APPENDIX 9 - continued

Print No: 0013

Macropathology and histopathology - individual findings for animals killed after 4 weeks of treatment

Group	: 1	2	3	4
Compound	: Control	Aspergillus niger (ARD-1)		
Dosage (mg/kg/day)	: 0	500	1500	5000

Printed: 10-JAN-00
Page: 77

Schedule number: GSB 061

ANIMAL NUMBER: 0077 SEX: FEMALE DOSE GROUP: 4 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 06-MAY-99 STUDY DAY OF DEATH: 30 STUDY WEEK OF DEATH: 5 TERMINAL BODY WEIGHT: 262.6 GRAMS

PATHOLOGY OBSERVATIONS

NECROPSY	HISTOPATHOLOGY
	CAECUM : -INFLAMMATORY CELL INFILTRATE IN LAMINA PROPRIA,-SLIGHT
	KIDNEYS : -CORTICO-MEDULLARY MINERALISATION,-MODERATE -MEDULLARY MINERALISATION,-SLIGHT
L N MANDIBULAR : -AREA(S) OF CHANGE; MULTIPLE PUNCTATE, DARK FOCI.	L N MANDIBULAR : -HAEMORRHAGE,-SLIGHT

Macropathology and histopathology - individual findings for animals killed after 4 weeks of treatment

Group	: 1	2	3	4
Compound	: Control	Aspergillus niger (ARO-1)		
Dosage (mg/kg/day)	: 0	500	1500	5000

Schedule number: GSB 061

ANIMAL NUMBER: 0078 SEX: FEMALE DOSE GROUP: 4 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 06-MAY-99 STUDY DAY OF DEATH: 30 STUDY WEEK OF DEATH: 5 TERMINAL BODY WEIGHT: 233.3 GRAMS

P A T H O L O G Y O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
	CAECUM : -INFLAMMATORY CELL INFILTRATE IN LAMINA PROPRIA,-SLIGHT
	PARATHYROIDS : >TISSUE MISSING
	SALIVARY GLANDS : -LYMPHOCYTIC INFILTRATION,-MINIMAL
	THYMUS : -LYMPHOCYTOLYSIS,-SLIGHT

*** ANIMAL HAS NO GROSS OBSERVATIONS RECORDED ***

Macropathology and histopathology - individual findings for animals killed after 4 weeks of treatment

Group	1	2	3	4
Compound	Control	Aspergillus niger (ARO-1)		
Dosage (mg/kg/day)	0	500	1500	5000

Schedule number: GSB 061

ANIMAL NUMBER: 0079 SEX: FEMALE DOSE GROUP: 4 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 06-MAY-99 STUDY DAY OF DEATH: 30 STUDY WEEK OF DEATH: 5 TERMINAL BODY WEIGHT: 237.0 GRAMS

PATHOLOGY OBSERVATIONS

NECROPSY	HISTOPATHOLOGY
	CAECUM : -INFLAMMATORY CELL INFILTRATE IN LAMINA PROPRIA,-SLIGHT
	KIDNEYS : -CORTICO-MEDULLARY MINERALISATION,-MINIMAL
	LIVER X 5 : -FOCI OF LEUCOCYTES WITHIN SINUSOIDS,-MINIMAL
	THYMUS : -LYMPHOCYTOLYSIS,-SLIGHT
THYROIDS : -NOT EVIDENT; LEFT.	THYROIDS : >NO SIGNIFICANT LESION

Macropathology and histopathology - individual findings for animals killed after 4 weeks of treatment

Group	: 1	2	3	4	Printed: 10-JAN-00
Compound	: Control	Aspergillus niger (ARO-1)			Page: 80
Dosage (mg/kg/day)	: 0	500	1500	5000	

Schedule number: GSB 061

ANIMAL NUMBER: 0080 SEX: FEMALE DOSE GROUP: 4 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 06-MAY-99 STUDY DAY OF DEATH: 30 STUDY WEEK OF DEATH: 5 TERMINAL BODY WEIGHT: 242.0 GRAMS

P A T H O L O G Y O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
	CAECUM : -INFLAMMATORY CELL INFILTRATE IN LAMINA PROPRIA,-SLIGHT
	THYMUS : -LYMPHOCYTOLYSIS,-SLIGHT
	UTERUS : -ENDOMETRIAL GLAND HYPERPLASIA,-SLIGHT

*** ANIMAL HAS NO GROSS OBSERVATIONS RECORDED ***

Study Number : GSB/061

CONFIDENTIAL

Huntingdon Life Sciences

PROTOCOL

ENZYME PREPARATION FROM

ASPERGILLUS NIGER (ARO-1)

TOXICITY STUDY BY

ORAL GAVAGE ADMINISTRATION TO

CD RATS FOR 4 WEEKS

Sponsor

Gist-Brocades BV
CT&S/REG
PO Box 1
Wateringseweg 1
NL-2600 MA Delft
THE NETHERLANDS

Research Laboratory

Huntingdon Life Sciences Ltd
PO Box 2
Huntingdon
Cambridgeshire
PE18 6ES
ENGLAND

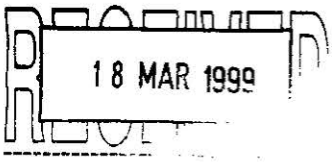
Total number of pages: 25

Final Protocol

CONTACT DETAILS

Sponsor's Monitoring Scientist	:	Mrs R. Hempenius
Addressee/s of samples		
Formulation analysis	:	P.P.J.M. Snuverink Gist Brocades BV Department CT&S/Reg A. Fleminglaan 1 2613 AX Delft The Netherlands

Study Number : GSB/061



Huntingdon Life Sciences

PROTOCOL APPROVAL

ENZYME PREPARATION FROM

ASPERGILLUS NIGER (ARO-1)

TOXICITY STUDY BY

ORAL GAVAGE ADMINISTRATION TO

(b) (6) RATS FOR 4 WEEKS

(b) (6) ..... 10 March 1999

S. Cooper. B.Sc. C.Biol., M.I.Biol. Date
Study Director,
Huntingdon Life Sciences Ltd.

The signature of the Study Director confirms this protocol as the working document for the study. Any changes made subsequent to the date of the Study Director's signature will be documented in formal amendments.

(b) (6) ..... 10 March 1999

A. Broadmeadow, B.Tech., Dip.R.C.Path(Tox)., C.Biol., M.I.Biol. Date
Management.
Huntingdon Life Sciences Ltd.

(b) (6) .. 16 March 1999

Mrs R. Hempenius Date
Sponsor,
Gist-Brocades BV.

Please sign both copies of this page, retain one for your records and return one to the Study Director at Huntingdon Life Sciences.

Huntingdon Life Sciences

ENZYME PREPARATION FROM

ASPERGILLUS NIGER (ARO-1)

TOXICITY STUDY BY

ORAL GAVAGE ADMINISTRATION TO

CD RATS FOR 4 WEEKS

Enquiry Number: 16098D

Number of pages for internal distribution: 22

This working document is approved for circulation and use:

(b) (6)

Study Director

10 March 1999
Date

Primary location of study

Eye Research Centre
Eye
Suffolk

Building Number: 2

All procedures to be performed at the above site unless otherwise detailed below.

Location of specific tasks

Quality control of dosage form	:	Sponsor.
Histology	:	To be documented in the raw data and included in the final report.

CONTENTS

1. INTRODUCTION

Management of study

Study Director	:	S. Cooper.
Monitoring Toxicologist	:	P. Aughton.
In the temporary absence of the Study Director, the scientific responsibilities will be taken over by the Monitoring Toxicologist; other items of routine study management should be referred to the following person in the first instance.	:	P. Knights.

Objective

Assessment of systemic toxic potential in a 4 week oral gavage study in CD Rats.

Regulatory compliance

The study will be performed in compliance with the following regulations or guidelines:

Food and Drug Administration for the USA

Good Laboratory Practice

The study will be conducted in compliance with principles of Good Laboratory Practice Standards as set forth in:

The UK Good Laboratory Practice Regulations 1997 (Statutory Instrument No 654).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

EC Council Directive 87/18/EEC of 18 December 1986 (Official Journal No L 15/29).

Animal model	:	CD Rats, accepted by regulatory agencies, background data available.
Route	:	Oral Gavage, to simulate the conditions of human exposure during use of the test substance.

Treatment groups and dosages

Group	:	1	2	3	4
Compound	:	Control		Aspergillus niger (ARO-1)	
Dosage (mg/kg/day)	:	0	500	1500	5000

2. STUDY SCHEDULE AND STRUCTURE

2.1. Duration of treatment

Minimum period : 4 weeks.

The treatment period may be extended, with the Sponsor's consent, to incorporate any additional observations considered necessary; documented in an amendment to protocol.

Throughout the necropsy period treatment will continue and serial observations will be recorded at appropriate intervals (Section 4.3). Data for any additional complete weeks before commencement of necropsies will be included in the final report.

2.2. Scheduled time plan

(to be up-dated as required in an amendment to protocol)

Sample of Aspergillus niger (ARO-1) arrived	:	23 December 1998	
Animals to arrive	:		
Treatment to commence	:		
Terminal sacrifice to commence	:		
Histopathology to be completed	:		(estimated)
Draft report to be issued	:		(estimated)

2.3. Identity of treatment groups

(to be selected from 90 animals ordered)

Group	Treatment	Dosage (mg/kg/day) #	Number of animals	
			Male	Female
1	Control	0	10	10
2	Aspergillus niger (ARO-1)	500	10	10
3	Aspergillus niger (ARO-1)	1500	10	10
4	Aspergillus niger (ARO-1)	5000	10	10

Expressed in terms of the test substance as supplied.

Group	Cage numbers		Animal numbers	
	Male	Female	Male	Female
1	1-2	9-10	1-10	41-50
2	3-4	11-12	11-20	51-60
3	5-6	13-14	21-30	61-70
4	7-8	15-16	31-40	71-80

3. TEST SUBSTANCE AND FORMULATION

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work etc. Act 1974, and the Control of Substances Hazardous to Health Regulations 1994, it is a condition of undertaking the study that the Sponsor shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to Huntingdon Life Sciences. The Sponsor shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

Information received will be used to set the Huntingdon Life Sciences Hazard Class, which determines safety precautions taken in the workplace.

Huntingdon Life Sciences Hazard Class: 2

3.1. Test substance

Sponsor's identification	:	Aspergillus niger (ARO-1)
Storage conditions	:	Deep-frozen (approximately -20°C) and protected from light.
Sponsor's responsibilities	:	Documentation of methods of synthesis, fabrication or derivation. Stability data. Certificate of analysis which will be included in the final report.
Certificate of analysis details	:	Test substance identity. Batch number. Purity. Composition. Other appropriate characteristics. Current expiry date.

3.2. Formulation

Treatment		
Group 1, Control	:	Vehicle.
Group 2	:	Aspergillus niger (ARO-1); 50 mg/ml.
Group 3	:	Aspergillus niger (ARO-1); 150 mg/ml.
Group 4	:	Aspergillus niger (ARO-1); 500 mg/ml.
Conversion factor	:	The test substance will be used as supplied.
Vehicle	:	Water obtained be reverse osmosis.
Method of preparation	:	Will be documented in the study data and included in the final report.
Frequency of preparation	:	Weekly. Formulations will be divided into daily aliquots and stored refrigerated (approximately 4°C) before use.

3.3. Quality control of dosage form

Liquid formulation	:	At specified intervals during treatment, the test formulations will be analysed for achieved concentration of the test substance.
Analysis	:	To be performed by the Sponsor.

Homogeneity and stability

Information provided by the Sponsor, indicates that formulations are stable for 15 days when stored refrigerated (approximately 4°C).

The test substance forms a solution in water, therefore homogeneity assessment is not required.

Achieved concentration

Sampling and determination	:	Weeks 1, 3. Four samples (nominally 10 ml accurately weighed) from all groups. The samples will be deep-frozen (approximately -20°C) and sent to the Sponsor for analysis.

4. ANIMAL MANAGEMENT

4.1. Animals - supply, acclimatisation and allocation

4.1.1. Animals

Species	:	Rat.
Strain	:	Crl:CD® BR.
Age ordered	:	28 ± 2 days.
Weight range ordered	:	To be within an 11 g range for each sex.
Supplier	:	Charles River (UK) Limited.

4.1.2. Acclimatisation

Duration	:	At least 7 days before commencement of treatment.
Husbandry conditions	:	Refer to Section 4.2.

4.1.3. Allocation to treatment groups

Allocation	:	On arrival.
Method	:	Random.
Cage distribution	:	To equalise environmental influences between groups.

4.1.4. Identification

Numbering	:	Unique for each animal within study.
Method	:	Tail tattoo.
Cage labels	:	Uniquely identifying the occupants.

4.1.5. Animal replacement

10 spare animals will be ordered to replace any individuals rejected during the acclimatisation period.

Replacement before treatment	:	Ill-health. Abnormalities. Bodyweight range extremes.
Replacement during treatment	:	None scheduled.

4.2. Animals - housing, diet and water supply

4.2.1. Environmental control

Rodent facility	:	Limited access - to minimise entry of external biological and chemical agents.
Air supply	:	Filtered, not recirculated.
Temperature	:	Maintained within the range of 19-25°C.
Relative humidity	:	Maintained within the range of 40-70%.

Monitored continuously or daily. Excursions outside these ranges documented in the study data.

Lighting	:	12 hours light : 12 hours dark.
Alarm systems	:	Activated on ventilation failure and when temperature/humidity limits exceeded.
Electricity supply	:	Public supply with automatic stand-by generators.

4.2.2. Animal accommodation

Animals per cage	:	Five of the same sex, unless reduced by mortality or isolation.
Cage material	:	Polypropylene or stainless steel.
Cage flooring	:	Stainless steel grid.

The cages will be suspended above absorbent paper. The latter will be changed at appropriate intervals each week; cages, cage-trays, food hoppers and water bottles will be changed at appropriate intervals. Precise details of caging will be included in the final report.

4.2.3. Diet and water supply

Copies of all certificates of analysis are stored in the archives.

Diet supply

Diet name	:	Rat and Mouse No. 1 Maintenance Diet.
Diet type	:	Pelleted diet.
Availability	:	Non-restricted.

Certification	:	Before delivery each batch of diet is analysed by the supplier for various nutritional components and chemical and microbiological contaminants. Supplier's analytical certificates are scrutinised and approved before any batch of diet is released for use.

This diet contains no added antibiotic or other chemotherapeutic or prophylactic agent.

Water supply

Supply	:	Public drinking water.
Regulatory agency	:	U.K. Department of the Environment.
Availability	:	Non-restricted via polyethylene or polycarbonate bottles with sipper tubes (except if urine collection is performed).
Certification	:	Certificates of analysis are routinely received from the supplier.

4.2.4. Contaminants assay

It is the Sponsor's responsibility to advise Huntingdon Life Sciences of any specific contaminants likely to prejudice the outcome of the study. Analyses for such contaminants may be performed if requested by the Sponsor.

4.3. Animals - procedures

For the 4 weeks of treatment precise day numbers, where quoted, may be varied by not more than 2 days. Examinations scheduled for before termination of treatment will be undertaken during the last scheduled week of treatment unless otherwise specified. The precise times of all examinations will be included in the final report.

4.3.1. Administration

Route	:	Oral gavage.
Treated at	:	Constant dosages in mg/kg/day.
Volume dosage	:	10 ml/kg/day.
Individual dose volume	:	Calculated from the most recently recorded scheduled bodyweight.
Controls (Group 1)	:	Vehicle at the same volume dosage as treated groups.
Frequency	:	Once daily at approximately the same time each day.
Sequence	:	By group.
Formulation	:	A daily record of the usage of formulation will be maintained based on weights. This balance is compared with the expected usage as a check of correct administration.

4.3.2. Clinical observations

Animals and their cages	:	Inspected at least twice daily for evidence of reaction to treatment or ill-health.
Deviations from normal recorded at the time in respect of	:	Nature and severity. Date and time of onset. Duration and progress of the observed condition.
Physical examination	:	Once each week for all animals.

In addition detailed observations will be made in association with dosing according to the following schedule and frequency:

Minimum schedule	:	1. Week 1 - daily. 2. Weeks 2 to 4 - twice weekly (middle and end of week).

Frequency	:	1. Pre-dose observation. 2. As each animal is returned to its home cage. 3. At the end of dosing each group. 4. Between 1 and 2 hours after completion of dosing all groups. 5. As late as possible in the working day.

The above schedule will be amended, as necessary, in the light of signs observed.

During the acclimatisation period, observations of the animals and their cages will be recorded at least once per day.

4.3.3. Mortality

Debilitated animals	:	Observed carefully, may be isolated to prevent cannibalism.
Premature sacrifice	:	Animals may be killed on humane grounds or if considered *in extremis*.

Where practicable blood samples will be taken before death, as specified in Section 4.3.7. Where possible, samples will be analysed for the parameters specified in Sections 6.1 and 6.2.

Animals found dead, killed *in extremis* or on humane grounds	:	A necropsy is performed as soon as possible. Animals found outside the normal workday will be preserved in a refrigerator (approximately 4°C) provided for this purpose.

4.3.4. Bodyweight

Bodyweight recording	:	Day that treatment commences. Each week. At necropsy.

More frequent weighing may be performed to aid the monitoring of the condition of animals displaying ill-health. These data will be retained in the archives.

4.3.5. Food consumption

Food consumption recording	:	Weekly.
Food supplied	:	At intervals each week.
Food spilled	:	Recorded at cage cleaning.
Food remaining	:	Recorded at end of study week.

4.3.6. Other observations

Animals will be subject to procedures as specified in Section 5. Investigations will be performed in the following weeks:

Examination	Week	Animals
Ophthalmic examination	Pretreatment	All animals.
	4	Groups 1 and 4.

4.3.7. Biosampling

Investigations will be performed as follows:

Blood samples - Haematology/Blood Chemistry

Examination	Week	Animals
Haematology	4	All animals.
Blood chemistry	4	All animals.

Conditions	:	Following overnight deprivation of food (not decedents). Samples collected under light general anaesthesia.
Anaesthetic	:	Isoflurane.
Sample site	:	Retro-orbital sinus.
Anticoagulant/ Sample volume	:	EDTA/0.5 ml (Haematology). Citrate/0.5 ml (Coagulation). Lithium heparin/1.0 ml (Blood chemistry).
Analysis	:	Sections 6.1 and 6.2.

4.4. Animals - termination

All animals will be subject to terminal investigations (Section 7). The sequence in which the animals are killed after completion of treatment will allow satisfactory inter-group comparison.

5. OTHER OBSERVATIONS

5.1. Ophthalmic examination

Ophthalmic examinations will be performed as follows:

Week	Animals
Pretreatment	All animals.
4	All animals of Groups 1 and 4.

Both eyes will be examined using a binocular indirect ophthalmoscope after induction of mydriasis with 0.5 % tropicamide. The structures examined will include, but not necessarily be restricted to, the following:

Adnexa
Conjunctiva
Cornea and sclera
Anterior chamber and iris (pupil dilated)
Lens and vitreous
Ocular fundus

At the discretion of the examining veterinary surgeon, a slit-lamp biomicroscope may be used to evaluate and define any lesions identified.

