemoglobin level (12.9 vs 13.2 g %). Significantly more patients in the suture group were subjected to further surgical therapy (by 4-month to 12-month review), the most frequent indications for surgery being menstrual disorders and cervical stenosis. Although the average cone length in the suture group and the pack group was identical, the mean length of

Single (patient) blinded randomized controlled study	“ferric subsulfate” vs. Standard suture technique (N=100)		the hospital) occurred more than twice as often in the suture group (n=16 vs. n=7; P = 0.07). 4. Monsel’s solution group had a higher incidence of requiring blood transfusion (5% vs 4%; p > 0.05).	the cone specimen in those patients in the pack group who did develop stenosis (as judged by the inability to sound the os) was significantly greater than in those patients who did not.
Doyle et al. 1992 Does application of Monsel's solution after loop diathermy excision of the transformation zone reduce post-operative discharge? Results of a prospective randomised controlled trial. Single (patient) blinded randomized controlled trial	Monsel's Solution applied to cervical wound vs. control (no extra treatment)	Women undergoing large Loop excision (diathermy) of the Transformation Zone (LLETZ) of the cervix.	Application of Monsel’s solution did not reduce the quantity or duration of post diathermy discharge by a large amount - Log of mean total blood loss (SD) as per patient reporting using a “sanitary pad chart adapted from Higham et al. (1990):* 3.942 (1.3) in Monsel group vs. 4.14 (1.2) in control group - Excision >20mm in size had moderate-severe operative blood loss than those <20mm *Higham J. M., Shaw R. & O’Brien P.M.S. (1990) A comparison of a new pictorial self-assessment of the severity of menorrhagia compared to the standard alkaline haematin method. Br J Obstet Gynaecol 97, 734-739	Authors concluded that the degree of post-surgical vaginal discharge (after day 14) was common in their study (>66% of women). They propose that secondary infection of the cervical crater is the responsible pathology behind the high prevalence of prolong post loop diathermy excision discharge.

APPENDIX 2: ASSESSMENT OF VAGINAL BLEEDING SCORE ON A SANITARY PAD USING A 5-LEVEL PICTOGRAM IN WOMEN FOLLOWING CERVICAL BIOPSIES

Assessment of vaginal bleeding score on a sanitary pad using a 5-level pictogram in women following cervical biopsies (Hilal 2016)



Tab 5

Lorcaserin Hydrochloride

Tab 5a

FDA Evaluation of Lorcaserin Hydrochloride

DATE: May 2, 2022

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Physician, Pharmacy Compounding Review Team (PCRT),
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THROUGH: Charles Ganley, MD, Office Director, OND Office of Specialty Medicine
Frances Gail Bormel, R.Ph, J.D. Office Director, OCQC, CDER Compliance

TO: Pharmacy Compounding Advisory Committee

SUBJECT: Evaluation of Lorcaserin Hydrochloride for the Withdrawn or Removed List

I. INTRODUCTION

Section 503A of the Federal Food, Drug, and Cosmetic Act (FD&C Act) describes the conditions that must be satisfied for human drug products compounded by a licensed pharmacist in a State-licensed pharmacy or Federal facility, or by a licensed physician, to be exempt from three sections of the FD&C Act: sections 505 (concerning the approval of new drugs under new drug applications (NDAs) or abbreviated new drug applications (ANDAs)), 502(f)(1) (concerning the labeling of drugs with adequate directions for use), and 501(a)(2)(B) (concerning current good manufacturing practice). One of the conditions that must be satisfied to qualify for the exemptions under section 503A is that the licensed pharmacist or licensed physician "does not compound a drug product that appears on a list published by the Secretary in the Federal Register of drug products that have been withdrawn or removed from the market because such drug products or components of such drug products have been found to be unsafe or not effective."[1]