Pretreatment, rejected animals will be replaced with animals, with no adverse ocular abnormality, selected from spare animals from the same batch.

If treatment-related changes are suspected the examination will be extended to all animals of all groups and documented in an amendment to protocol.

Representative photographs will be taken of any unusual or treatment-related findings, if considered appropriate, and documented in an amendment to protocol.

6. CLINICAL PATHOLOGY

6.1. Haematology, peripheral blood

Blood sample analysis will be performed on the following occasion(s):

Week	Animals
4	All animals.

All samples will be examined for the following characteristics:

1) Using EDTA as anticoagulant -

Packed cell volume
Haemoglobin concentration
Erythrocyte count
Total leucocyte count
Differential leucocyte count
Abnormalities of the blood film
Platelet count
Mean cell haemoglobin
Mean cell volume
Mean cell haemoglobin concentration

2) Using citrate as anticoagulant -

Prothrombin time
Activated partial thromboplastin time

6.2. Blood chemistry

Blood sample analysis will be performed on samples obtained from the same animals and at the same time as for haematology.

All samples will be examined for the following characteristics:

Using lithium heparin as anticoagulant -

Alkaline phosphatase
Alanine amino-transferase
Aspartate amino-transferase
Gamma glutamyl transpeptidase
Ornithine carbamyl transferase
Glucose
Bilirubin - total
Cholesterol - total

Triglycerides
Creatinine
Urea
Total protein
Albumin - by chemical assay
Albumin/globulin ratio
Sodium
Potassium
Chloride
Calcium
Phosphorus

6.3. Urinalysis

Urinalysis may be incorporated into the study design, if required by the Sponsor, in the light of expected or observed toxicity of the test material.

7. NECROPSY AND HISTOLOGY

7.1. Method of kill

Method	:	Carbon dioxide.
Sequence	:	To allow satisfactory inter-group comparison.

7.2. Macroscopic Pathology

(Table 1)

Complete	:	All animals.
Checks	:	Retained tissues.
Photography	:	Unusual or suspected treatment-related findings; at the discretion of the necropsy supervisor or Study Director.
Special requirements	:	Retain lymph nodes adjacent to masses (where appropriate).

7.3. Organ weights

(Table 1)

Data collection	:	For bilateral organs, left and right organs will be weighed together unless otherwise specified on the Pathology Procedures Table.
Data presentation	:	Absolute. Adjusted for terminal bodyweight.

7.4. Fixation

(Table 1)

Standard	:	10% Neutral Buffered Formalin.
Others	:	Testes and epididymides: Initially in Bouin's fluid. Eyes: In Davidson's fluid.

7.5. Histology

(Table 1 and Section 8.1)

Processing - Full List	:	All animals killed or dying prematurely. All terminal animals of Groups 1 and 4.
Processing - Abnormalities only	:	All terminal animals of Groups 2 and 3.
Routine staining	:	4-5 µm sections stained with haematoxylin and eosin, except testis which is stained using a standard PAS method.
Special staining	:	None. In the event that special histology staining is required, then this work will be carried out at Huntingdon Research Centre.

TABLE 1-Pathology procedures

Tissue	Weigh	Fix	Light microscopy
Abnormalities		*	*
Adrenals		*	*
Aorta - thoracic		*	*
Brain	*	*	*
Caecum		*	*
Colon		*	*
Duodenum		*	*
Epididymides	*	*	*
Eyes		*	*
Femur		c)	*
Head		b)	#
Heart	*	*	*
Ileum		*	*
Jejunum		*	*
Kidneys	*	*	*
Lachrymal glands		*	*
Liver	*	*	*
Lungs (including bronchi)	*	*	*
Lymph nodes - mandibular		*	*
- mesenteric		*	*
- regional to masses		*	*
Mammary area - caudal		*	*
Oesophagus		*	*
Ovaries	*	*	*
Pancreas		*	*
Pituitary		*	*
Prostate		*	*
Rectum		*	*
Salivary glands		*	*
Sciatic nerves		*	†
Seminal vesicles		*	*
Skeletal muscle - thigh		*	†
Spinal cord		*	*
Spleen	*	*	*
Sternum		*	*
Stomach		*	*
Testes	*	*	*
Thymus	*	*	*
Thyroid with parathyroids	a)	*	*
Tongue		*	*
Trachea		*	*
Urinary bladder		*	*
Uterus with cervix	*	*	*
Vagina		*	*

a) Weighed after partial fixation.
b) Including nasal cavity, paranasal sinuses and nasopharynx.
c) Both hindlimbs retained, one sectioned where appropriate.
* Organs weighed, samples fixed or sections examined microscopically.
# Examined if effects suspected during the study.
† Only one examined.

The tissues subjected to histological processing will include the following regions:

Tissue		Regions to be examined
Adrenals	:	cortex and medulla.
Brain	:	cerebellum, cerebrum and midbrain.
Femur	:	longitudinal section through joint, to include articular surface, epiphysial plate and bone marrow.
Heart	:	including auricular and ventricular regions.
Kidneys	:	including cortex, medulla and papilla regions.
Liver	:	section from all main lobes.
Lungs	:	section from two major lobes, to include bronchi.
Mammary area	:	includes overlying skin.
Spinal cord	:	transverse and longitudinal sections at the cervical level.
Sternum	:	includes bone marrow.
Stomach	:	keratinised, glandular and antrum.
Thyroid	:	includes parathyroid in section, where possible.
Uterus	:	uterus section separate from cervix section.

For bilateral organs sections of both the left and right organs will be examined, unless otherwise specified on the Pathology Procedures Table.

A single section will be prepared from each of the remaining tissues required for microscopic pathology.

8. PATHOLOGY

8.1. Light microscopy

Category	Animals	Tissues
Premature deaths	All from all groups.	All specified in Table 1.
Terminal sacrifice	All animals of Groups 1 and 4.	All specified in Table 1.
Terminal sacrifice	All animals of Groups 2 and 3.	Abnormalities only.

Recording	:	Correlation of masses.
Peer Review	:	Carried out by a reviewing pathologist to Internationally accepted standards.

8.2. Extension of initial examination

At the discretion of the pathologist, further processing and staining techniques may be used to evaluate individual lesions. Details of these techniques will be documented and retained in the archives.

Light microscopy may be extended, following consultation with the Sponsor, as follows:

from all animals of Groups 2 and 3 killed at terminal sacrifice for tissues considered to exhibit a reaction to treatment in Group 4.

Any such requirement will be documented in an amendment to the protocol.

Tissues displaying treatment-related change may be further examined using additional processing or staining techniques.

8.3. Photomicrography

(Optional)

Images	:	Illustration of major lesions after consultation with the Sponsor; taken by a Pathologist.
Report	:	Full photomicrographic report if required.

9. DATA TREATMENT

9.1. Food conversion efficiency

The group mean food conversion efficiency of each sex, expressed as bodyweight gain per unit of food consumed as a percentage, will be calculated for each week of the study.

9.2. Statistical analysis

Data-types

The following data types will be analysed at each timepoint separately:-

bodyweight, using gains over appropriate study periods.
blood chemistry and haematology.
organ weights, both absolute and adjusted for terminal bodyweight.
pathological findings, for the number of animals with and without each finding.

Methods

For categorical data, the proportion of animals will be analysed using Fisher's Exact test for each treated group versus the control.

For continuous data, (excluding clinical pathology data), Bartlett's test will first be applied to test the homogeneity of variance between the groups. Using tests dependent on the outcome of Bartlett's test, treated groups will then be compared with the control group, incorporating adjustment for multiple comparisons where necessary. For clinical pathology data, Students t-test will be applied.

10. REPORTING

Study progress	:	Periodic verbal and written updates on study progress will be provided by the Study Director. Status reports will be sent weekly until termination of the in-life phase.
Draft final report	:	For review by the Sponsor.
Authorised final report	:	After approval from the Sponsor.

Routinely reports are supplied on A4 paper. The following numbers of reports are supplied.

Type of report	**Printing**	**Number of copies**	
		Bound	**Unbound**
Draft report	Double-sided	0	2
Authorised final	Double-sided	1	0
	Single-sided	0	1
Photographic report (if any)	Single-sided	1	0

Any additions or corrections to an authorised final report will be documented as a formal addendum/amendment to the final report.

11. QUALITY ASSURANCE AND ARCHIVING PROCEDURES

11.1. Quality Assurance

Protocol check	:	Authorised protocol and any amendments.
Procedure inspections	:	Critical phases of this study (study based) and routine procedures on representative studies (process based).
Study audit	:	The GLP aspects of the management and conduct of this study.
Report review (Final report)	:	Following issue of the draft report to the Sponsor.
Report of QA findings	:	To Study Director and management promptly on completion of each QA action.

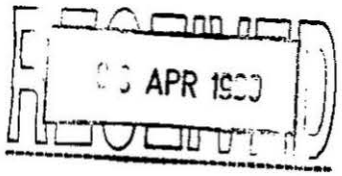
11.2. Archives

All experimental data arising from the study (including documentary raw data, specimens, records, other materials; collectively defined as the "materials") will remain the property of the Sponsor.

Huntingdon Life Sciences shall retain the materials in its archive for a period of 10 years from the date of issue of the final report. After such time, the Sponsor will be contacted and their advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Study Number : GSB/061
Protocol Amendment Number : 1

Huntingdon Life Sciences



ENZYME PREPARATION FROM

ASPERGILLUS NIGER (ARO-1)

TOXICITY STUDY BY

ORAL GAVAGE ADMINISTRATION TO

CD RATS FOR 4 WEEKS

Total number of pages: 5

Number of pages for internal distribution: 5

Study Director : S. Cooper, B.Sc., C.Biol., M.I.Biol.

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

FIRST AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: (b) (6) Date: 30 March 1999
(Study Director)

For the Sponsor (b) (6)

Approved by: Date: 6 April 1999

Huntingdon Life Sciences

ENZYME PREPARATION FROM

ASPERGILLUS NIGER (ARO-1)

TOXICITY STUDY BY

ORAL GAVAGE ADMINISTRATION TO

CD RATS FOR 4 WEEKS

Reason for amendment : Primary location of study: This study will now be housed in Building Number 4.

: Section 2.2: The scheduled time plan is updated.

: Section 3.1: Sponsor's identification of test material is amended in line with the format specified in the protocol title.

: Section 4.2.1: Section amended due to the change of location of study; Building 4 is a fully barriered unit.

: Section 4.2.3: Availability of diet supply amended to reflect the fasting of the animals prior to blood sampling.

Amendments

Primary location of study

Eye Research Centre
Eye
Suffolk

Building Number: ~~2~~ 4

All procedures to be performed at the above site unless otherwise detailed below.

2. STUDY SCHEDULE AND STRUCTURE

2.2 Scheduled time plan
(to be up-dated as required in an amendment to protocol)

Sample of Aspergillus niger (ARO-1) arrived	:	23 December 1998	
Animals to arrive	:	24 March 1999	
Treatment to commence	:	7 April 1999	
Terminal sacrifice to commence	:	5 May 1999	
Histopathology to be completed	:	w/e 2 July 1999	(estimated)
Draft report to be issued	:	w/e 23 July 1999	(estimated)

3. TEST SUBSTANCE AND FORMULATION

3.1 Test substance

Sponsor's identification	:	Enzyme preparation from Aspergillus niger (ARO-1)
Storage conditions	:	Deep-frozen (approximately -20°C) and protected from light.
Sponsor's responsibilities	:	Documentation of methods of synthesis, fabrication or derivation. Stability data. Certificate of analysis which will be included in the final report.
Certificate of analysis details	:	Test substance identity. Batch number. Purity. Composition. Other appropriate characteristics. Current expiry date.

4. ANIMAL MANAGEMENT

4.2 Animals - housing, diet and water supply

4.2.1 Environmental control

Rodent facility	:	Full barrier ~~Limited access~~ - to minimise entry of external biological and chemical agents.
Air supply	:	Filtered, not recirculated.
Temperature	:	Maintained within the range of 19-25°C.
Relative humidity	:	Maintained within the range of 40-70%.

Monitored continuously or daily. Excursions outside these ranges documented in the study data.

Lighting	:	12 hours light : 12 hours dark.
Alarm systems	:	Activated on ventilation failure and when temperature/humidity limits exceeded.
Electricity supply	:	Public supply with automatic stand-by generators.

4.2.3 Diet and water supply

Copies of all certificates of analysis are stored in the archives.

Diet supply

Diet name	:	Rat and Mouse No. 1 Maintenance Diet.
Diet type	:	Pelleted diet.
Availability	:	Non-restricted except overnight before blood sampling.
Certification	:	Before delivery each batch of diet is analysed by the supplier for various nutritional components and chemical and microbiological contaminants. Supplier's analytical certificates are scrutinised and approved before any batch of diet is released for use.

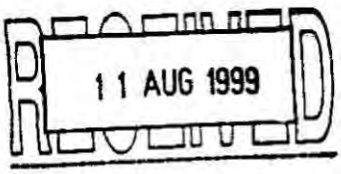
This diet contains no added antibiotic or other chemotherapeutic or prophylactic agent.

Water supply

Supply	:	Public drinking water.
Regulatory agency	:	U.K. Department of the Environment.
Availability	:	Non-restricted via polyethylene or polycarbonate bottles with sipper tubes (except if urine collection is performed).
Certification	:	Certificates of analysis are routinely received from the supplier.

Study Number : GSB/061
Protocol Amendment Number : 2

Huntingdon Life Sciences



ENZYME PREPARATION FROM ASPERGILLUS NIGER (ARO-1) TOXICITY STUDY BY ORAL GAVAGE ADMINISTRATION TO CD RATS FOR 4 WEEKS

Total number of pages: 3

Number of pages for internal distribution: 3

Study Director : S. Cooper, B.Sc., C.Biol., M.I.Biol.

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

SECOND AMENDMENT APPROVAL

(b) (6)

For Huntingdo

Authorised by:
(Study Director

Date: 27 July 1999

(b) (6)

For the Sponso

Approved by: _____ Date: 9 August 1999.

Huntingdon Life Sciences

ENZYME PREPARATION FROM

ASPERGILLUS NIGER (ARO-1)

TOXICITY STUDY BY

ORAL GAVAGE ADMINISTRATION TO

CD RATS FOR 4 WEEKS

Reason for amendment : Section 2.2 : The scheduled timeplan is updated due to the trackdown histopathological examination.

: Sections 7.5 and 8.1 : Following consultation with the Sponsor regarding the micropathological changes seen in the Group 4 females, light microscopy will be extended as follows : Caecums will be taken to slide and examined for Group 2 and 3 females.

Amendments

2. STUDY SCHEDULE AND STRUCTURE

2.2 Scheduled time plan

(to be up-dated as required in an amendment to protocol)

Sample of Aspergillus niger (ARO-1) arrived	:	23 December 1998	
Animals to arrive	:	24 March 1999	
Treatment to commence	:	7 April 1999	
Terminal sacrifice to commence	:	5 May 1999	
Histopathology to be completed	:	w/b 2 August 1999	(estimated)
Draft report to be issued	:	w/b 9 August 1999	(estimated)

7.5 Histology

(Table 1 and Section 8.1)

Processing - Full List	:	All animals killed or dying prematurely. All terminal animals of Groups 1 and 4.
Processing – Abnormalities only	:	All male animals of Groups 2 and 3.
Abnormalities and caecums		All female animals of Groups 2 and 3.
Routine staining	:	4-5 µm sections stained with haematoxylin and eosin, except testis which is stained using a standard PAS method.
Special staining	:	None. In the event that special histology staining is required, then this work will be carried out at Huntingdon Research Centre.

8. PATHOLOGY

8.1 Light microscopy

Category	Animals	Tissues
Premature deaths	All from all groups.	All specified in Table 1.
Terminal sacrifice	All animals of Groups 1 and 4.	All specified in Table 1.
Terminal sacrifice	All males of Groups 2 and 3.	Abnormalities only.
Terminal sacrifice	All females of Groups 2 and 3.	Abnormalities and caecums.

Recording	:	Correlation of masses.
Peer Review	:	Carried out by a reviewing pathologist to Internationally accepted standards.

Annex 9

Beta-glucosidase Ames test

Gist-brocades

PO Box 1
2600 MA Delft
The Netherlands

rapportnummer: 15.747

datum: 23-6-1999

ENZYME PREPARATION FROM ASPERGILLUS NIGER (ARO-1) BACTERIAL MUTATION ASSAY

STUDY NR. GSB058/992604

auteur(s) : K. May
experimenteel werk: Huntingdon Life Sciences Ltd. Eye, Suffolk, IP23 7 PX, England

trefwoorden: **Toxicology**
Mutagenicity
Beta-D-Glucosidase
Aspergillus-niger
ARO-1
GLP
GLP-9708

verzendlijst

1. R. A. Hempenius 2. F.G. Honig 3. Archiefrapportage (2x)	

s.v.p. na gebruik retour R&D-archief

handtekening auteur Datum manuscript: 19-5-99 Huntingdon Life Sciences Ltd. Eye, Suffolk, England	handtekening afd.chef

QAU ASSURANCE UNIT

QAU STATEMENT

Title report	: Enzyme preparation from *Aspergillus niger* (ARO-1) Bacerial mutation assay
Report date	: 19 May 1999
Submitted by	: Huntingdon Life Sciences Eye Suffolk IP23 7PX England
Author/Study Director	: K. May
Study no.	: GSB058/992604

This report has not been checked by Gist-brocades's QAU.
Reliance was placed on the QUALITY ASSURANCE STATEMENT in report no. GSB058/992604, page 4, by H. Comb, Principal Auditor, Department of Quality Assurance, Huntingdon Life Sciences Ltd.

Quality Assurance Unit
Gist-Brocades BV

Date 17-6-1999

(b) (6)

F.G. Honig

(b) (6)

ENZYME PREPARATION FROM *ASPERGILLUS NIGER* (ARO-1)

BACTERIAL MUTATION ASSAY

Sponsor

Gist-brocades BV,
Central Technology & Services,
Wateringseweg 1,
PO Box 1,
2600 MA Delft,
THE NETHERLANDS

Research Laboratory

Huntingdon Life Sciences Ltd.,
Eye,
Suffolk,
IP23 7PX,
ENGLAND

Report issued 19 May 1999

CONTENTS

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice Standards, with the exception stated below, and I consider the data generated to be valid.

The UK Good Laboratory Practice Regulations 1997 (Statutory Instrument No 654).

EC Council Directive 87/18/EEC of 18 December 1986 (Official Journal No L 15/29) and, from 1 May 1999, EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No L 77/8).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

In line with normal practice in this type of short-term study, the protocol did not require analysis of the dose form.

(b) (6)

....

19 May 1999

Kenneth May, B.Sc., C.Biol., M.I.Biol.,
Study Director,
Huntingdon Life Sciences Ltd.

Date

QUALITY ASSURANCE STATEMENT

The following inspections and audits have been carried out in relation to this study:

Study Phase	Date of Inspection	Date of Reporting
Protocol Audit	3 March 1999	3 March 1999
Process Based Inspections		
Formulation	3 November 1998	3 November 1998
S9 Preparation	10 November 1998	10 November 1998
Treatment	17 December 1998	17 December 1998
Plate Scoring	22 December 1998	22 December 1998
Report Audit	26 April 1999	26 April 1999

Protocol Audit: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

Process based inspections: At or about the time this study was in progress inspections of routine and repetitive procedures employed on this type of study were carried out. These were conducted and reported to appropriate Company Management as indicated above.

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results to reflect the raw data.

(b) (6)

14 May 1999

Helen Comb, B.Sc.,
Principal Auditor,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

Date

SUMMARY

In this *in vitro* assessment of the mutagenic potential of Enzyme preparation from *Aspergillus niger* (ARO-1), histidine dependent auxotrophic mutants of *Salmonella typhimurium*, strains TA1535, TA1537, TA98 and TA100, and a tryptophan dependent mutant of *Escherichia coli*, strain CM891 (WP2uvrA/pKM101), were exposed to the test substance diluted in purified water, which was also used as a negative control.

Two independent mutation tests, using the treat-and-plate method, were performed in the presence and absence of liver preparations from Aroclor 1254-induced rats (S9 mix), following preliminary tests to determine toxicity and to confirm that the test substance did not inhibit the activity of the S9 mix at the concentrations tested.

Concentrations of up to 10 mg/ml were tested in the main mutation tests. This is higher than the standard limit concentration recommended in the regulatory guidelines this assay follows, and has been selected in order to compensate for the relatively short exposure time of the test method employed. Other concentrations used were a series of dilutions of the highest concentration (separated by *ca* half-$\log_{10}$ intervals). No signs of toxicity were observed towards the tester strains in either mutation test.

No evidence of mutagenic activity was seen at any concentration of Enzyme preparation from *Aspergillus niger* (ARO-1) in either mutation test.

The concurrent positive controls demonstrated the sensitivity of the assay and the metabolising activity of the liver preparations.

It is concluded that the Enzyme preparation from *Aspergillus niger* (ARO-1) shows no evidence of mutagenic activity in this bacterial system.

INTRODUCTION

This report describes a study designed to assess the mutagenic potential of Enzyme preparation from *Aspergillus niger* (ARO-1) in a bacterial system. The study was conducted in compliance with the following guidelines:

> OECD Guidelines for Testing of Chemicals. (1997) No. 471: Genetic Toxicology: Bacterial Reverse Mutation Test.
>
> EEC Annex to Directive 92/69/EEC. (1992) Part B : Methods for Determination of Toxicity, B.13. Other effects - Mutagenicity: *Escherichia coli* - Reverse Mutation Assay. *O.J.* No. L 383 A, 157.
>
> EEC Annex to Directive 92/69/EEC. (1992) Part B : Methods for Determination of Toxicity, B.14. Other effects - Mutagenicity: *Salmonella typhimurium* - Reverse Mutation Assay. *O.J.* No. L 383 A, 160.
>
> US EPA Office of Prevention, Pesticides and Toxic Substances (1998) Health Effects Test Guidelines No. OPPTS 870.5100: "Bacterial Reverse Mutation Test".

The method described was also designed to comply with ICH (1996 & 1997), and followed the recommendations of the United Kingdom Environmental Mutagen Society (Gatehouse *et al* 1990).

The *in vitro* technique described by Ames and his co-workers, (Ames, McCann and Yamasaki 1975, Maron and Ames 1983) enables the mutagenic effect of a test substance to be determined by exposing specially selected strains of *Salmonella typhimurium* to the test substance. Normally *S. typhimurium* is capable of synthesising the essential amino acid, histidine, but the mutant strains used in this test are incapable of this function. When these strains are exposed to a mutagen, reverse mutation to the original histidine independent form takes place in a proportion of the population. These are referred to as revertants, and are readily detected by their ability to grow and form colonies on a histidine deficient medium (supplemented with biotin, since these strains are also incapable of biotin synthesis).

A technique based on similar principles has also been described by Green (1984). This system employs mutant strains of *Escherichia coli* which are incapable of synthesising the amino acid tryptophan required for growth.

It was anticipated that the test substance might contain free amino-acids, including histidine and tryptophan, which could interfere with the mechanism of the plate incorporation assay. A liquid culture treat-and-plate assay was therefore employed to overcome this problem.

The strains used carry additional mutations which render them more sensitive to mutagens. The *S. typhimurium* strains have a defective cell coat which allows greater permeability of test substances into the cell. All the strains are deficient in normal DNA repair processes. In addition three of them possess a plasmid (pKM101) which introduces an error-prone repair process, resulting in increased sensitivity to some mutagens.

Many substances do not exert a mutagenic effect until they have been metabolised by enzyme systems not available in the bacterial cell. Therefore the bacteria and test substance are incubated in both the absence and presence of a supplemented liver fraction (S9 mix) prepared from rats previously treated with a substance (Aroclor 1254) known to induce a high level of enzymic activity.

The protocol was approved by Huntingdon Life Sciences Management on 15 February 1999, the Sponsor on 22 February 1999 and by the Study Director on 2 March 1999.

The experimental phase of the study was conducted between 4 and 29 March 1999.

TEST SUBSTANCE

Identity:	Enzyme preparation from *Aspergillus niger* (ARO-1)
Appearance:	Brown liquid
Storage conditions:	*ca* –20°C
Lot number:	RER 710
Expiry date:	October 1999 (provisional)
Purity:	9.1% (w/w) dry matter
Date received:	23 December 1998

EXPERIMENTAL PROCEDURE

BACTERIAL STRAINS

The following strains were used:-

S. typhimurium TA1535: contains a histidine missense mutation (*his*G46) but is also deficient in a DNA repair system (*uvr*B) and has a defective lipopolysaccharide coat on the cell wall (*rfa* mutation). It is reverted by many agents causing base-pair substitutions, but is not sensitive to frameshift mutagens.

S. typhimurium TA100: is the same as TA1535 but contains a resistance transfer factor conferring ampicillin resistance and increasing sensitivity to some mutagens (plasmid pKM101). In addition to base-pair substitutions, it is also able to detect certain frameshift mutagens.

S. typhimurium TA1537: bears a histidine frameshift mutation (*his*C3076). Like TA1535, it is defective in a DNA repair system and lipopolysaccharide coat. It is sensitive to agents causing frameshift mutations involving insertion or deletion of a single base-pair.

S. typhimurium TA98: contains another histidine frameshift mutation (*his*D3052). Again it has a defective DNA repair system and lipopolysaccharide coat but also contains the pKM101 plasmid. It is reverted by agents causing deletion of two adjacent base-pairs (double frameshift mutations), but not by simple alkylating agents causing base-pair substitutions.

E. coli CM891: (WP2uvrA/pKM101) contains an ochre mutation. It is reverted by many agents causing A-T base-pair substitutions at the *trp*E locus or by G-C base-pair substitutions in transfer RNA loci elsewhere in the chromosome. It is also deficient in a DNA repair system (*uvr*A), and is more readily reverted by certain mutagens than its parent strain WP2. It also contains the pKM101 plasmid.

The strains of *S. typhimurium* were obtained from Professor B.N. Ames, University of California, Berkeley, California, USA.

The strain of *E. coli* was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland.

Batches of the strains were obtained from master stocks held in liquid nitrogen. The test batches were aliquots of nutrient broth cultures and were stored at -80°C. Dimethyl sulphoxide (DMSO) was added to the cultures at 8% v/v as a cryopreservative. Each batch of frozen strain was tested, where applicable, for cell membrane permeability (*rfa* mutation), sensitivity to UV light and the pKM101 plasmid which confers resistance to ampicillin. The responses of the strains to a series of diagnostic mutagens were also assessed.

For use in tests, an aliquot of frozen culture was added to 25 ml of nutrient broth and incubated, with shaking, at 37°C for 10 hours. These cultures provided at least 10^9 cells per ml which were measured by spreading aliquots (0.1 ml) of a 10^{-6} dilution of the overnight cultures on the surface of plates of nutrient agar.

POSITIVE CONTROLS

In the absence of S9 mix

Identity:	*N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG)
Supplier:	Sigma Chemical
Lot number:	67F-3700
Purity:	98%
Appearance:	Pale yellow crystalline powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	5 µg/plate for strain TA1535 3 µg/plate for strain TA100 2 µg/plate for strain CM891

Identity:	9-Aminoacridine
Supplier:	Sigma Chemical
Batch number:	106F-06681
Purity:	> 97%
Appearance:	Yellow powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	80 µg/plate for strain TA1537

Identity:	2-Nitrofluorene
Supplier:	Aldrich Chemical Company
Batch number:	80501-24227
Purity:	98%
Appearance:	Beige powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	1 µg/plate for strain TA98

In the presence of S9 mix

Identity:	2-Aminoanthracene
Supplier:	Aldrich Chemical Company
Batch number:	52234-024
Purity:	96%
Appearance:	Green powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	2 µg/plate for strain TA1535 10 µg/plate for strain CM891

Identity:	Benzo[a]pyrene
Supplier:	Aldrich Chemical Company
Batch number:	07778-105
Purity:	98%
Appearance:	Yellow powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	5 µg/plate for strains TA1537, TA98 and TA100

PREPARATION OF S9 FRACTION

Species:	Rat
Sex:	Male
Strain:	Sprague-Dawley derived
Source:	Harlan Olac Ltd
Age:	7-8 weeks
Weight:	<300 g

S9 fraction was prepared from a group of *ca* 10 animals. Mixed function oxidase systems in the rat livers were stimulated by Aroclor 1254, administered as a single intra-peritoneal injection in Arachis oil at a dosage of 500 mg/kg bodyweight. On the fifth day after injection, following an overnight starvation, the rats were killed by cervical dislocation and their livers aseptically removed.

The following steps were carried out at 0-4°C under aseptic conditions. The livers were placed in 0.15 M KCl (3 ml KCl : 1 g liver) before being transferred to an Ultra-Turrax homogeniser. Following preparation, the homogenate was centrifuged at 9000 *g* for 10 minutes. The supernatant fraction (S9 fraction) was dispensed into aliquots and stored at -80°C until required. Each batch of S9 fraction was tested for sterility and efficacy.

Date of preparation: 18 November 1998

PREPARATION OF S9 MIX

S9 mix contained: S9 fraction (10% v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium orthophosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADP (4 mM). All the cofactors were filter-sterilised before use.

SELECTION OF SOLVENT AND FORMULATION OF TEST SUBSTANCE

The test substance was supplied as a frozen aqueous solution. Individual aliquots of the test substance were thawed on the day of test, sterilised by membrane filtration (0.2 µm pore) and diluted as necessary in purified water (obtained by reverse osmosis of tap-water).

All concentrations cited in this report are expressed in terms of the dry matter content of the Enzyme preparation from *Aspergillus niger* (ARO-1) sample as received (9.1% w/w).

MUTATION TEST PROCEDURE

Preliminary toxicity test

The test substance was added to cultures of the five tester strains at five concentrations separated by *ca* half-log intervals. The highest final concentration of Enzyme preparation from *Aspergillus niger* (ARO-1) tested was 10 mg/ml. The negative control was purified water.

An aliquot of 0.3 ml of a 10 hour bacterial culture and 1.5 ml S9 mix or 1.5 ml 0.1 M sodium orthophosphate buffer (pH 7.4) were placed in centrifuge tubes. An aliquot of 0.3 ml of the test solution was added. This is equivalent to pooling three treatment mixtures. One centrifuge tube was prepared for each concentration. Following addition of the test solution, the mixture was incubated at 37°C for 1 hour with shaking. The mixture was then centrifuged at 1000 *g* for 20 minutes, the supernatant decanted and the pellet resuspended in 3 ml of 0.1 M sodium orthophosphate buffer (pH 7.4). The suspension was divided into three equal volumes and 2 ml of molten agar containing 0.5mM histidine/biotin/tryptophan added. The mixture was thoroughly shaken and overlaid onto previously prepared petri dishes containing 25 ml minimal agar. Each petri dish was individually labelled with a unique code corresponding to a sheet, identifying the dish's contents. Plates were also prepared without the addition of bacteria in order to assess the sterility of the test substance, S9 mix and sodium orthophosphate buffer. All plates were incubated at 37°C for *ca* 72 hours. After this period the appearance of the background bacterial lawn was examined and revertant colonies counted using a Domino automated colony counter.

Any toxic effects of the test substance would be detected by a substantial reduction in revertant colony counts or by the absence of a complete background bacterial lawn. In the absence of any toxic effects the top concentration normally used in the main tests would be the same as that used in the preliminary test. If toxic effects were observed a lower concentration may be chosen. It should be ensured that if a lower concentration was chosen, signs of bacterial inhibition are present at the top concentration. Ideally a minimum of three non-toxic concentrations should be obtained.

Test for effect of protease activity on S9 mix.

The test substance was added to cultures of strain TA98 at three concentrations separated by two-fold intervals. The highest final concentration of Enzyme preparation from *Aspergillus niger* (ARO-1) tested was 10 mg/ml as it was known from the preliminary toxicity test that the test substance was non-toxic at this concentration. An aliquot of 0.3 ml of a 10 hour bacterial culture and 1.4 ml S9 mix were placed in centrifuge tubes. An aliquot of 0.3 ml of the test solution was added. 2-Aminoanthracene (0.1 ml at a concentration of 150 µg/ml) was added to all tubes. The contents of each tube were mixed thoroughly. Two further tubes containing purified water instead of Enzyme preparation from *Aspergillus niger* (ARO-1) were also included; in one, the S9 mix was replaced by 0.1 M sodium orthophosphate buffer (pH 7.4). The mixture was incubated at 37°C for 1 hour with shaking. The mixture was then centrifuged at 1000 *g* for 20 minutes, the supernatant decanted and the pellet resuspended in 3 ml of 0.1 M sodium orthophosphate buffer (pH 7.4). The suspension was divided into three equal volumes and 2 ml of molten agar containing 0.5mM histidine/biotin/tryptophan added. The mixture was thoroughly shaken and overlaid onto previously prepared petri dishes containing 25 ml minimal agar. Each petri dish was individually labelled with a unique code corresponding to a sheet, identifying the dish's contents. All plates were incubated at 37°C for *ca* 72 hours. After this period the appearance of the background bacterial lawn was examined and revertant colonies counted using a Domino automated colony counter.

If the number of revertants obtained from cultures treated with 2-aminoanthracene, S9 mix and test substance is significantly smaller than the number of revertants obtained from cultures treated with 2-aminoanthracene and S-9 mix only, it is assumed that the test substance inhibits the activity of the S9 mix. If this were the case, all subsequent tests would be performed using aliquots of the test substance which have been inactivated.

First main mutation test

The test substance was added to cultures of the five tester strains at five concentrations separated by *ca* half-log intervals. The highest final concentration of Enzyme preparation from *Aspergillus niger* (ARO-1) tested was again 10 mg/ml. The negative control was purified water. The appropriate positive controls were also included.

An aliquot of 0.3 ml of a 10 hour bacterial culture and 1.5 ml S9 mix or 1.5 ml 0.1 M sodium orthophosphate buffer (pH 7.4) were placed in centrifuge tubes. An aliquot of 0.3 ml of the test solution was added. This is equivalent to pooling three treatment mixtures. One centrifuge tube was prepared for each concentration. Following addition of the test solution, the mixture was incubated at 37°C for 1 hour with shaking. The mixture was then centrifuged at 1000 *g* for 20 minutes, the supernatant decanted and the pellet resuspended in 3 ml of 0.1 M sodium orthophosphate buffer (pH 7.4). The suspension was divided into three equal volumes and 2 ml of molten agar containing 0.5mM histidine/biotin/tryptophan added. The mixture was thoroughly shaken and overlaid onto previously prepared petri dishes containing 25 ml minimal agar. Each petri dish was individually labelled with a unique code corresponding to a sheet, identifying the dish's contents. Plates were also prepared without the addition of bacteria in order to assess the sterility of the test substance, S9 mix and sodium orthophosphate buffer. All plates were incubated at 37°C for *ca* 72 hours. After this period the appearance of the background bacterial lawn was examined and revertant colonies counted using a Domino automated colony counter.

Second main mutation test

As a clear negative response was obtained in the first test, the second test was an exact repeat of the first.

STABILITY AND FORMULATION ANALYSIS

The stability of the test substance and the stability of the test substance in the solvent were not determined as part of this study. Analysis of achieved concentration was not performed as part of this study.

ASSESSMENT OF RESULTS

For a test to be considered valid the mean of the solvent control revertant colony numbers for each strain should lie in the range stated in the appropriate Standard Operating Procedure. Also, the positive control compounds must cause at least a doubling of mean revertant colony numbers over the negative control.

The mean number of revertant colonies for all treatment groups were compared with those obtained for the solvent control groups. The mutagenic activity of a test substance was assessed by applying the following criteria:

a) If treatment with a test substance produces an increase in revertant colony numbers of at least twice the concurrent solvent controls, with some evidence of a positive dose-relationship, in two separate experiments, with any bacterial strain either in the presence or absence of S9 mix, it is considered to show evidence of mutagenic activity in this test system. No statistical analysis is performed.

b) If treatment with a test substance does not produce reproducible increases of at least 1.5 times the concurrent solvent controls in either mutation test it is considered to show no evidence of mutagenic activity in this test system. No statistical analysis is performed.

c) If the results obtained fail to satisfy the criteria for a clear "positive" or "negative" response given in paragraphs a) and b), additional testing may be performed in order to resolve the issue of the test substance's mutagenic activity in this test system. Should an increase in revertant colony numbers then be observed which satisfies paragraph (a) the substance is considered to show evidence of mutagenic activity in this test system. No statistical analysis is performed.

   If no clear "positive" response can be obtained, the test data may be subjected to analysis to determine the statistical significance of any observed increases in revertant colony numbers. The statistical procedures used will be those described by Mahon *et al* (1989) and will usually be analysis of variance followed by Dunnett's test.

MAINTENANCE OF RECORDS

All experimental data arising from the study (including documentary raw data, records and other materials; collectively defined as the "materials") will remain the property of the Sponsor.

Huntingdon Life Sciences shall retain the materials in its archive for a period of five years from the date of issue of the final report. After such time, the Sponsor will be contacted and their advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials, subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences shall also retain a copy of the final report in its archive indefinitely.

RESULTS

The results obtained with Enzyme preparation from *Aspergillus niger* (ARO-1) and positive control compounds are presented in Tables 1 to 13. The mean values quoted have been corrected to the nearest whole number.

The absence of colonies on sterility check plates confirmed the absence of microbial contamination.

The total colony counts on nutrient agar plates (see Tables) confirmed the viability and high cell density of the cultures of the individual organisms.

The mean revertant colony counts for the solvent controls were within the ranges stated in the appropriate Standard Operating Procedure or quoted by Gatehouse *et al* (1990). Appropriate positive control chemicals (with S9 mix where required) induced substantial increases in revertant colony numbers with all strains, confirming sensitivity of the cultures and activity of the S9 mix.

PRELIMINARY TOXICITY TEST

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to Enzyme preparation from *Aspergillus niger* (ARO-1) at any concentration in either the presence or absence of S9 mix.