Section 503B of the FD&C Act describes the conditions that must be satisfied for a drug compounded for human use by or under the direct supervision of a licensed pharmacist in a registered outsourcing facility to be exempt from three sections of the FD&C Act: sections 502(f)(1), 505, and 582 (concerning drug supply chain security). One of the conditions in section 503B of the FD&C Act that must be satisfied to qualify for the exemptions is that "[t]he drug does not appear on a list published by the Secretary of drugs that have been withdrawn or

[1] Section 503A(b)(1)(C).

removed from the market because such drugs or components of such drugs have been found to be unsafe or not effective."[2]

FDA has established a list of drug products that were withdrawn or removed from the market because such drug products or components of such drug products have been found to be unsafe or not effective, and which may not be compounded under the exemptions provided by section 503A(a) or section 503B(a) of the FD&C Act (the Withdrawn or Removed List).[3]

The Office of Compounding Quality and Compliance (OCQC) asked the Office of New Drugs (OND) to provide input on whether lorcaserin hydrochloride should be included on the Withdrawn or Removed List.

II. BACKGROUND

Lorcaserin hydrochloride is a selective agonist of the 5-hydroxytryptamine (5-HT) 2C receptors (Tak and Lee 2021). BELVIQ (lorcaserin hydrochloride) tablets, 10 mg, was the subject of NDA 022529, and BELVIQ XR (lorcaserin hydrochloride) extended-release tablets, 20 mg, was the subject of NDA 208524, both held by Eisai Inc. (Eisai), and initially approved on June 27, 2012, and July 15, 2016, respectively. BELVIQ and BELVIQ XR were indicated as an adjunct to a reduced-calorie diet and increased physical activity for chronic weight management in adults with an initial body mass index (BMI) of:

- 30 kilograms per square meter (kg/m^2)or greater (obese); or
- 27 kg/m^2 or greater (overweight) in the presence of at least one weight-related comorbid condition (e.g., hypertension, dyslipidemia, type 2 diabetes).[4] (86 FR 12697, March 4, 2021)

A. Regulatory History

1. United States

In 2012, when approving lorcaserin hydrochloride for use in adults, FDA required the drug manufacturer, Eisai, to conduct a randomized, double-blind, placebo-controlled clinical trial to evaluate the risk of cardiovascular problems associated with the use of lorcaserin hydrochloride. The Cardiovascular and Metabolic Effects of Lorcaserin in Overweight and Obese Patients-Thrombolysis in Myocardial Infarction 61 (CAMELLIA-TIMI 61) clinical trial was conducted to fulfill this requirement (85 FR 58063, September 17, 2020). Eisai collected primary outcome measure data between January 24, 2014, and May 14, 2018[5]

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[2] Section 503B(a)(4).
[3] See 21 CFR 216.24.
[4] See the NDA 022529 and NDA 208524 labels, which are available through the FDA Online Label Repository at https://labels.fda.gov/.
[5] This clinical trial can be found at NIH U.S. National Library of Medicine, www.ClinicalTrials.gov, search term "lorcaserin." NCT 02019264. Accessed Oct. 15, 2021.

Although the primary outcome measure of the CAMELLIA-TIMI 61 clinical trial was to evaluate the risk of cardiovascular problems associated with the use of lorcaserin hydrochloride, FDA's analysis of the trial results suggested an imbalance in cancer in humans. Imbalance in cancer means that more subjects taking lorcaserin hydrochloride were diagnosed with cancer compared to subjects taking placebo. Additional evidence would be necessary to investigate this signal; however, the Agency determined that it is unlikely that the necessary safety endpoints (i.e., cancer and reproductive safety) can be readily or ethically investigated in a clinical trial (86 FR 12697, March 4, 2021).

In a drug safety communication issued on January 14, 2020, the FDA alerted the public that results from a clinical trial assessing the risk of heart-related problems showed a possible increased risk of cancer with lorcaserin hydrochloride (86 FR 12697, March 4, 2021).