No visible thinning of the background lawn of non-revertant cells was obtained following exposure to Enzyme preparation from *Aspergillus niger* (ARO-1). A top exposure concentration of 10 mg/ml was therefore selected for use in the test for effect of protease activity on S9 mix.

TEST FOR EFFECT OF PROTEASE ACTIVITY ON S9 MIX

A small (*ca* 10%) reduction in the numbers of revertants induced by 2-aminoanthracene was obtained following exposure to Enzyme preparation from *Aspergillus niger* (ARO-1), indicating that the activity of the S9 mix was slightly inhibited. It was decided, however, that this small degree of inhibition did not present a significant technical problem. A top exposure concentration of 10 mg/ml, with no requirement for inactivation, was therefore selected for use in the main tests.

FIRST MAIN TEST

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to Enzyme preparation from *Aspergillus niger* (ARO-1) at any concentration in either the presence or absence of S9 mix.

No visible thinning of the background lawn of non-revertant cells was obtained following exposure to Enzyme preparation from *Aspergillus niger* (ARO-1).

SECOND MAIN TEST

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to Enzyme preparation from *Aspergillus niger* (ARO-1) at any concentration in either the presence or absence of S9 mix.

No visible thinning of the background lawn of non-revertant cells was obtained following exposure to Enzyme preparation from *Aspergillus niger* (ARO-1).

CONCLUSION

It is concluded that the Enzyme preparation from *Aspergillus niger* (ARO-1) shows no evidence of mutagenic activity in this bacterial system.

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TABLE 1

Results obtained with *S. typhimurium* TA98: preliminary test

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	Enzyme prep. from *Aspergillus niger* (ARO-1) (10000 µg/ml); sterility check		-	0	0	0	0	0
3		(10000 µg/ml)	+	22	19	19	20	2
4	Enzyme preparation	(3000 µg/ml)	+	24	24	19	22	3
5	from *Aspergillus niger*	(1000 µg/ml)	+	27	20	24	24	4
6	(ARO-1)	(300 µg/ml)	+	17	27	24	23	5
7		(100 µg/ml)	+	17	24	19	20	4
8	Purified water		+	28	29	24	27	3
9		(10000 µg/ml)	-	23	20	19	21	2
10	Enzyme preparation	(3000 µg/ml)	-	26	17	17	20	5
11	from *Aspergillus niger*	(1000 µg/ml)	-	24	26	20	23	3
12	(ARO-1)	(300 µg/ml)	-	20	16	17	18	2
13		(100 µg/ml)	-	22	21	28	24	4
14	Purified water		-	26	28	22	25	3
15	Benzo[a]pyrene (5 µg/plate)		+	160	146	154	153	7
16	2-Nitrofluorene (1 µg/plate)		-	208	182	204	198	14
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	117	116	115	116	1

* Except plates Nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 2

Results obtained with *E. coli* CM891: preliminary test

Plate No.	Addition	S9 mix + present - absent	Revertant colony counts* and means				
			A	B	C	Mean	sd
1+	None; S9 mix sterility check	+	0	0	0	0	0
1 -	None; buffer sterility check	-	0	0	0	0	0
2	Enzyme prep. from *Aspergillus niger* (ARO-1) (10000 µg/ml); sterility check	-	0	0	0	0	0
3	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	+	166	119	137	141	24
4	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	+	124	148	151	141	15
5	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	+	153	150	126	143	15
6	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	+	132	121	136	130	8
7	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	+	137	154	148	146	9
8	Purified water	+	143	157	131	144	13
9	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	-	145	126	103	125	21
10	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	-	130	133	109	124	13
11	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	-	123	114	117	118	5
12	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	-	104	128	137	123	17
13	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	-	144	146	122	137	13
14	Purified water	-	136	128	150	138	11
15	2-Aminoanthracene (10 µg/plate)	+	334	318	385	346	35
16	ENNG (2 µg/plate)	-	493	496	467	485	16
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar	-	143	139	141	141	2

* Except plates Nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 3

Results of test for effect of protease activity on S9 mix

Plate No.	Addition* (µg)		S9 mix + present - absent	TA98 Revertant colony counts and means A	B	C	Mean	sd
1	Enzyme preparation from	(10000)	+	1732	1670	1664	1689	38
2	*Aspergillus niger*	(5000)	+	1527	1857	1767	1717	171
3	(ARO-1)	(2500)	+	1886	1691	1674	1750	118
4	Purified water		+	2017	1918	1740	1892	140
5	Purified water		-	29	35	31	32	3

* 2-Aminoanthracene (5 µg) present in all treatments.

TABLE 4

Results obtained with *S. typhimurium* TA98: main test 1

Plate No.	Addition	S9 mix + present - absent	Revertant colony counts* and means				
			A	B	C	Mean	sd
1+	None; S9 mix sterility check	+	0	0	0	0	0
1 -	None; buffer sterility check	-	0	0	0	0	0
2	Enzyme prep. from *Aspergillus niger* (ARO-1) (10000 µg/ml); sterility check	-	0	0	0	0	0
3	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	+	27	19	23	23	4
4	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	+	19	22	23	21	2
5	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	+	20	22	20	21	1
6	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	+	22	27	23	24	3
7	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	+	24	24	26	25	1
8	Purified water	+	26	24	31	27	4
9	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	-	24	22	26	24	2
10	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	-	20	22	23	22	2
11	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	-	24	23	24	24	1
12	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	-	24	20	24	23	2
13	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	-	23	20	26	23	3
14	Purified water	-	29	23	24	25	3
15	Benzo[a]pyrene (5 µg/plate)	+	170	181	151	167	15
16	2-Nitrofluorene (1 µg/plate)	-	158	170	189	172	16
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar	-	129	109	130	123	12

* Except plates Nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 5

Results obtained with *S. typhimurium* TA98: main test 2

Plate No.	Addition	S9 mix + present - absent	Revertant colony counts* and means				
			A	B	C	Mean	sd
1+	None; S9 mix sterility check	+	0	0	0	0	0
1 -	None; buffer sterility check	-	0	0	0	0	0
2	Enzyme prep. from *Aspergillus niger* (ARO-1) (10000 µg/ml); sterility check	-	0	0	0	0	0
3	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	+	21	21	22	21	1
4	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	+	22	23	27	24	3
5	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	+	21	28	20	23	4
6	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	+	22	26	24	24	2
7	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	+	23	17	21	20	3
8	Purified water	+	29	24	31	28	4
9	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	-	22	19	26	22	4
10	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	-	23	21	22	22	1
11	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	-	19	19	26	21	4
12	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	-	19	20	20	20	1
13	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	-	14	22	24	20	5
14	Purified water	-	28	21	22	24	4
15	Benzo[a]pyrene (5 µg/plate)	+	169	183	166	173	9
16	2-Nitrofluorene (1 µg/plate)	-	244	233	194	224	26
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar	-	114	111	104	110	5

* Except plates Nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 6

Results obtained with *S. typhimurium* TA100: main test 1

Plate No.	Addition	S9 mix + present - absent	Revertant colony counts* and means				
			A	B	C	Mean	sd
1+	None; S9 mix sterility check	+	0	0	0	0	0
1 -	None; buffer sterility check	-	0	0	0	0	0
2	Enzyme prep. from *Aspergillus niger* (ARO-1) (10000 µg/ml); sterility check	-	0	0	0	0	0
3	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	+	75	86	93	85	9
4	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	+	97	116	89	101	14
5	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	+	95	88	86	90	5
6	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	+	89	72	82	81	9
7	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	+	109	86	88	94	13
8	Purified water	+	71	73	97	80	14
9	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	-	71	68	84	74	9
10	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	-	73	86	103	87	15
11	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	-	87	89	86	87	2
12	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	-	116	78	77	90	22
13	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	-	104	82	73	86	16
14	Purified water	-	81	96	115	97	17
15	Benzo[a]pyrene (5 µg/plate)	+	331	317	393	347	40
16	ENNG (3 µg/plate)	-	255	285	271	270	15
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar	-	128	116	123	122	6

* Except plates Nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 7

Results obtained with *S. typhimurium* TA100: main test 2

Plate No.	Addition	S9 mix + present - absent	Revertant colony counts* and means				
			A	B	C	Mean	sd
1+	None; S9 mix sterility check	+	0	0	0	0	0
1 -	None; buffer sterility check	-	0	0	0	0	0
2	Enzyme prep. from *Aspergillus niger* (ARO-1) (10000 µg/ml); sterility check	-	0	0	0	0	0
3	(10000 µg/ml)	+	68	82	85	78	9
4	Enzyme preparation (3000 µg/ml)	+	93	100	71	88	15
5	from *Aspergillus* (1000 µg/ml)	+	75	89	75	80	8
6	*niger* (ARO-1) (300 µg/ml)	+	68	88	71	76	11
7	(100 µg/ml)	+	101	71	71	81	17
8	Purified water	+	90	90	74	85	9
9	(10000 µg/ml)	-	66	90	79	78	12
10	Enzyme preparation (3000 µg/ml)	-	80	90	59	76	16
11	from *Aspergillus* (1000 µg/ml)	-	71	96	96	88	14
12	*niger* (ARO-1) (300 µg/ml)	-	96	90	67	84	15
13	(100 µg/ml)	-	95	64	71	77	16
14	Purified water	-	97	70	88	85	14
15	Benzo[a]pyrene (5 µg/plate)	+	300	270	350	307	40
16	ENNG (3 µg/plate)	-	350	321	365	345	22
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar	-	167	151	161	160	8

* Except plates Nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 8

Results obtained with *S. typhimurium* TA1535: main test 1

Plate No.	Addition	S9 mix + present - absent	Revertant colony counts* and means				
			A	B	C	Mean	sd
1+	None; S9 mix sterility check	+	0	0	0	0	0
1 -	None; buffer sterility check	-	0	0	0	0	0
2	Enzyme prep. from *Aspergillus niger* (ARO-1) (10000 µg/ml); sterility check	-	0	0	0	0	0
3	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	+	13	13	9	12	2
4	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	+	9	12	19	13	5
5	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	+	8	14	14	12	3
6	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	+	15	15	14	15	1
7	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	+	13	13	13	13	0
8	Purified water	+	14	17	12	14	3
9	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	-	13	9	14	12	3
10	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	-	17	9	10	12	4
11	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	-	13	10	10	11	2
12	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	-	13	16	14	14	2
13	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	-	14	12	12	13	1
14	Purified water	-	10	14	13	12	2
15	2-Aminoanthracene (2 µg/plate)	+	147	106	119	124	21
16	ENNG (5 µg/plate)	-	474	447	486	469	20
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar	-	165	147	138	150	14

* Except plates Nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 9

Results obtained with *S. typhimurium* TA1535: main test 2

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	Enzyme prep. from *Aspergillus niger* (ARO-1) (10000 µg/ml); sterility check		-	0	0	0	0	0
3		(10000 µg/ml)	+	17	17	16	17	1
4	Enzyme preparation	(3000 µg/ml)	+	26	22	16	21	5
5	from *Aspergillus*	(1000 µg/ml)	+	24	14	16	18	5
6	*niger* (ARO-1)	(300 µg/ml)	+	21	19	19	20	1
7		(100 µg/ml)	+	16	19	15	17	2
8	Purified water		+	21	15	22	19	4
9		(10000 µg/ml)	-	13	16	19	16	3
10	Enzyme preparation	(3000 µg/ml)	-	23	13	15	17	5
11	from *Aspergillus*	(1000 µg/ml)	-	21	10	20	17	6
12	*niger* (ARO-1)	(300 µg/ml)	-	20	17	20	19	2
13		(100 µg/ml)	-	13	17	15	15	2
14	Purified water		-	21	16	17	18	3
15	2-Aminoanthracene (2 µg/plate)		+	165	140	158	154	13
16	ENNG (5 µg/plate)		-	455	402	385	414	37
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	159	155	164	159	5

* Except plates Nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 10

Results obtained with *S. typhimurium* TA1537: main test 1

Plate No.	Addition	S9 mix + present - absent	Revertant colony counts* and means				
			A	B	C	Mean	sd
1+	None; S9 mix sterility check	+	0	0	0	0	0
1 -	None; buffer sterility check	-	0	0	0	0	0
2	Enzyme prep. from *Aspergillus niger* (ARO-1) (10000 µg/ml); sterility check	-	0	0	0	0	0
3	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	+	9	8	7	8	1
4	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	+	10	7	7	8	2
5	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	+	8	8	7	8	1
6	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	+	12	7	8	9	3
7	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	+	9	7	10	9	2
8	Purified water	+	10	7	7	8	2
9	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	-	7	3	8	6	3
10	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	-	5	8	12	8	4
11	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	-	14	9	7	10	4
12	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	-	8	6	9	8	2
13	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	-	7	8	13	9	3
14	Purified water	-	6	9	9	8	2
15	Benzo[a]pyrene (5 µg/plate)	+	94	117	114	108	13
16	9-Aminoacridine (80 µg/plate)	-	176	159	160	165	10
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar	-	133	124	157	138	17

* Except plates Nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 11

Results obtained with *S. typhimurium* TA1537: main test 2

Plate No.	Addition	S9 mix + present - absent	Revertant colony counts* and means				
			A	B	C	Mean	sd
1+	None; S9 mix sterility check	+	0	0	0	0	0
1 -	None; buffer sterility check	-	0	0	0	0	0
2	Enzyme prep. from *Aspergillus niger* (ARO-1) (10000 µg/ml); sterility check	-	0	0	0	0	0
3	(10000 µg/ml)	+	9	6	7	7	2
4	Enzyme preparation (3000 µg/ml)	+	7	12	8	9	3
5	from *Aspergillus* (1000 µg/ml)	+	9	10	6	8	2
6	*niger* (ARO-1) (300 µg/ml)	+	7	8	10	8	2
7	(100 µg/ml)	+	6	10	9	8	2
8	Purified water	+	9	12	10	10	2
9	(10000 µg/ml)	-	5	8	6	6	2
10	Enzyme preparation (3000 µg/ml)	-	12	6	6	8	3
11	from *Aspergillus* (1000 µg/ml)	-	8	10	9	9	1
12	*niger* (ARO-1) (300 µg/ml)	-	9	10	10	10	1
13	(100 µg/ml)	-	6	8	8	7	1
14	Purified water	-	6	10	9	8	2
15	Benzo[a]pyrene (5 µg/plate)	+	85	111	93	96	13
16	9-Aminoacridine (80 µg/plate)	-	136	122	102	120	17
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar	-	104	114	116	111	6

* Except plates Nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 12

Results obtained with *E.coli* CM891: main test 1

Plate No.	Addition	S9 mix + present - absent	Revertant colony counts* and means				
			A	B	C	Mean	sd
1+	None; S9 mix sterility check	+	0	0	0	0	0
1 -	None; buffer sterility check	-	0	0	0	0	0
2	Enzyme prep. from *Aspergillus niger* (ARO-1) (10000 µg/ml); sterility check	-	0	0	0	0	0
3	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	+	117	118	104	113	8
4	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	+	96	94	110	100	9
5	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	+	110	126	128	121	10
6	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	+	123	125	102	117	13
7	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	+	121	114	114	116	4
8	Purified water	+	107	126	95	109	16
9	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	-	95	118	122	112	15
10	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	-	115	122	112	116	5
11	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	-	100	123	97	107	14
12	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	-	88	99	102	96	7
13	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	-	111	116	88	105	15
14	Purified water	-	111	90	97	99	11
15	2-Aminoanthracene (10 µg/plate)	+	212	269	231	237	29
16	ENNG (2 µg/plate)	-	883	818	887	863	39
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar	-	126	129	165	140	22

* Except plates Nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 13

Results obtained with *E. coli* CM891: main test 2

Plate No.	Addition	S9 mix + present - absent	Revertant colony counts* and means				
			A	B	C	Mean	sd
1+	None; S9 mix sterility check	+	0	0	0	0	0
1 -	None; buffer sterility check	-	0	0	0	0	0
2	Enzyme prep. from *Aspergillus niger* (ARO-1) (10000 µg/ml); sterility check	-	0	0	0	0	0
3	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	+	110	88	109	102	12
4	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	+	90	114	115	106	14
5	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	+	123	129	106	119	12
6	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	+	114	89	109	104	13
7	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	+	123	104	107	111	10
8	Purified water	+	86	106	110	101	13
9	Enzyme preparation from *Aspergillus niger* (ARO-1) (10000 µg/ml)	-	111	85	81	92	16
10	Enzyme preparation from *Aspergillus niger* (ARO-1) (3000 µg/ml)	-	88	88	124	100	21
11	Enzyme preparation from *Aspergillus niger* (ARO-1) (1000 µg/ml)	-	81	118	117	105	21
12	Enzyme preparation from *Aspergillus niger* (ARO-1) (300 µg/ml)	-	125	103	102	110	13
13	Enzyme preparation from *Aspergillus niger* (ARO-1) (100 µg/ml)	-	104	71	89	88	17
14	Purified water	-	73	103	104	93	18
15	2-Aminoanthracene (10 µg/plate)	+	282	233	244	253	26
16	ENNG (2 µg/plate)	-	703	810	794	769	58
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar	-	151	145	147	148	3

* Except plates Nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

APPENDIX 1

Historical control data

Presented below are the historical control data from the period 1 April 1997 to 31 December 1998.

Purified water solvent controls

Strain	TA100		TA1535		CM891		TA98		TA1537	
S9 mix	-	+	-	+	-	+	-	+	-	+
Minimum	80	80	11	10	87	91	25	27	8	7
Maximum	89	91	13	12	102	102	29	30	10	10
Mean	84.8	87.0	12.0	11.4	95.6	96.3	27.3	28.7	8.6	8.2

Positive controls

Strain	TA100		TA1535		CM891		TA98		TA1537	
S9 mix	-	+	-	+	-	+	-	+	- (80 µg)	+
Minimum	265	273	455	97	480	209	97	109	165	103
Maximum	449	336	874	137	736	287	144	134	238	116
Mean	336.4	299.4	609.2	116.2	573.8	240.2	117.6	117.8	199.2	108.0

APPENDIX 2

Gist-brocades
BIOTECHNOLOGY CONTRIBUTING TO FOOD, HEALTH AND THE ENVIRONMENT

WATERINGSEWEG 1
PO BOX 1
2600 MA DELFT
THE NETHERLANDS
TELEPHONE (015)2799111
TELEFAX (015)2793482
TELEX 38103 GIBN NL
CABLES GISTBROCADES

CERTIFICATE OF ANALYSIS

name of the product	:	Enzyme preparation from *Aspergillus niger* ARO-1
batch no.	:	RER 710
GLP-archive no.	:	GLP-9708
status	:	ISO 9002
date of manufacture	:	July 1997
date of expiration	:	October 1999 (provisional)
active component(s)	:	pectinase, apiosidase, arabinofuranosidase, β-glucosidase, rhamnosidase
date of issue	:	22 February 1999

analysis type	method no.	result	specification
1 Pectinase activity	CQA 4 005 00	31,600 AVJP/g	record
2 β-D-glucosidase activity	CQA 4 046 00	1750 BDG/g	record
3 Apiosidase activity	R+D 4483 3/10/94	498 nK/g	record
4 Arabinofuranosidase activity	CQA 4 054 00	14,800 ARF/g	record
5 Rhamnosidase activity	CQA 4 106 00	84 RHU/g	record
6 Chlorogenase activity	CQA 4 135 00	14.8 µmol/h/g	record
7 Stability at 21°C during 48 hrs (50, 150 and 500 mg/ ml in water)	CQA 4 046 00	> 90%	record
8 Stability at 4°C during 15 days (50, 150 and 500 mg/ ml in water)	CQA 4 046 00	> 90%	record
9 Description	CQA 7 022 00	clear liquid	record
10 Colour	CQA 7 022 00	brown	record
11 Dry matter	60335	9.1% (w/w)	record
12 Ashes	60328	0.3% (w/w)	record
13 Total protein (N*6.25)	60055	5.1% (w/w)	record
14 Total carbohydrates	calculate	3.7% (w/w)	record
15 Total organic solids	SOP 869/E	8.8% (w/w)	record
16 Antifoam	W 0660.A	PEG: 15 mg/kg PPG: 46 mg/kg	record
17 Heavy metals (as Pb)	FCC IV	< 30 mg/kg	≤ 30 mg/kg

Study Manager

Release: yes / ~~no~~

(b) (6)

date: 22-02-1999

GIST-BROCADES B.V.
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DELFT NR 27704
ESTABLISHED AT DELFT

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TELEPHONE (015)2799111
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TELEX 38103 GBI NL
CABLES GISTBROCADES

CERTIFICATE OF ANALYSIS

name of the product	:	Enzyme preparation from *Aspergillus niger* ARO-1
batch no.	:	RER 710
GLP-archive no.	:	GLP-9708
status	:	ISO 9002
date of manufacture	:	July 1997
date of expiration	:	October 1999 (provisional)
active component(s)	:	pectinase, apiosidase, arabinofuranosidase, β-D-glucosidase, rhamnosidase
date of issue	:	22 February 1999

analysis type	method no.	result	specification
18 Lead	60401	< 5 mg/kg	≤ 5 mg/kg
19 Arsenic	61748	< 3 mg/kg	≤ 3 mg/kg
20 Cadmium	60988	< 0.5 mg/kg	≤ 0.5 mg/kg
21 Mercury	61748	< 0.5 mg/kg	≤ 0.5 mg/kg
22 Antimicrobial activity	69811	absent[1]	absent by test
23 Standard plate count	69814	< 5 CFU/g	< 10^4 CFU/g
24 Coliforms	69817	< 10 CFU/g	< 30 CFU/g
25 Salmonella	69825	absent/25 g	absent by test
26 Escherichia coli	69849	absent/25 g	absent by test
27 *Staphylococcus aureus*	69803	absent/g	absent by test
28 Aflatoxin B1	71 3360.00	absent by test	absent by test
29 T2 toxin	71 3361.00	absent by test	absent by test
30 Ochratoxin A	71 3362.00	absent by test	absent by test
31 Zearalenone	71 3363.00	absent by test	absent by test

Study Manager

Release: yes / no

(b) (6)

date: 22-02-1999

remark: [1] one with Ø = 18 mm

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Annex 10

Beta-glucosidase Chromosome Aberration Test

Gist-brocades

PO Box 1
2600 MA Delft
The Netherlands

rapportnummer: 15.749

datum: 23-6-1999

ENZYME PREPARATION FROM *ASPERGILLUS NIGER* (ARO-1) *IN VITRO* MAMMALIAN CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES

STUDY NR. GSB059/992952

auteur(s) : C.E. Mason
experimenteel werk: Huntingdon Life Sciences Ltd., Eye, Suffolk, IP23 7 PX, England

trefwoorden:
Toxicology
Mutagenicity
Chromosome-Aberration
in-vitro
Beta-D-Glucosidase
ARO-1
GLP
GLP-9708

verzendlijst

1. R. A. Hempenius 2. F.G. Honig 3. Archiefrapportage (2x)	

s.v.p. na gebruik retour R&D-archief

handtekening auteur Datum manuscript: 3-6-1999 Huntingdon Life Sciences Ltd., Eye, Suffolk, England	handtekening afd.chef

QUALITY ASSURANCE UNIT

QAU STATEMENT

Title report	: Enzyme preparation from *Aspergillus niger* (ARO-1) *In vitro* mammalian chromosome aberration test in human lymphocytes
Report date	: 4 June 1999
Submitted by	: Huntingdon Life Sciences Ltd. Eye Suffolk IP23 7PX England
Author/Study Director	: C.E. Mason
Study no.	: GSB059/992952

This report has not been checked by Gist-brocades's QAU.
Reliance was placed on the QUALITY ASSURANCE STATEMENT in report no. GSB059/992952, page 5, by H. Comb, Principal Auditor, Department of Quality Assurance, Huntingdon Life Sciences Ltd.

Quality Assurance Unit
Gist-Brocades BV

Date 17-6-1999

(b) (6)

F.G. Honig

(b) (6)

ENZYME PREPARATION FROM *ASPERGILLUS NIGER* (ARO-1)

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES

Sponsor

Gist-brocades BV,
Central Technology & Services,
Wateringseweg 1,
PO Box 1,
2600 MA Delft,
THE NETHERLANDS.

Research Laboratory

Huntingdon Life Sciences Ltd.,
Eye,
Suffolk,
IP23 7PX,
ENGLAND.

Report issued 3 June 1999

CONTENTS

CONTENTS (continued)

Page

APPENDICES

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid.

The United Kingdom Good Laboratory Practice Regulations 1997, Statutory Instrument No. 654.

EC Council Directive, 87/18/EEC of 18 December 1986, (No. L15/29) and from 1 May 1999, EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No. L77/8).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

In line with normal practice in this type of short-term study, the protocol did not require analysis of the dose form.

(b) (6)

....

3rd June 1999

C. E. Mason, B.Sc. (Hons.),
Study Director,
Department of Genetic Toxicology,
Huntingdon Life Sciences Ltd.

Date

QUALITY ASSURANCE STATEMENT

The following have been inspected or audited in relation to this study:

Study Phases Inspected	Date of Inspection	Date of Reporting
Protocol Audit	3 March 1999	3 March 1999
Process Based Inspections		
S-9 preparation	18 November 1998	19 November 1998
Culture establishment	4 January 1999	4 January 1999
Treatment	6 January 1999	7 January 1999
Harvesting and slide preparation	22 January 1999	22 January 1999
Slide scoring	9 February 1999	9 February 1999
Formulation	8 March 1999	8 March 1999
Report Audit	27 April 1999	27 April 1999

Protocol Audit: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

Process Based Inspections: At or about the time this study was in progress inspections of other routine and repetitive procedures employed on this type of study were carried out. These were promptly reported to appropriate Company Management

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results of this study to reflect the raw data.

(b) (6)

2 JUNE 1999

Helen Comb, B.Sc.,
Principal Auditor,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

Date

SUMMARY

A study was performed to assess the ability of Enzyme preparation from *Aspergillus niger* (ARO-1) to induce chromosomal aberrations in human lymphocytes cultured *in vitro.*

Human lymphocytes, in whole blood culture, were stimulated to divide by addition of phytohaemagglutinin, and exposed to the test substance both in the presence and absence of S9 mix derived from rat livers. Solvent and positive control cultures were also prepared. Two hours before the end of the incubation period, cell division was arrested using Colcemid®, the cells harvested and slides prepared, so that metaphase cells could be examined for chromosomal damage.

In order to assess the toxicity of Enzyme preparation from *Aspergillus niger* (ARO-1) to cultured human lymphocytes, the mitotic index was calculated for all cultures treated with the test substance and the solvent control. On the basis of these data, the following concentrations were selected for metaphase analysis:

First test

Without S9 mix - 3 hours treatment, 17 hours recovery: 1250, 2500 and 5000 µg/ml.

With S9 mix - 3 hours treatment, 17 hours recovery: 1250, 2500 and 5000 µg/ml.

Second test

Without S9 mix - 20 hours continuous treatment: 1000, 2000 and 3000 µg/ml.

With S9 mix - 3 hours treatment, 17 hours recovery: 1250, 2500 and 5000 µg/ml.

In both the absence and presence of S9 mix, Enzyme preparation from *Aspergillus niger* (ARO-1) caused no statistically significant increase in the proportion of metaphase figures containing chromosomal aberrations, at any dose level, when compared with the solvent control, in either test.

A quantitative analysis for polyploidy was made in cultures treated with the negative control and highest dose level. No increases in the proportion of polyploid cells were seen.

All positive control compounds caused large, statistically significant increases in the proportion of aberrant cells, demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

It is concluded that Enzyme preparation from *Aspergillus niger* (ARO-1) has shown no evidence of clastogenic activity in this *in vitro* cytogenetic test system.

INTRODUCTION

This report describes a study designed to assess the ability of Enzyme preparation from *Aspergillus niger* (ARO-1) to cause chromosomal aberrations in human lymphocytes cultured *in vitro*.

The study was conducted in compliance with the following guideline:

> OECD Guideline for the Testing of Chemicals. (1997) Genetic Toxicology: *In Vitro* Mammalian Chromosome Aberration Test, Guideline 473.

Human lymphocytes have been used in this type of study for a number of years (Evans and O'Riordan 1975, Scott, Dean, Danford and Kirkland 1990). They are cultured *in vitro* but do not divide unless stimulated to do so. This is achieved by adding phytohaemagglutinin (PHA) to the culture which results in a high mitotic yield (Nowell 1960).

In this study, blood taken from healthy male donors was pooled and diluted with tissue culture medium. The cultures were incubated in the presence of PHA before being treated with the test substance. Following treatment the cells were arrested at metaphase using the mitotic inhibitor, Colcemid®. Chromosomes in these metaphase cells were then examined for the presence of chromosome aberrations. The best estimate of the aberration frequency is at the first cell division after initiation of treatment since certain types of damage may be lost during subsequent cell divisions. In this laboratory the cell cycle time for human lymphocytes in whole blood culture is approximately 13-14 hours.

The study was performed on two separate occasions. In the first test, a three hour treatment was used in both the presence and the absence of S9 mix. In the second test, a continuous treatment was used without S9 mix, and the test with S9 mix was a repeat of the first test.

Aberrations were scored according to the classification of the ISCN (1985). Traditionally gaps have been excluded from the quantitation of chromosome aberrations. Some gaps, however, have been shown to be real discontinuities in DNA (Heddle and Bodycote 1970, Satya-Prakash, Hsu and Pathak 1981). In this study the total number of cells containing aberrations both with and without gaps has been calculated.

Many substances do not exert a mutagenic effect until they have been metabolised by enzyme systems that are not found in cultured cells. Therefore the cultures and test substance were incubated in both the absence and presence of a supplemented liver fraction (S9 mix) prepared from rats previously treated with a substance (Aroclor 1254) known to induce a high level of enzymic activity (Maron and Ames 1983, Natarajan *et al.* 1976).

The protocol was approved by Huntingdon Life Sciences Management on 24 February 1998, by the Sponsor on 25 February 1998 and by the Study Director on 1 March 1999.

The experimental phase of the study was conducted between 2 March 1999 and 6 April 1999.

TEST SUBSTANCE

Identity:	Enzyme preparation from *Aspergillus niger* (ARO-1)
Appearance:	Brown liquid
Storage conditions:	*ca* -20°C
Batch number:	RER 710
Expiry:	October 1999 (provisional)
Dry matter:	9.1% (w/w)
Date received:	23 December 1998

EXPERIMENTAL PROCEDURE

CULTURE OF LYMPHOCYTES

Human blood was collected aseptically from healthy male donors, pooled and diluted with RPMI 1640 tissue culture medium (Life Technologies) supplemented with 10% foetal calf serum (Globepharm), 1 unit/ml Heparin (CP Pharmaceuticals Ltd.), 20 I.U./ml penicillin/20 µg/ml streptomycin (Imperial) and 2.0 mM glutamine (Imperial). Aliquots (0.4 ml blood: 4.5 ml medium: 0.1 ml phytohaemagglutinin (Gibco) of the cell suspension were placed in sterile universal containers and incubated at 37°C in for approximately 48 hours. The cultures were gently shaken daily to resuspend the cells.

POSITIVE CONTROLS

In the absence of S9 mix

Identity:	Mitomycin C
Supplier:	Sigma Chemical Co Ltd
Appearance:	Blue powder
Batch number:	68H2521
Solvent:	Sterile purified water
Final concentration:	0.1 µg/ml

In the presence of S9 mix

Identity:	Cyclophosphamide
Supplier:	Asta Medica Ltd
Appearance:	White powder
Batch number:	6093491
Solvent:	Sterile purified water
Final concentration:	6 µg/ml

PREPARATION OF S9 FRACTION

Species:	Rat
Sex:	Male
Strain:	Sprague-Dawley derived
Source:	Charles River UK
Age:	7 - 8 weeks
Weight:	<300 g

S9 fraction was prepared from a group of *ca.* 10 animals. Mixed function oxidase systems in the rat livers were stimulated by Aroclor 1254, administered as a single intraperitoneal injection in corn oil at a dosage of 500 mg/kg bodyweight. On the fifth day after injection, following an overnight starvation, the rats were killed and their livers aseptically removed.

The following steps were carried out at 0 - 4°C under aseptic conditions. The livers were placed in 0.15 M KCl (3 ml KCl: 1 g liver) before being transferred to a homogeniser. Following preparation, the homogenates were centrifuged at 9000 *g* for 10 minutes. The supernatant fraction (S9 fraction) was dispensed into aliquots and stored at -80°C or below until required.

PREPARATION OF S9 MIX

S9 mix contained: S9 fraction (10% v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium orthophosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADP (4 mM). All the cofactors were filter-sterilised before use.

SELECTION OF SOLVENT AND FORMULATION OF TEST SUBSTANCE

Information supplied by the study sponsor indicated that the purity of the active ingredient (expressed in terms of % dry matter) was 9.1%, i.e. the active ingredient was present in the material supplied at a concentration of 91 mg/ml. Enzyme preparation from *Aspergillus niger* (ARO-1) was diluted to 50 mg/ml in culture medium, and serial dilutions were freshly prepared in culture medium before addition to the cultures.

TREATMENT OF CELLS WITH TEST SUBSTANCE - FIRST TEST

After approximately 48 hours, 500 µl aliquots of Enzyme preparation from *Aspergillus niger* (ARO-1) were added to one set of duplicate cultures to give final concentrations of 39.1, 78.1, 156.3, 312.5, 625, 1250, 2500 and 5000 µg/ml. Culture medium, the solvent control, in 500 µl aliquots, was added to two cultures. Mitomycin C, at a final concentration of 0.1 µg/ml, was added to duplicate cultures.

Immediately before treatment of the second set of cultures, 1 ml of medium was removed from each culture and discarded. This was replaced with 1 ml of S9 mix, followed by 500 µl aliquots of the various dilutions of Enzyme preparation from *Aspergillus niger* (ARO-1), giving the same series of final concentrations as above. Culture medium (500 µl) was added to two cultures. Cyclophosphamide was added to duplicate cultures at a final concentration of 6 µg/ml.

Three hours after dosing, the cultures were centrifuged at 1400 rpm for 5 minutes and the cell pellets resuspended in fresh medium. They were then incubated for a further 17 hours.

HARVESTING AND FIXATION

Two hours before the cells were harvested, mitotic activity was arrested by addition of Colcemid® (Sigma) to each culture at a final concentration of 0.1 µg/ml. After 2 hours incubation, each cell suspension was transferred to a conical centrifuge tube and centrifuged for 5 minutes at 1400 r.p.m. The cell pellets were treated with a hypotonic solution (0.075M KCl prewarmed at 37°C). After a 10 minute period of hypotonic incubation at 37°C, the suspensions were centrifuged at 1400 r.p.m. for 5 minutes and the cell pellets fixed by addition of freshly prepared cold fixative (3 parts methanol : 1 part glacial acetic acid). The fixative was replaced several times.

SLIDE PREPARATION

The pellets were resuspended, then centrifuged at 1400 r.p.m. for 5 minutes and finally resuspended in a small volume of fresh fixative. A few drops of the cell suspensions were dropped onto pre-cleaned microscope slides which were then allowed to air-dry. The slides were then stained in 10% Giemsa, prepared in buffered water (pH 6.8). After rinsing in buffered water the slides were left to air-dry and then mounted in DPX.

MICROSCOPIC EXAMINATION

The prepared slides were examined by light microscopy using a low power objective. The proportion of mitotic cells per 1000 cells in each culture was recorded except for positive control treated cultures. From these results the dose level causing a decrease in mitotic index of approximately 50% of the solvent control value or, if there was no decrease, the maximum achievable concentration was used as the highest dose level for the metaphase analysis. The intermediate and low dose levels were also selected.

The selected slides were then coded. Metaphase cells were identified using a low power objective and examined at a magnification of x1000 using an oil immersion objective. One hundred metaphase figures were examined, where possible, from each culture. Chromosome aberrations were scored according to the classification of the ISCN (1985). Only cells with 44 - 48 chromosomes were analysed. Polyploid and endoreduplicated cells were noted when seen. The vernier readings of all aberrant metaphase figures were recorded.

The incidence of polyploid metaphase cells, out of 500 metaphase cells, was determined quantitatively for negative control cultures and cultures treated with the highest dose level of the test substance used in the analysis for chromosomal aberrations.

The number of aberrant metaphase cells in each treatment group was compared with the solvent control value using Fisher's test (Fisher 1973).

SECOND TEST

Cultures were initiated and maintained as previously described. In this second test a continuous treatment was used in the absence of S9 mix. In the presence of S9 mix, a three hour treatment was used, as in the first test. The harvest time was at 20 hours for both parts of the test. Concentrations of Enzyme preparation from *Aspergillus niger* (ARO-1) were as follows:

Without S9 mix:	250, 500, 750, 1000, 2000, 3000, 4000 and 5000 µg/ml.
With S9 mix:	312.5, 625, 1250, 2500 and 5000 µg/ml.

Duplicate cultures were used for each treatment and two cultures were treated with the solvent control. Positive control cultures were treated as in the first test.

Three hours after dosing, the cultures containing S9 mix were centrifuged and the cell pellets resuspended in fresh medium. They were then incubated for a further 17 hours. Cultures treated in the absence of S9 mix were incubated for 20 hours.

All cultures were treated with Colcemid®, at a final concentration of 0.1 µg/ml, two hours before the end of the incubation period. They were then harvested, fixed and the slides prepared as previously described. The slides were then examined microscopically as previously described.

STABILITY AND FORMULATION ANALYSIS

The stability of the test substance and of the test substance in the solvent were not determined as part of this study. Analysis of achieved concentration was not performed as part of this study.

ASSESSMENT OF RESULTS

An assay is considered to be acceptable if the negative and positive control values lie within the current historical control range.

The test substance is considered to cause a positive response if the following conditions are met:

Statistically significant increases ($P<0.01$) in the frequency of metaphases with aberrant chromosomes (excluding gaps) are observed at one or more test concentration.

The increases exceed the negative control range of this laboratory, taken at the 99% confidence limit.

The increases are reproducible between replicate cultures.

The increases are not associated with large changes in osmolality of the treatment medium or extreme toxicity.