In a drug safety communication issued on February 13, 2020, the FDA announced it had asked Eisai to voluntarily withdraw lorcaserin hydrochloride from the U.S. market. On the same day, Eisai submitted a request to the FDA to withdraw approval of NDA 022529 for BELVIQ and NDA 208524 for BELVIQ XR under 21 CFR 314.150(d) and waived its opportunity for a hearing (86 FR 12697, March 4, 2021).

On September 17, 2020, FDA published a Federal Register notice (85 FR 58063, September 17, 2020),[6] withdrawing approval of the applications for lorcaserin hydrochloride tablets, 10 mg and extended-release tablets, 20 mg, effective September 17, 2020. Lorcaserin hydrochloride tablets, 10 mg and lorcaserin hydrochloride extended-release tablets, 20 mg, were removed from the "Orange Book: Approved Drug Products with Therapeutic Equivalence Evaluations" (86 FR 12697, March 4, 2021).

On March 4, 2021, FDA published a notice in the Federal Register announcing that BELVIQ (lorcaserin hydrochloride) tablets, 10 mg and BELVIQ XR (lorcaserin hydrochloride) extended-release tablets, 20 mg were withdrawn from sale for reasons of safety or effectiveness and that the Agency would not accept or approve ANDAs for lorcaserin hydrochloride that refer to this drug product (86 FR 12697, March 4, 2021). FDA assessed that the risk outweighs any potential benefits for the indications (adjunct treatment for chronic weight management in obese and overweight adults) for these products based on the clinical findings from the CAMELLIA-TIMI 61 trial suggesting an imbalance in cancer along with corroborating evidence from animal models.

The Outsourcing Facility Product Report database was searched and identified no reports of lorcaserin hydrochloride use in compounding by outsourcing facilities during 2019 and 2020.[7]

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[6] See 85 FR 58063, available at https://www.federalregister.gov/documents/2020/09/17/2020-20458/eisai-inc-withdrawal-of-approval-of-two-new-drug-application-for-belviq-lorcaserin-hydrochloride-and.
[7] Lorcaserin hydrochloride, Outsourcing Facility Product Report. https://www.accessdata.fda.gov/scripts/cder/outsourcingfacility/index.cfm. Accessed on Oct. 15, 2021.

2. Foreign Countries

Lorcaserin hydrochloride failed to gain approval from the European Medical Agency (EMA). On May 3, 2013, Arena Pharmaceuticals officially notified the EMA Committee for Medicinal Products for Human Use (CHMP) that it wished to withdraw its application for marketing authorization for Belviq (lorcaserin hydrochloride). Although Belviq showed a modest benefit in weight loss, the CHMP was concerned about the potential risk of tumors, particularly with long-term use. Other safety concerns included the potential risk of psychiatric disorders (such as depression) and valvulopathy. Therefore, at the time of the withdrawal, the CHMP believed that the benefits of Belviq did not outweigh its risks.[8]

In 2013, ANVISA, the Brazilian regulatory agency controlling pharmaceutical products and active pharmaceutical ingredients (APIs), suspended the manufacturing, import, commercialization, manipulation, and use of the API lorcaserin hydrochloride.[9]

On February 2, 2018, Eisai cancelled its new drug submission for lorcaserin hydrochloride to Health Canada (the organization that regulates medications in Canada). Health Canada identified some open questions about the data that precluded issuing an approval and Eisai decided to withdraw the submission.[10]

B. Summary

Lorcaserin hydrochloride was approved by FDA as an adjunct treatment for chronic weight management in certain overweight and obese adults. However, the FDA analysis of the CAMELLIA-TIMI 61 clinical trial revealed an increased risk of malignancy with the use of lorcaserin hydrochloride. This primary safety concern led to the withdrawal of lorcaserin hydrochloride from the U.S. market.