Evidence of a dose-relationship is considered to support the conclusion.

A negative response is claimed if no statistically significant increases in the number of aberrant cells above concurrent control frequencies are observed, at any dose level.

A further evaluation may be carried out if the above criteria for a positive or a negative response are not met.

MAINTENANCE OF RECORDS

All experimental data arising from the study (including documentary raw data, specimens, records and other materials; collectively defined as the "materials") will remain the property of the Sponsor.

Huntingdon Life Sciences shall retain the materials in its archive for a period of five years from the date of issue of the final report. After such time, the Sponsor will be contacted and their advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials, subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences shall also retain a copy of the final report in its archive indefinitely.

RESULTS

FIRST TEST

Toxicity data

Mitotic indices of cultured human lymphocytes treated with Enzyme preparation from *Aspergillus niger* (ARO-1) are shown in Table 2.

In the absence of S9 mix, Enzyme preparation from *Aspergillus niger* (ARO-1) caused a reduction in the mitotic index to 91 % of the solvent control value at 5000 µg/ml. The dose levels selected for the metaphase analysis were 1250, 2500 and 5000 µg/ml.

In the presence of S9 mix, Enzyme preparation from *Aspergillus niger* (ARO-1) did not cause significant reductions in the mitotic index at any concentration tested. The dose levels selected for the metaphase analysis were 1250, 2500 and 5000 µg/ml.

The quantitative analysis for polyploidy showed no increase in the number of polyploid metaphase figures when compared to the solvent control.

Metaphase analysis

The effects of Enzyme preparation from *Aspergillus niger* (ARO-1) on the chromosomes of cultured human lymphocytes are shown in Table 3 and summarised in Table 1.

In both the absence and the presence of S9 mix, Enzyme preparation from *Aspergillus niger* (ARO-1) caused no statistically significant increases in the proportion of cells with chromosomal aberrations at any dose level, when compared with the solvent control.

Both positive control compounds, mitomycin C and cyclophosphamide, caused large, statistically significant increases ($P<0.001$) in the proportion of aberrant cells. This demonstrated the efficacy of the S9 mix and the sensitivity of the test system.

SECOND TEST

Toxicity data

Mitotic indices of cultured human lymphocytes treated with Enzyme preparation from *Aspergillus niger* (ARO-1) are shown in Table 4.

In the absence of S9 mix, Enzyme preparation from *Aspergillus niger* (ARO-1) caused a reduction in the mitotic index to 52 % of the solvent control value at 3000 µg/ml. The dose levels selected for the metaphase analysis were 1000, 2000 and 3000 µg/ml.

In the presence of S9 mix, Enzyme preparation from *Aspergillus niger* (ARO-1) did not cause significant reductions in the mitotic index at any concentration tested. The dose levels selected for the metaphase analysis were 1250, 2500 and 5000 µg/ml.

The quantitative analysis for polyploidy showed no increase in the number of polyploid metaphase cells when compared to the solvent control.

Metaphase analysis

The effects of Enzyme preparation from *Aspergillus niger* (ARO-1) on the chromosomes of cultured human lymphocytes are shown in Table 5 and summarised in Table 1.

In both the absence and the presence of S9 mix, Enzyme preparation from *Aspergillus niger* (ARO-1) caused no statistically significant increases in the proportion of cells with chromosomal aberrations at any dose level, when compared with the solvent control.

Both positive control compounds, mitomycin C and cyclophosphamide, caused large, statistically significant increases ($P<0.001$) in the proportion of aberrant cells.

CONCLUSION

It is concluded that Enzyme preparation from *Aspergillus niger* (ARO-1) has shown no evidence of clastogenic activity in this *in vitro* cytogenetic test system.

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TABLE 1

Summary of Results

Test 1

Exposure period (hours)	S9 mix	Concentration of *Aspergillus niger* (ARO-1) (µg/ml)	Cells with aberrations Excluding gaps			Cells with aberrations Including gaps			Relative Mitotic Index (%)
			Individual values (%)		Mean (%)	Individual values (%)		Mean (%)	
3	-	0 (Culture medium)	0	1	0.5	0	2	1.0	100
		1250	0	1	0.5	2	2	2.0	98
		2500	0	2	1.0	1	2	1.5	98
		5000	2	2	2.0	3	3	3.0	91
		0.1 (Mitomycin C)	21	14	17.5***	22	16	19.0***	-
3	+	0 (Culture medium)	0	1	0.5	1	1	1.0	100
		1250	0	1	0.5	1	1	1.0	190
		2500	0	0	0.0	0	0	0.0	173
		5000	0	2	1.0	0	2	1.0	169
		6 (Cyclophosphamide)	17	13	15.0***	21	22	21.5***	-

Test 2

Exposure period (hours)	S9 mix	Concentration of *Aspergillus niger* (ARO-1) (µg/ml)	Cells with aberrations Excluding gaps			Cells with aberrations Including gaps			Relative Mitotic Index (%)
			Individual values (%)		Mean (%)	Individual values (%)		Mean (%)	
20	-	0 (Culture medium)	0	0	0.0	2	3	2.5	100
		1000	1	1	1.0	5	5	5.0	77
		2000	1	2	1.5	6	8	7.0	63
		3000	0	2	1.0	5	5	5.0	52
		0.1 (Mitomycin C)	26	28	27.0***	31	34	32.5***	-
3	+	0 (Culture medium)	0	0	0.0	0	2	1.0	100
		1250	0	0	0.0	2	0	1.0	146
		2500	0	0	0.0	2	0	1.0	146
		5000	1	0	0.5	2	2	2.0	88
		6 (Cyclophosphamide)	20	19	19.5***	25	23	24.0***	-

*** P<0.001
** P<0.01
Otherwise P>0.01

Aspergillus niger (ARO-1) - Enzyme preparation from *Aspergillus niger* (ARO-1)

TABLE 2

Mitotic index data - first test

Without S9 mix, 3 hours treatment and 17 hours recovery

Concentration of *Aspergillus niger*(ARO-1) (µg/ml)	Mitotic index		Relative mitotic index (%)	Polyploidy	
	Incidence	% Mean		Incidence	% Mean
0 (Culture medium)	109/1000 133/1000	12.1	100	0/500 1/500	0.1
39.1	112/1000 119/1000	11.6	96		
78.1	119/1000 108/1000	11.4	94		
156.3	122/1000 104/1000	11.3	93		
312.5	105/1000 132/1000	11.9	98		
625	103/1000 125/1000	11.4	94		
1250	122/1000 113/1000	11.8	98		
2500	105/1000 131/1000	11.8	98		
5000	91/1000 129/1000	11.0	91	0/500 0/500	0.0

Aspergillus niger (ARO-1) - Enzyme preparation from *Aspergillus niger* (ARO-1)

TABLE 2

Mitotic index data - first test (continued)

With S9 mix, 3 hours treatment and 17 hours recovery

Concentration of *Aspergillus niger*(ARO-1) (µg/ml)	Mitotic index		Relative mitotic index (%)	Polyploidy	
	Incidence	% Mean		Incidence	% Mean
0 (Culture medium)	53/1000 48/1000	5.1	100	1/500 0/500	0.1
39.1	61/1000 61/1000	6.1	120		
78.1	81/1000 55/1000	6.8	133		
156.3	53/1000 62/1000	5.8	114		
312.5	89/1000 75/1000	8.2	161		
625	88/1000 84/1000	8.6	169		
1250	103/1000 91/1000	9.7	190		
2500	79/1000 96/1000	8.8	173		
5000	76/1000 96/1000	8.6	169	1/500 1/500	0.2

Aspergillus niger (ARO-1) - Enzyme preparation from *Aspergillus niger* (ARO-1)

TABLE 3

Metaphase analysis data - first test

Without S9 mix, 3 hours treatment and 17 hours recovery

Concentration of *Aspergillus niger* (ARO-1) (µg/ml)	No. cells examined	Aberrations						No. Of aberrant cells				Relative MI %
		Chromatid type		Chromosome type		Others	Gaps	Exc. gaps	Mean %	Inc. gaps	Mean %	
		ctb	cte	csb	cse		ctg csg					
0	100							0	0.5	0	1.0	100
(Culture medium)	100	1					2	1		2		
1250	100						2	0	0.5	2	2.0	98
	100	1					1	1		2		
2500	100						1	0	1.0	1	1.5	98
	100	2						2		2		
5000	100	2					1	2	2.0	3	3.0	91
	100	1		1			1	2		3		
0.1	100	17	2	3			2	21	17.5	22	19.0	-
Mitomycin C	100	12	1	5			2 1	14	***	16	***	

ctb	Chromatid break	cte	Chromatid exchange
csb	Chromosome break	cse	Chromosome exchange
ctg	Chromatid gap	csg	Chromosome gap
		others	Cells with greater than 8 aberrations, pulverised cells and pulverised chromosomes

***	P<0.001
**	P<0.01
Otherwise	P>0.01

Aspergillus niger (ARO-1) - Enzyme preparation from *Aspergillus niger* (ARO-1)

TABLE 3

Metaphase analysis data - first test (continued)

With S9 mix, 3 hours treatment and 17 hours recovery

Concentration of *Aspergillus niger* (ARO-1) (µg/ml)	No. cells examined	Aberrations				No. Of aberrant cells				Relative MI %
		Chromatid type		Chromosome type		Others	Gaps	Exc. gaps	Mean %	Inc. gaps	Mean %	
		ctb	cte	csb	cse		ctg csg					
0	100						1	0	0.5	1	1.0	100
(Culture medium)	100	1						1		1		
1250	100						1	0	0.5	1	1.0	190
	100			1				1		1		
2500	100							0	0.0	0	0.0	173
	100							0		0		
5000	100							0	1.0	0	1.0	169
	100	1		1				2		2		
6	100	19		1			6	17	15.0	21	21.5	-
Cyclophosphamide	100	14	1				10	13	***	22	***	

ctb	Chromatid break	cte	Chromatid exchange
csb	Chromosome break	cse	Chromosome exchange
ctg	Chromatid gap	csg	Chromosome gap
		others	Cells with greater than 8 aberrations, pulverised cells and pulverised chromosomes

***	P<0.001
**	P<0.01
Otherwise	P>0.01

Aspergillus niger (ARO-1) - Enzyme preparation from *Aspergillus niger* (ARO-1)

TABLE 4

Mitotic index data - second test

Without S9 mix, 20 hours continuous treatment

Concentration of *Aspergillus niger*(ARO-1) (µg/ml)	Mitotic index		Relative mitotic index (%)	Polyploidy	
	Incidence	% Mean		Incidence	% Mean
0 (Culture medium)	106/1000 118/1000	11.2	100	1/500 0/500	0.1
250	103/1000 103/1000	10.3	92		
500	89/1000 93/1000	9.1	81		
750	100/1000 99/1000	10.0	89		
1000	89/1000 83/1000	8.6	77		
2000	73/1000 69/1000	7.1	63		
3000	55/1000 60/1000	5.8	52	0/500 1/500	0.1
4000	28/1000 27/1000	2.8	25		
5000	15/1000 13/1000	1.4	13		

Aspergillus niger (ARO-1) - Enzyme preparation from *Aspergillus niger* (ARO-1)

TABLE 4

Mitotic index data - second test (continued)

With S9 mix, 3 hours treatment and 17 hours recovery

Concentration of *Aspergillus niger*(ARO-1) (µg/ml)	Mitotic index		Relative mitotic index (%)	Polyploidy	
	Incidence	% Mean		Incidence	% Mean
0 (Culture medium)	86/1000 65/1000	7.6	100	0/500 0/500	0.0
312.5	101/1000 111/1000	10.6	139		
625	122/1000 112/1000	11.7	154		
1250	110/1000 111/1000	11.1	146		
2500	114/1000 107/1000	11.1	146		
5000	72/1000 61/1000	6.7	88	1/500 2/500	0.3

Aspergillus niger (ARO-1) - Enzyme preparation from *Aspergillus niger* (ARO-1)

TABLE 5

Metaphase analysis data - second test

Without S9 mix, 20 hours continuous treatment

Concentration of *Aspergillus niger* (ARO-1) (µg/ml)	No. cells examined	Aberrations						No. Of aberrant cells				Relative MI %
		Chromatid type		Chromosome type		Others	Gaps	Exc. gaps	Mean %	Inc. gaps	Mean %	
		ctb	cte	csb	cse		ctg csg					
0	100						2	0	0.0	2	2.5	100
(Culture medium)	100						3	0		3		
1000	100	1					4	1	1.0	5	5.0	77
	100	1					3 1	1		5		
2000	100		1	1			6	1	1.5	6	7.0	63
	100	2					6	2		8		
3000	100						5	0	1.0	5	5.0	52
	100	1		1			4	2		5		
0.1	100	21	4	7			8	26	27.0	31	32.5	-
Mitomycin C	100	25	8	6			10 1	28	***	34	***	

ctb	Chromatid break	cte	Chromatid exchange
csb	Chromosome break	cse	Chromosome exchange
ctg	Chromatid gap	csg	Chromosome gap
		others	Cells with greater than 8 aberrations, pulverised cells and pulverised chromosomes

***	P<0.001
**	P<0.01
Otherwise	P>0.01

Aspergillus niger (ARO-1) - Enzyme preparation from *Aspergillus niger* (ARO-1)

TABLE 5

Metaphase analysis data - second test (continued)

With S9 mix, 3 hours treatment and 17 hours recovery

Concentration of *Aspergillus niger* (ARO-1) (µg/ml)	No. cells examined	Aberrations				No. of aberrant cells				Relative MI %
		Chromatid type		Chromosome type			Gaps	Exc. Gaps	Mean %	Inc. gaps	Mean %	
		ctb	cte	csb	cse		ctg csg					
0	100							0	0.0	0	1.0	100
(Culture medium)	100						2	0		2		
1250	100						2	0	0.0	2	1.0	146
	100							0		0		
2500	100						2	0	0.0	2	1.0	146
	100							0		0		
5000	100	1					1	1	0.5	2	2.0	88
	100						2	0		2		
6	100	19	1	2			11	20	19.5	25	24.0	-
Cyclophosphamide	100	22					7	19	***	23	***	

ctb	Chromatid break	cte	Chromatid exchange
csb	Chromosome break	cse	Chromosome exchange
ctg	Chromatid gap	csg	Chromosome gap
		others	Cells with greater than 8 aberrations, pulverised cells and pulverised chromosomes

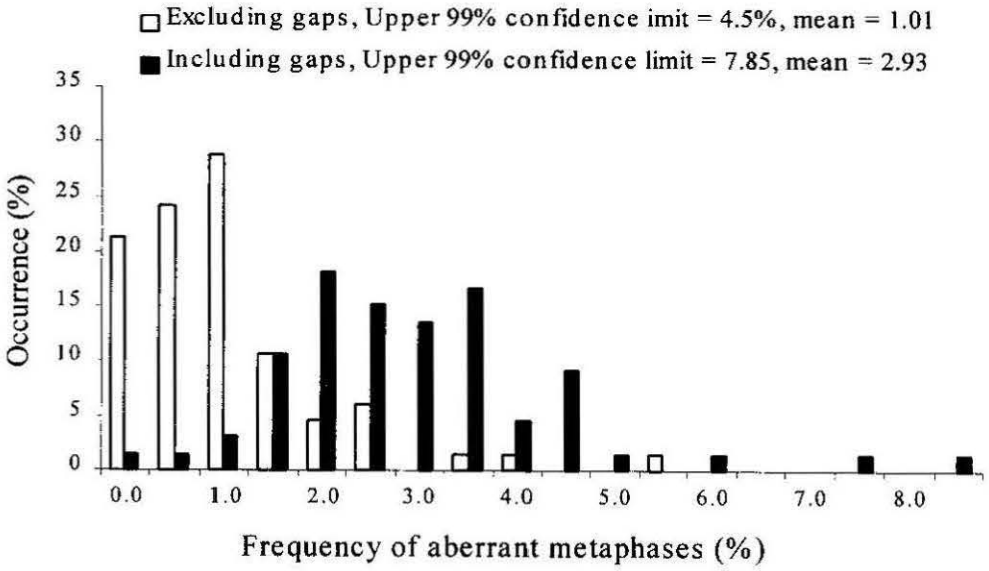
***	P<0.001
**	P<0.01
Otherwise	P>0.01

Aspergillus niger (ARO-1) - Enzyme preparation from *Aspergillus niger* (ARO-1)

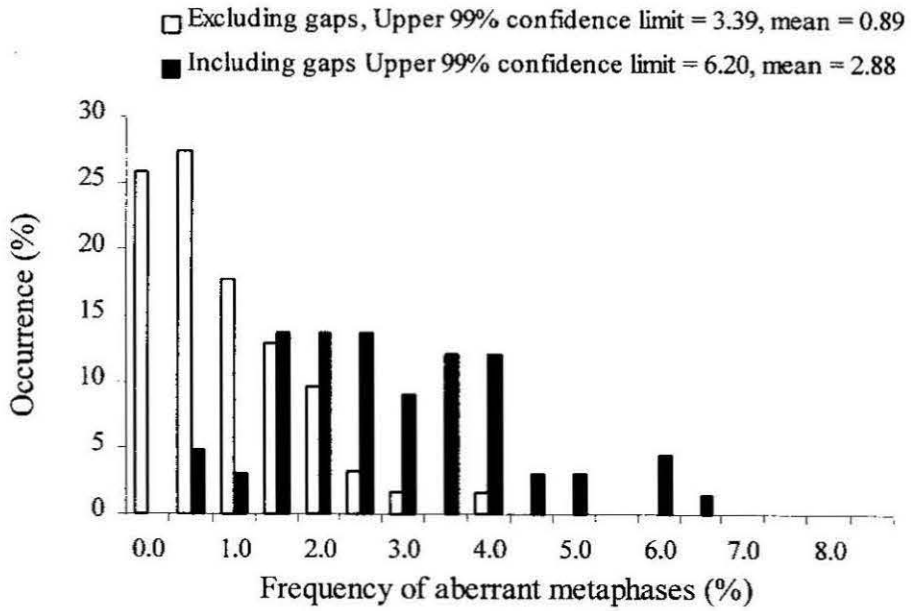
APPENDIX 1

Historical negative control data (January 1997 - December 1998)