III. CDER EVALUATION OF LORCASERIN HYDROCHLORIDE FOR THE WITHDRAWN OR REMOVED LIST

The Office of Compounding Quality and Compliance (OCQC) asked the Office of New Drugs' (OND) Pharmacy Compounding Review Team (PCRT) in consultation with the Division of Diabetes, Lipid Disorders, and Obesity (DDLO) to provide input on three questions related to whether lorcaserin hydrochloride should be included on the Withdrawn or Removed List and, if yes, how the drug should be described on the list. The three questions and OND's responses are provided here.

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[8] Withdrawal of the marketing authorization application for Belviq (lorcaserin), European Medicines Agency. https://www.ema.europa.eu/en/documents/medicine-qa/questions-answers-withdrawal-marketing-authorisation-application-belviq_en.pdf. Accessed on Oct. 15, 2021.

[9] Brazil: ANVISA, See English language translation (using Google or Edge web browser) for lorcaserin information on ANVISA website that is in Portuguese http://antigo.anvisa.gov.br/informacoes-tecnicas13/-/asset_publisher/WvKKx2fhdjM2/content/a-gerencia-de-farmacovigilancia-alerta-para-o-risco-potencial-de-ocorrencia-de-neoplasias-com-o-uso-do-medicamento-belviq-lorcasserina-/33868?p_p_auth=ehviQcAD&inheritRedirect=false. Accessed on February 02, 2022.

[10] Regulatory Decision Summary – lorcaserin hydrochloride (*Belviq) – Health Canada. https://hpr-rps.hres.ca/reg-content/regulatory-decision-summary-detailTwo.php?lang=en&linkID=RDS00370. Access on Oct. 15, 2021.

Question 1:
Based on the information available, do you agree that lorcaserin hydrochloride drug products were withdrawn or removed from the market for safety reasons? Please summarize the basis for the withdrawal or removal of the drug from the market citing available evidence where appropriate.

OND response:
Yes, we agree that lorcaserin hydrochloride drug products were withdrawn from the market for safety reasons. The primary safety concern for lorcaserin hydrochloride is the increased risk of malignancy observed in both postmarketing trial data from the CAMELLIA-TIMI 61 trial as well as nonclinical studies (86 FR 12697, March 4, 2021) as explained below.

The CAMELLIA-TIMI 61 trial, a postmarketing study involving 12,000 overweight or obese subjects with or at high risk for atherosclerotic vascular disease, evaluated the risk for pulmonary hypertension and valvular heart defects associated with lorcaserin hydrochloride treatment (Bohula et al. 2018). Concern for these conditions stemmed from the occurrence of cardiovascular side effects during treatment with other FDA-approved medications for weight loss. Those medications had similar mechanisms of action to lorcaserin hydrochloride (serotonin agonists) and were previously withdrawn from the market (Scheen 2011). Lorcaserin hydrochloride is a selective agonist of the 5-HT 2C receptors (Tak and Lee 2021). Non-specific serotonin agonists that stimulate the peripheral serotonin 2B receptor are believed to stimulate mitotic activity and subsequent cell overgrowth within the heart valve leaflets (Rothman et al. 2000). Because lorcaserin hydrochloride has a high selectivity for 5-HT2c receptor, it was unclear whether lorcaserin hydrochloride caused pulmonary hypertension or valvular heart defects. In the CAMELLIA-TIMI 61 trial, neither pulmonary hypertension nor valvular heart defects occurred at an increased rate in patients treated with lorcaserin hydrochloride compared to placebo (Bohula et al. 2018b). However, in an article describing how the FDA analyzed the data from the CAMELLIA-TIMI 61 trial, the authors reported that when compared to the placebo group, the group treated with lorcaserin hydrochloride had more:

- Total cancers;
- Patients with cancer;
- Cancer deaths;
- Patients with multiple primary tumors; and
- Patients with metastatic disease.