Without S9 mix



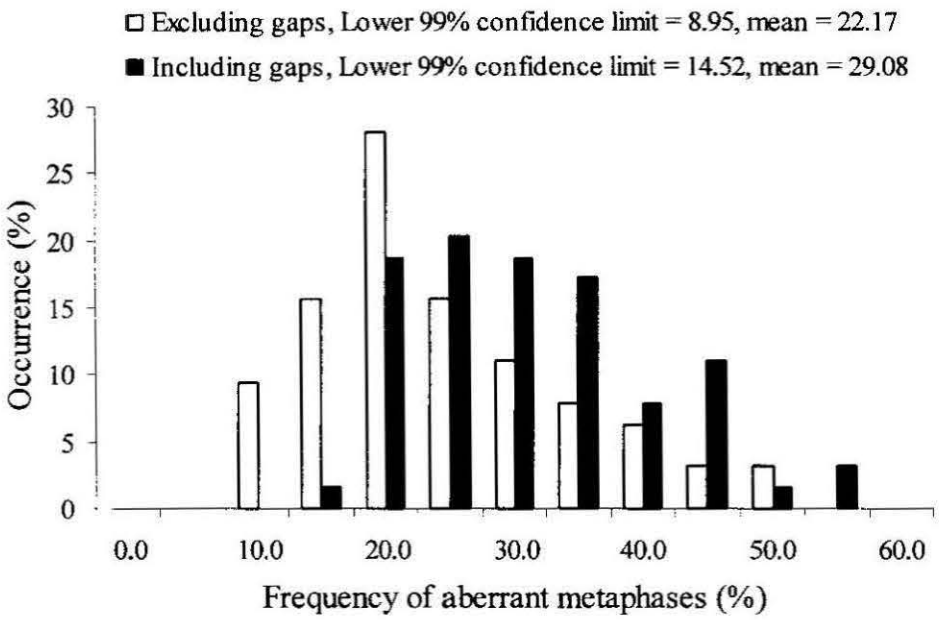
With S9 mix



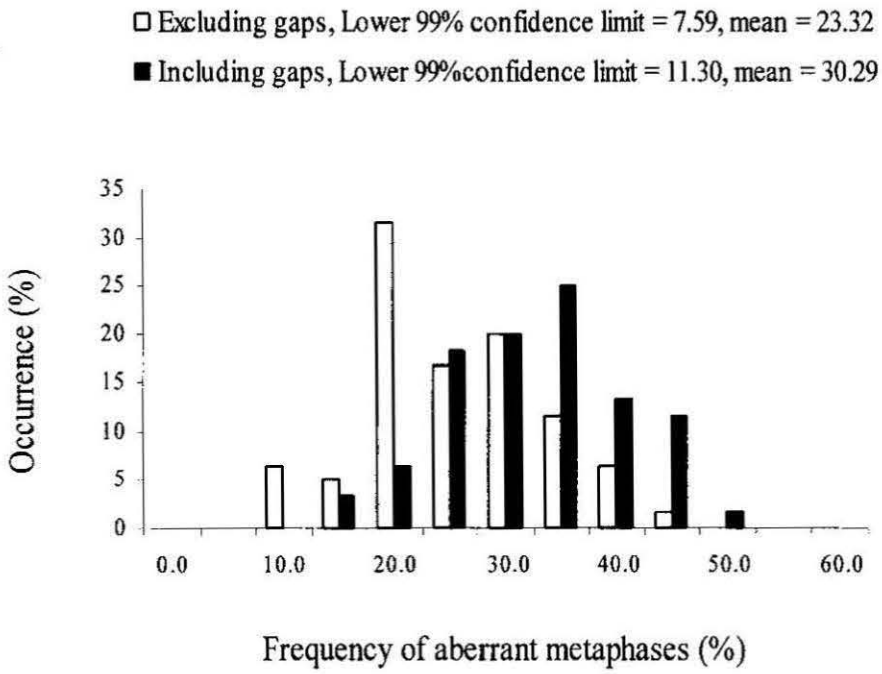
APPENDIX 2

Historical positive control data (January 1997 – December 1998)

Without S9 mix



With S9 mix



APPENDIX 3

Gist-brocades
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CERTIFICATE OF ANALYSIS

name of the product	:	Enzyme preparation from *Aspergillus niger* ARO-1
batch no.	:	RER 710
GLP-archive no.	:	GLP-9708
status	:	ISO 9002
date of manufacture	:	July 1997
date of expiration	:	October 1999 (provisional)
active component(s)	:	pectinase, apiosidase, arabinofuranosidase, β-glucosidase, rhamnosidase
date of issue	:	22 February 1999

analysis type	method no.	result	specification
1 Pectinase activity	CQA 4 005 00	31,600 AVJP/g	record
2 β-D-glucosidase activity	CQA 4 046 00	1750 BDG/g	record
3 Apiosidase activity	R+D 4483 3/10/94	498 nK/g	record
4 Arabinofuranosidase activity	CQA 4 054 00	14,800 ARF/g	record
5 Rhamnosidase activity	CQA 4 106 00	84 RHU/g	record
6 Chlorogenase activity	CQA 4 135 00	14.8 µmol/h/g	record
7 Stability at 21°C during 48 hrs (50, 150 and 500 mg/ ml in water)	CQA 4 046 00	> 90%	record
8 Stability at 4°C during 15 days (50, 150 and 500 mg/ ml in water)	CQA 4 046 00	> 90%	record
9 Description	CQA 7 022 00	clear liquid	record
10 Colour	CQA 7 022 00	brown	record
11 Dry matter	60335	9.1% (w/w)	record
12 Ashes	60328	0.3% (w/w)	record
13 Total protein (N*6.25)	60055	5.1% (w/w)	record
14 Total carbohydrates	calculate	3.7% (w/w)	record
15 Total organic solids	SOP 869/E	8.8% (w/w)	record
16 Antifoam	W 0660.A	PEG: 15 mg/kg PPG: 46 mg/kg	record
17 Heavy metals (as Pb)	FCC IV	< 30 mg/kg	≤ 30 mg/kg

Study Manager

Release: ~~yes~~ / ~~no~~

(b) (6)

date: 22-02-1999

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CERTIFICATE OF ANALYSIS

name of the product	:	Enzyme preparation from *Aspergillus niger* ARO-1
batch no.	:	RER 710
GLP-archive no.	:	GLP-9708
status	:	ISO 9002
date of manufacture	:	July 1997
date of expiration	:	October 1999 (provisional)
active component(s)	:	pectinase, apiosidase, arabinofuranosidase, β-D-glucosidase, rhamnosidase
date of issue	:	22 February 1999

analysis type	method no.	result	specification
18 Lead	60401	< 5 mg/kg	≤ 5 mg/kg
19 Arsenic	61748	< 3 mg/kg	≤ 3 mg/kg
20 Cadmium	60988	< 0.5 mg/kg	≤ 0.5 mg/kg
21 Mercury	61748	< 0.5 mg/kg	≤ 0.5 mg/kg
22 Antimicrobial activity	69811	absent[1]	absent by test
23 Standard plate count	69814	< 5 CFU/g	< 10^4 CFU/g
24 Coliforms	69817	< 10 CFU/g	< 30 CFU/g
25 Salmonella	69825	absent/25 g	absent by test
26 Escherichia coli	69849	absent/25 g	absent by test
27 *Staphylococcus aureus*	69803	absent/g	absent by test
28 Aflatoxin B1	7I 3360.00	absent by test	absent by test
29 T2 toxin	7I 3361.00	absent by test	absent by test
30 Ochratoxin A	7I 3362.00	absent by test	absent by test
31 Zearalenone	7I 3363.00	absent by test	absent by test

Study Manager

remark: [1] one with Ø = 18 mm

Release: yes / no

(b) (6)

date: 22-02-1999

Viebrock, Lauren

From: Yingling, Gary L. <gary.yingling@morganlewis.com>
Sent: Friday, June 01, 2018 3:18 PM
To: Viebrock, Lauren
Cc: Vaughn, Jessica L.; Montaguti, Paola
Subject: FW: Response to GRN 750
Attachments: Letter to Lauren VieBrock re GRN 750 Response to FDA Questions Re DSM with Attachments.pdf

Lauren: Attached you will find a letter with the FDA questions, DSM's responses and attachments. Please do not hesitate to contact me if you have any questions. gary

Gary L. Yingling
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June 1, 2018

VIA ELECTRONIC MAIL

Lauren VieBrock, Ph.D.
Consumer Safety Officer/Microbiology Reviewer
US Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Biotechnology and GRAS Notice Review
5001 Campus Drive
College Park, MD 20740

Re: GRN 750: Response to FDA Request for Additional Information

Dear Dr. VieBrock:

On behalf of our client, DSM Food Specialties ("DSM"), we submit this response to questions raised by the U.S. Food and Drug Administration ("FDA" or "Agency") in an email on May 17, 2018, regarding GRAS Notification 750 for the use of beta-glucosidase in brewing of fermented beverages such as beer. We have included FDA's comment in bold italics, followed by the DSM response.

1. ***Part 1.8 "Exemptions from FOIA Disclosure" states that the notice does not contain any confidential information. However, information in Annex 7 (pages 97 and 185 of the PDF), Annex 8 (pages 212 and 393), Annex 9 (page 429), and Annex 10 (page 465) are labeled as confidential. Please clarify whether you consider this information to be confidential***.

DSM does not consider these annexes to be confidential and it was not DSM's intent to label these as confidential. Further review of the annexes reveal that only a select number of pages in each of the listed annexes were marked confidential by the test labs that prepared the reports. The pages from each of the annexes that were marked confidential are being resubmitted as part of this response, with the confidential label removed. These pages can be found in Appendix 1, separated by their corresponding annexes.

2. ***Please address the following regarding beta-glucosidase amino acid sequence:***
    a. ***On page 27 of the notice you state that the exact amino acid sequence of the beta-glucosidase was not determined. Please confirm that the sequence analyses (for homology to know allergens and toxin, etc.) were performed using the amino acid sequence of the beta-glucosidase***

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from A. niger that is publicly available, and not the sequence of the beta-glucosidase described in GRN 750. Please provide rationale for using the published sequence.

b. Please confirm that homology of the beta-glucosidase amino acid sequence to known toxins has been assessed (i.e. using UNIPROT or Toxic Exposure Databases). If significant homologies were identified, please indicate your reasoning as to why they do not raise safety concerns.

Beta-glucosidase from *Aspergillus niger* (ARO) is a "classical" (non-recombinant) enzyme, and therefore no characterization at the molecular level has been performed on this enzyme (neither genome sequencing, targeted gene sequencing, nor mass spectrometric analysis of the amino acid sequence of the protein). Based on the taxonomic identification (*Aspergillus niger* v. Tieghem), we have selected a representative *Aspergillus niger* beta-glucosidase sequence deposited in the public sequence databases for the *in silico* allergenicity screening (see Annex 6 of the dossier).

Beta-glucosidases from *Aspergillus niger* have a defined length of 860 amino acids. When arbitrarily selecting and aligning a set of 9 *A. niger* beta-glucosidase sequences, all proved to be highly homologous; the sequence that was used for the *in silico* allergenicity screening was also used as input for searching the Toxic Exposome Database T3DB for significant homologies to known toxins. No significant hits (e-value <1) were found.

3. Per the GRAS Final rule, the regulatory text requires taxonomic information beyond genus and species, such as variety or strain, "when applicable" for a source microorganism such as those used to produce enzyme preparations. Examples of when information such as variety or strain would be applicable are those microbial sources, such as some fungi, for which there are multiple strains or subspecies that have different properties with respect to the ability to produce toxins, antibiotics, or other substances that are not suitable for use in food." Please provide more information on the A. niger strains used, including the parent strain name and lineage/source, as well as confirmation that the final product strain does not contain any functional or transferable antibiotic resistance genes.

The ARO strain is a historical, classical strain for which the original source is no longer known. The strain was deposited in DSM's internal strain collection in June 1992, and no classical strain improvement or genetic engineering has been applied to this strain. In 1997, taxonomic identification using the techniques in use at the time (phenotypic and biochemical tests) assigned the strain to *Aspergillus niger* v. Tieghem. So far, we did not perform taxonomic identification of this strain using state-of-the-art molecular biology techniques.

Based on the extensive knowledge available on *Aspergillus niger*, and the fact that no genetic engineering has been applied, we have no reason to believe that the ARO strain contains resistance genes against antibiotics of clinical relevance. On the other hand, the strain may have the potential to produce the two mycotoxins, ochratoxin A and fumonisin B (Pel et al., 2007). Absence of mycotoxins in the enzyme preparation has been checked and confirmed, and is provided in the certificates of analysis located in Appendix 2 of this response.

4. ***Please provide data and/or information from three or more batches to show consistency with the set specifications and to support the statement that mycotoxins are absent from the final enzyme preparation.***

The absence of mycotoxins in food enzyme was confirmed in three commercial batches described in the dossier and can be found the above referenced certificates of analysis provided in Appendix 2.

5. ***Please provide the rationale and basis for your GRAS conclusion using only the information that is published or publicly available, as unpublished studies cannot be used as pivotal studies for safety conclusion. Please see below for FDA's viewpoint with respect to using the unpublished studies with pectinase enzyme preparation as a basis for GRAS conclusion for beta-glucosidase.***

On page 27 of the notice, DSM states:
"The toxicological studies were conducted for the pectinase enzyme preparation. However, since the DSM pectinase enzyme complex contains substantial amounts of beta-glucosidase as side activity, these studies are considered appropriate to assess the safety of beta-glucosidase."

DSM goes onto state further:
"The Margin of Safety is calculated by dividing the NOAEL derived from a sub-chronic toxicity study by the Estimated Daily Intake. To derive the NOAEL, an additional safety factor 6 was used to account for the duration of exposure in toxicological studies (factor 3 is taken for extrapolation from subacute to sub-chronic study and factor 2 from sub-chronic to chronic study, therefore a factor 6 is applied to extrapolate from subacute to chronic study[6])."

Footnote 6 states:
"EFSA 2012 Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels, and Units in the absence of measured data."

FDA notes the following regarding the use of the discussed toxicological studies:

a. ***Since the reference for the EFSA Guidance was not given in the notice, we presume that DSM was referring to EFSA publication ESFA J. 2012; 10(3): 2579. According to this document:***

   "The EFSA Scientific Committee is not in a position to propose default values to extrapolate from subacute to chronic duration because … there is less confidence that toxic effect identified in 90-day or chronic studies will be apparent in studies of shorter duration."

   While the EFSA Scientific Committee does conclude that uncertainty factor (UF) of 2 can be used for the extrapolation from subchronic to chronic study duration, the Committee still recommends "using the overall default UF of 100 (10x10) for inter and intra species extrapolation."

b. A number of assumptions are required in order to extrapolate toxicity or lack thereof from studies using a crude extract to a purified substance. For example, contaminants in a crude extract may mask a potentially harmful effects of a substance. Thus, simply standardizing the NOAEL based on one specific biological activity (i.e. glucosidase activity) between the crude and the purified substance may be considered extremely speculative as a given enzyme activity is perhaps just one of many factors that potentially could influence toxicity of a biological substance.

These and other information suggest that different pectinase preparations have significantly different contaminating substances and activities. Therefore, it is difficult, if not impossible, to quantitatively extrapolate toxicological properties from a crude preparation to a purified enzyme. While the unpublished toxicological studies using pectinase preparations may be used to corroborate the safety of glucosidase, it would be considered beyond accepted toxicological principles to extrapolate the NOAEL from studies on less pure form of glucosidase to obtain margin of exposure/safety for the purified glucosidase.

Beta-glucosidase is one of the activities present in the pectinase complex fermentation medium produced by a selected classical strain of *Aspergillus niger* (ARO). The same strain is also currently used to produce pectinase. The pectinase complex fermentation medium contains substantial amounts of beta-glucosidase and can therefore be standardized on beta-glucosidase activity to the final enzyme preparation. The final beta-glucosidase enzyme preparation is therefore not a purified enzyme it is a crude enzyme preparation, standardized on beta-glucosidase activity. Therefore, the toxicological studies performed using this pectinase complex are relevant to evaluate the safety of beta-glucosidase.

In order to determine the Margin of Safety of food enzymes, uncertainty factors are applied, where appropriate, to cover the uncertainty and variability arising from inter-species differences, intra-species differences, duration of toxicological studies, use of LOAEL when NOAEL is absent, severity of the adverse effects, etc.

DSM is confident that the beta-glucosidase preparation is safe, among other considerations, based on the following:

- *Aspergillus niger* has a long history of use in food industry. Since the 1960s it has been used for the production of a large number of food enzymes (Bennet, 1985a, 1985b; Schuster et al, 2002).

- The safety of several food enzymes produced with *Aspergillus niger* has been assessed in toxicological studies, which typically include an oral sub-chronic (90-day) toxicity study and 2 in vitro genotoxicity studies, and reviewed by JECFA and by official authorities of many countries, such as the USA, Canada, France, Denmark and Australia, resulting in the approval of the use of the enzymes for use in food processing.

- The outcome of the standard toxicity package, consisting of an oral sub-chronic (90-day) toxicity study in rats and two in vitro genotoxicity tests, performed by the food industry on hundreds of food enzymes shows no toxicological potential. These studies have been performed with enzyme-batches from wild-type strains, classical mutants and genetically modified strains. The cumulated evidence from oral toxicity studies performed on mammals shows that enzymes are virtually non-toxic. In such studies, the occasionally observed effects were considered not to be of any toxicological relevance. Data of these toxicological studies are also described in literature (e.g. Pariza and Johnson, 2001; Pariza and Cook, 2010; Olempska-Beer et al., 2006).

- The safety of the DSM *Aspergillus niger* enzyme production strains have been extensively studied and confirmed with several standard packages of toxicological studies which included 90-day oral studies. In all cases the NOAEL was the highest dose level tested (van Dijck, 2003).

- The beta-glucosidase object of this dossier is produced by a selected strain of *Aspergillus niger* which concurrently produces the pectinase activity that is object of the GRAS Notice 89.

Based on all the above considerations, the Margin of Safety is calculated by dividing the NOAEL derived from the oral 28-day toxicity study of beta-glucosidase by the Estimated Daily Intake (EDI).

Margin of Safety = NOAEL/EDI	440/0.378 = 1164
	440/0.126 = 3492

The calculated MoS ranges between 1164 and 3492 is therefore considered sufficiently high to ensure consumers safety.

As previously explained, the beta-glucosidase described in this dossier is part of a pectinase complex fermentation medium that contains the pectinase, which is object of the GRAS Notice 89. Therefore, the background of impurities that may originate from the production strain is the same when producing beta-glucosidase and pectinase.

The final product (beta-glucosidase enzyme preparation) is represented by the pectinase complex fermentation medium standardized on beta-glucosidase activity, to which formulating agents are added. This final product (beta-glucosidase enzyme preparation) is not a purified enzyme. Therefore, DSM believes that the toxicological studies performed with the pectinase complex are relevant to evaluate the safety of the final product (beta-glucosidase enzyme preparation).

* * *

In addition to our above responses, we would also like to note, due to an administrative error, section 6.1 of the Notice (Page 26) was not included in the final submitted document. Therefore, we are providing this page, which includes a description on the safety of the production strain, in Appendix 3 of this response.

We hope that these responses adequately address the concerns of the Agency. However, if additional research or clarification is needed, please do not hesitate to contact me. I can be reached by email at gary.yingling@morganlewis.com or by phone at (202) 739-5610.