Overall, 7.7% of participants in the lorcaserin hydrochloride group and 7.1% in the placebo group were diagnosed with cancer. Rates were notably higher in the lorcaserin hydrochloride group than in the placebo group for colorectal cancer, pancreatic cancer, and lung cancer. The number of patients with a new cancer diagnosis was similar in the lorcaserin hydrochloride and placebo groups within the first 180 days. Cancer risk was elevated among patients in the lorcaserin hydrochloride group for all latency periods beyond 180 days (Sharretts et al. 2020).

Lorcaserin hydrochloride had a malignancy signal in nonclinical studies as well. In carcinogenicity studies in rats, female and male rats treated with lorcaserin hydrochloride had increased incidence of multiple types of malignancies. The types of malignancies included

astrocytoma, malignant schwannoma, subcutis fibroma, squamous carcinoma of the skin, and mammary fibroadenoma and adenocarcinoma in male rats. Benign and malignant mammary neoplasms dominated the outcome in female rats.[11]

In summary, the nonclinical data and the analysis of the CAMELLIA-TIMI 61 clinical trial suggests an increased risk of malignancy with use of lorcaserin hydrochloride. While the excess cancer risk observed was small, it was difficult to mitigate this potential risk of clinically relevant malignancies against the uncertain clinical benefit of lorcaserin hydrochloride. The clinical findings corroborated by the evidence from the animal models informed the Agency's assessment that the risk outweighs any potential benefits for the current indications. These findings were considered clinically meaningful and could not be adequately addressed through labeling (86 FR 12697, March 4, 2021).

The increased risk of malignancy following treatment with lorcaserin hydrochloride was also evaluated in a recent systematic review and meta-analysis published after the FDA decided that the approvals of NDA 022529 and NDA 208524 were withdrawn for safety. In their discussion, the authors agreed with FDA's decision to withdraw lorcaserin hydrochloride from the market, stating that the benefit of lorcaserin hydrochloride (modest weight loss) did not outweigh the potential risk (malignant neoplasm) (de Andrade Mesquita et al. 2021).

> ***Question 2:***
> Do you have reason to expect that the safety issues that were identified as having been associated with lorcaserin hydrochloride would be restricted to certain formulations or do you expect these safety issues would extend to other drug products containing lorcaserin hydrochloride (e.g., other formulations, routes of administration, or dosage forms)?

OND response:
Lorcaserin hydrochloride was approved for use in adults in the oral tablet dosage form and marketed as Belviq (NDA 022529) and Belviq XR (NDA 208524). The CAMELLIA–TIMI 61 trial results suggested an imbalance in cancer, showing an increased risk of malignancy when oral lorcaserin hydrochloride formulations were used for chronic weight management in adults. Chance effect could not be ruled out and the imbalance persisted throughout multiple analysis approaches. Additional evidence would be necessary to investigate this signal; however, the Agency has determined that it is unlikely that the necessary safety endpoints (i.e., cancer and reproductive safety) can be readily or ethically investigated in a clinical trial (86 FR 12697, March 4, 2021). Although the mechanism by which lorcaserin hydrochloride is associated with malignancy is unknown, we are not aware of data or information suggesting that the increased risk of malignancy is restricted to particular drug products containing the API lorcaserin hydrochloride.

> ***Question 3:***
> Do you recommend that lorcaserin hydrochloride be included on the Withdrawn or Removed List and, if so, how should the entry be described? Please include your rationale for your recommendation.

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[11] See NONCLINICAL TOXICOLOGY from the NDA 022529 and NDA 208524 labels, which are available through the FDA Online Label Repository at https://labels.fda.gov/. Accessed on Nov 12, 2021.

OND response:
OND recommends that all drug products containing lorcaserin hydrochloride be included on the Withdrawn or Removed List. The rationale for this listing is discussed in questions 1 and 2.

We recommend that the following entry for lorcaserin hydrochloride be added to the Withdrawn or Removed List: *Lorcaserin hydrochloride: All drug products containing lorcaserin hydrochloride.*

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