Sincerely,

(b) (6)

Gary L. Yingling

Attachments

Appendix 1

Annex 7

ENZYME PREPARATION FROM ASPERGILLUS NIGER (ARO-1)

PRELIMINARY TOXICITY STUDY BY ORAL GAVAGE ADMINISTRATION TO CD RATS FOR 1 WEEK

Sponsor

Gist-Brocades BV
CT&S/REG
PO Box 1
Wateringseweg 1
NL-2600 MA Delft
The Netherlands

Research Laboratory

Huntingdon Life Sciences Ltd.
Eye
Suffolk
IP23 7PX
England

Draft: 23 February 1999
Final : 20 October 1999

Huntingdon Life Sciences

PROTOCOL

ENZYME PREPARATION FROM ASPERGILLUS NIGER (ARO-1)

PRELIMINARY TOXICITY STUDY BY

ORAL GAVAGE ADMINISTRATION TO CD RATS FOR

1 WEEK

Sponsor

Gist-Brocades BV
CT&S/REG
PO Box 1
Wateringseweg 1
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THE NETHERLANDS

Research Laboratory

Huntingdon Life Sciences Ltd
PO Box 2
Huntingdon
Cambridgeshire
PE18 6ES
ENGLAND

Total number of pages: 18

Final Protocol

Annex 8

ENZYME PREPARATION FROM ASPERGILLUS NIGER (ARO-1)

TOXICITY STUDY BY

ORAL GAVAGE ADMINISTRATION TO

CD RATS FOR 4 WEEKS

Sponsor

Gist-Brocades BV
CT&S/REG
PO Box 1
Wateringseweg 1
NL-2600 MA Delft
THE NETHERLANDS

Research Laboratory

Huntingdon Life Sciences Ltd.
Eye
Suffolk
IP23 7PX
England

Draft: 18 August 1999
Final: 25 January 2000

Huntingdon Life Sciences

PROTOCOL

ENZYME PREPARATION FROM

ASPERGILLUS NIGER (ARO-1)

TOXICITY STUDY BY

ORAL GAVAGE ADMINISTRATION TO

CD RATS FOR 4 WEEKS

Sponsor

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Research Laboratory

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ENGLAND

Total number of pages: 25

Final Protocol

Annex 9

ENZYME PREPARATION FROM *ASPERGILLUS NIGER* (ARO-1)

BACTERIAL MUTATION ASSAY

Sponsor

Gist-brocades BV,
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2600 MA Delft,
THE NETHERLANDS

Research Laboratory

Huntingdon Life Sciences Ltd.,
Eye,
Suffolk,
IP23 7PX,
ENGLAND

Report issued 19 May 1999

Annex 10

ENZYME PREPARATION FROM *ASPERGILLUS NIGER* (ARO-1)

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES

Sponsor

Gist-brocades BV,
Central Technology & Services,
Wateringseweg 1,
PO Box 1,
2600 MA Delft,
THE NETHERLANDS.

Research Laboratory

Huntingdon Life Sciences Ltd.,
Eye,
Suffolk,
IP23 7PX,
ENGLAND.

Report issued 3 June 1999

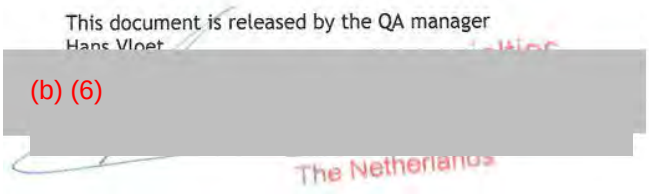
Appendix 2

CERTIFICATE OF ANALYSIS

RA-DLF-00073836

Name of the product	Beta-glucosidase
Batch	414032901
Date of issue	May 25, 2018

Parameter	Unit	Specification limits	Result
Activity	WBDG/g	2500 – 6500	6300
Lead	mg/kg	< 5	< 1
Coliforms	CFU/g	< 15	< 10
Salmonella	In 25 g	Absent	Absent
Escherichia coli	In 25 g	Absent	Absent
Antimicrobial activity	-	Absent by test	Absent by test
mycotoxins	-	Absent by test	Absent by test



Remarks (if any):

DSM Food Specialties B.V., Alexander Fleminglaan 1, 2613 AX Delft, the Netherlands

DSM Food Specialties B.V., Alexander Fleminglaan 1, 2613 AX Delft, the Netherlands

CERTIFICATE OF ANALYSIS

RA-DLF-00073838

Name of the product	Beta-glucosidase		
Batch	415092902		
Date of issue	May 25, 2018		
Parameter	**Unit**	**Specification limits**	**Result**
Activity	WBDG/g	2500 – 6500	3480
Lead	mg/kg	< 5	< 1
Coliforms	CFU/g	< 15	1
Salmonella	In 25 g	Absent	Absent
Escherichia coli	In 25 g	Absent	Absent
Antimicrobial activity	-	Absent by test	Absent by test
mycotoxins	-	Absent by test	Absent by test

Remarks (if any):

DSM Food Specialties B.V., Alexander Fleminglaan 1, 2613 AX Delft, the Netherlands

DSM Food Specialties B.V., Alexander Fleminglaan 1, 2613 AX Delft, the Netherlands

CERTIFICATE OF ANALYSIS

RA-DLF-00073837

Name of the product	Beta-glucosidase		
Batch	415062901		
Date of issue	May 25, 2018		
Parameter	**Unit**	**Specification limits**	**Result**
Activity	WBDG/g	2500 – 6500	2710
Lead	mg/kg	< 5	< 1
Coliforms	CFU/g	< 15	1
Salmonella	In 25 g	Absent	Absent
Escherichia coli	In 25 g	Absent	Absent
Antimicrobial activity	-	Absent by test	Absent by test
mycotoxins	-	Absent by test	Absent by test
This document is released by the QA manager Hans Vloet (b) (6) The Netherlands		Remarks (if any):	

DSM Food Specialties B.V., Alexander Fleminglaan 1, 2613 AX Delft, the Netherlands

DSM Food Specialties B.V., Alexander Fleminglaan 1, 2613 AX Delft, the Netherlands

Appendix 3

6.0 NARRATIVE

6.1 Safety of the Production Strain

The safety of the production organism is paramount to assessing the probable degree of safety for enzyme preparations to be used in food production. According to the International Food Biotechnology Council, food or food ingredients are safe to consume if they have been produced according to current Good Manufacturing Practices, from a nontoxigenic and nonpathogenic organism (Coulston and Kolbye, 1990a). A nontoxigenic organism is defined as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances" (Pariza and Foster, 1983).

Aspergillus niger is known to naturally occur in foods. The fungus is commonly present in products like rice, seeds, nuts, olives, and dried fruits.

For several decades, *Aspergillus niger* has been safely used in the commercial production of organic acids and various food enzymes, such as glucose oxidase, pectinase, alpha-amylase and glucoamylase. Industrial production of citric acid by *Aspergillus niger* has taken place since 1919 (Schuster *et al*., 2002).

This long experience of industrial use has resulted in a good knowledge of the characteristics of *Aspergillus niger* and understanding of the metabolic reactions.

The nonpathogenic nature has been confirmed by several experimental studies (Schuster *et al*., 2002). *Aspergillus niger* is therefore generally accepted as a nonpathogenic organism.

Even though products from *Aspergillus niger* have been used in food for many decades, there is no evidence that the industrial strains produce toxins under the routine conditions of industrial submerged fermentations. The safety has been confirmed by a large number of toxicological tests, as well as batch testing of the various end products for toxins.

The toxicological studies performed on various enzyme preparations from *Aspergillus niger* provided the basis for a safety evaluation by the Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO in 1988 (see Annex 4). Although not based on the results of the toxicological studies, JECFA first allocated a numerical Acceptable Daily Intake (ADI) to enzyme preparations of *Aspergillus niger*, based on the concern that some strains may produce unknown toxins. Two expert reports submitted to JECFA in 1988 concluded that the production of toxins was highly unlikely (see Annex 5). The long history of use as an enzyme source, the numerous toxicological studies and the two expert reports caused JECFA to review its decision in 1990 and change the ADI for enzyme preparations derived from *Aspergillus niger* into "not specified" (Joint FAO/WHO Expert Committee on Food Additives, 1990).

Viebrock, Lauren

From: Yingling, Gary L. <gary.yingling@morganlewis.com>
Sent: Tuesday, September 18, 2018 4:02 PM
To: Viebrock, Lauren
Cc: Vaughn, Jessica L.
Subject: FW: GRN 750 Questions
Attachments: Answers to FDA questions GRN 750 Sep 2018_MW_NvP_PM Annex 1.docx

Dear Dr Viebrock: Attached you will find a memo and a single annex responding to the four questions posed by the Agency concerning GRN 750. Please do not hesitate to contact me if there are any questions. gary

Gary L. Yingling
Morgan, Lewis & Bockius LLP
1111 Pennsylvania Avenue, NW | Washington, DC 20004-2541
Direct: +1.202.739.5610 | Main: +1.202.739.3000 | Fax: +1.202.739.3001
gary.yingling@morganlewis.com | www.morganlewis.com

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Wednesday, September 05, 2018 10:19 AM
To: Yingling, Gary L. <gary.yingling@morganlewis.com>
Subject: GRN 750 Questions

[EXTERNAL EMAIL]
Dear Mr. Yingling,

During our review of GRAS Notice No. 000750, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

Lauren VieBrock, Ph.D.
Consumer Safety Officer/Microbiology Reviewer
U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Biotechnology and GRAS Notice Review
(301) 796-7454

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Memo

[business group/legal entity]
[department]

[street address] [number]
[P.O. Box]
[postal code] [city]
[country]

September 18, 2018

Answers to FDA questions on the GRN 750 (beta-glucosidase from *A. niger*) received on September 5, 2018

Question 1
Per our phone conversation on June 27, 2018, we understand that the pectinase enzyme concentrate from GRN 89 is produced using the same *A. niger* production organism strain and manufacturing methods as that described in GRN 750 to produce beta-glucosidase. Please confirm that the difference in the two preparations is the standardization based on a given enzyme activity, i.e., pectinase or beta-glucosidase.

Answer:
DSM confirms that the pectinase enzyme preparation listed in GRN 89 is the same enzyme preparation as the beta-glucosidase enzyme preparation, which is the subject of GRN 750. This classical (non-recombinant) *A. niger* strain produces both enzyme activities, pectinase and beta-glucosidase and depending on the application, this enzyme preparation can be standardized on one of those enzymatic activities. DSM confirms that the manufacturing method is the same for both enzyme activities since it refers to the same enzyme preparation. The enzyme preparation produced by *A. niger* described in GRN 89 produces both pectinase and beta-glucosidase and is standardized on the pectinase activity, which is relevant for juice and wine applications. The enzyme preparation produced by *A. niger* described in GRN 750 is standardized on the beta-glucosidase activity which is relevant for brewing applications.

Question 2
In GRN 750, you refer to pectinase enzyme preparation from GRN 89 as having a side activity of the beta-glucoside. Please clarify and confirm that the pectinase enzyme preparation contains an additional enzyme component, i.e. beta-glucosidase that is responsible for the beta-glucoside activity, which is the subject of GRN 750.

Answer:
As it is explained in the answer to the question 1, both pectinase and beta-glucosidase enzyme activities are in fact present in the same enzymatic preparation produced using this classical (non-recombinant) *A. niger* strain. Therefore, the side activities of this enzyme preparation are the same for pectinase and beta-glucosidase. In other words, when the enzyme preparation is standardized as pectinase, beta-glucosidase is considered as side activity, when the enzyme is standardized as beta-glucosidase, pectinase is considered as the side activity. Please note that in the tox studies provided, the enzyme preparation was analyzed for both pectinase and beta-glucosidase enzyme activities.

Please find Certificate of analysis of tox batch for your convenience in attachment (page 33 of Annex 7 of originally submitted GRN 750).

Question 3
Based on the narrative in GRN 750, it was not clear that the pectinase enzyme preparation and the beta-glucosidase enzyme preparation were the same, making the scientific rationale for safety studies of one enzyme preparation substantiating the safety of another enzyme preparation. Please provide a clarifying narrative or a statement to explain how the safety studies demonstrating lack of toxicity of pectinase enzyme concentrate in GRN 750 supports the lack of toxicity of beta-glucosidase enzyme.

Answer:
As it is reported in the GRN 750, the safety studies were performed with a batch of the enzyme preparation that contains both pectinase and beta-glucosidase activities and that was measured for both enzyme activities (please see page 33 of Annex 7 of originally submitted GRN 750 for CoA of the tox batch). The Total Organic Solids content was also measured for the tox batch (please see CoA of tox batch). This measurement is a unique value in an enzyme preparation batch, independently of the enzyme activity measured. Since the Margin of Safety has been calculated by using parameters - No Observed Adverse Effect Level (NOAEL) and Estimated Daily Intake – which are expressed in terms of TOS (mg TOS/kg bodyweight/day and not in terms of enzyme activity, the Margin of Safety remain unchanged and it is not influenced by the enzyme activity measured in the enzyme preparation. Therefore, the results of the safety studies demonstrate the lack of toxicity of both pectinase and beta-glucosidase enzymes in this enzyme preparation.

Question 4
Please provide a narrative to demonstrate how the publicly available sequences for beta-glucosidase discussed in GRN 750 for allergenicity analyses are representative of all *A. niger* beta-glucosidases. Please include a discussion of the results of bioinformatic analyses of the representative beta-glucosidase sequences for allergenic potential. Please explain how the results of the analysis would support safety of the beta-glucosidase that is the subject of GRN 750.

Answer:
At the time of the submission of the GRN 750 to FDA, no characterization at the molecular level was available for the beta-glucosidase produced with this classical (non-recombinant) *A. niger* (ARO1) strain. Therefore, the data submitted were the results of the bioinformatic analysis performed on a representative beta-glucosidase sequence (from an *A. niger* type strain) deposited in the public sequence databases. Since the actual gene sequence of this classical (non-recombinant) *A. niger* ARO1 strain has now become available, we herewith submit an updated bioinformatic analysis performed on the actual deduced beta-glucosidase sequence of this enzyme preparation. The results are reported in Annex 1 to this letter.

The analysis was done by comparing the actual amino acid sequence of the beta-glucosidase produced with this classical (non-recombinant) *A. niger* strain that is the object of the GRN 750 with the amino acid sequences of known (food) allergens. The comparison was made in August 2018, using the database AllergenOnline™ (available at http://www.allergenonline.org/, last updated on 23 March 2018). The results indicate that two stretches of 80 amino acids could be identified with an identity of more than 35% to a protein sequence in the AllergenOnline database. The protein, Asp 14 from *A. niger*, is a non-food allergen. No identical stretches of 8 amino acids or more could be detected in the enzyme sequence as compared to the protein sequences in the AllergenOnline database. Based on these results, it is concluded that the beta-glucosidase protein of this enzyme preparation has no relevant matches with known food allergens and is not likely to produce an allergenic or sensitization response upon oral consumption.

Annex 1

Bioinformatic testing for putative allergenicity of *A. niger* ARO1 (DS06047) beta-glucosidase

Bioinformatics testing for putative allergenicity of *A. niger ARO1* - DS06047 beta-glucosidase

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

Applicant	Paola Montaguti
Project	FIAP
Project#	RD.0706.05
Scientist	Irsan Kooi
Date	15-08-2018

Used allergenicity database:

Database:	AllergenOnLine
Last updated:	23-03-2018
Link:	http://www.allergenonline.org/

Info of tested protein(s)

Enzyme name	Beta-glucosidase
Donor organism	N.A.
Production organism	*Aspergillus niger*

Protein sequence query

> Beta-glucosidase A. niger *ARO1* DS06047
MRFTLIEAVALTAVSLASADELAYSPPYYPSPWANGQGDWAQAYQRAVDIVSQMTLDEKVNLTTGTGWEL
ELCVGQTGGVPRLGVPGMCLQDSPLGVRDSDYNSAFPAGMNVAATWDKNLAYLRGKAMGQEFSDKGADIQ
LGPAAGPLGRSPDGGRNWEGFSPDPALSGVLFAETIKGIQDAGVVATAKHYIAYEQEHFRQAPEAQGYGF
NISESGSANLDDKTMHELYLWPFADAIRAGAGAVMCSYNQINNSYGCQNSYTLNKLLKAELGFQGFVMSD
WAAHHAGVSGALAGLDMSMPGDVDYDSGTSYWGTNLTISVLNGTVPQWRVDDMAVRIMAAYYKVGRDRLW
TPPNFSSWTRDEYGYKYYYVSEGPYEKVNQYVNVQRNHSELIRRIGADSTVLLKNDGALPLTGKERLVAL
IGEDAGSNPYGANGCSDRGCDNGTLAMGWGSGTANFPYLVTPEQAISNEVLKHKNGVFTATDNWAIDQIE
ALAKTASVSLVFVNADSGEGYINVDGNLGDRRNLTLWRNGDNVIKAAASNCNNTIVVIHSVGPVLVNEWY
DNPNVTAILWGGLPGQESGNSLADVLYGRVNPGAKSPFTWGKTREAYQDYLVTEPNNGNGAPQEDFVEGV
FIDYRGFDKRNETPIYEFGYGLSYTTFNYSNLEVQVLSAPAYEPASGETEAAPTFGEVGNASDYLYPSGL
QRITKFIYPWLNGTDLEASSGDASYGQDSSDYLPEGATDGSAQPILPAGGGPGGNPRLYDELIRVSVTIK
NTGKVAGDEVPQLVSKHEVRTRLNKANQSQYVSLGGPNEPKIVLRQFERITLQPSEETKWSTTLTRRDLA
NWNVEKQDWEITSYPKMVFVGSSSRKLPLRASLPTVH

Results

1. Blast against appropriate database
   As a control a blastP search is performed to verify the sequence and its origin.
   Best Blast hit is with A0A1L9NM10 - Beta-glucosidase of *Aspergillus tubingensis* CBS 134.48 gene: (ASPTUDRAFT_25807)

Table 1: Blast results

Query sequence	Besthit Id	Donor organism	database	%-age identity	description
Beta-glucosidase	A0A1L9NM10	*Aspergillus tubingensis*	UniProtKB	100 %	Beta-glucosidase

2. Check for signal sequence
   The signal sequence is removed upon secretion and is supposed to be degraded. Therefore, it is assumed that the signal sequence is not present in the product. The signal prediction is performed with SignalPv2.0.

Bioinformatics testing for putative allergenicity of *A. niger ARO1* - DS06047 beta-glucosidase

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

Table 2: Info signal sequence

Query sequence	signal	length	Signal sequence
Beta-glucosidase	yes	19	MRFTLIEAVALTAVSLASA

3. Full fasta alignment
Full fasta alignment is used to identify allergens that are highly similar to the query sequence. Matches with E -values > 1 are not likely to be related in evolution or structure while matches with E -values > 10^{-7} are not likely to share immunologic or allergic cross-reactivity.

No hits were found with e-values < 10-7. However, two hits were found (beta-xylosidase and xylosidase) mapping to one allergen (Asp n 14) with alignments just above (meaning poorer alignment) e-value 10-7.

Table 3: Overview of full fasta alignment

Query sequence	Allergen	Source link	Sequence length	E-score
Beta-glucosidase	Asp n 14	GI:4235093	804	2.7e-006
Beta glucosidase	Asp n 14	GI:2181180	804	6.7e-006

4. FASTA alignments for an 80 amino acids sliding window

Two hits (beta-xylosidase and xylosidase) were found with percentage identity just above thresholds (>35% identity)

Table 4: Hit info alignments for 80 aa sliding window

Query sequence	Allergen	Source link	%-age identity	Allergen host	Allergen type
Beta-glucosidase	Asp n 14	Asp n 14	36.30	*Aspergillus niger*	Non food
Beta-glucosidase	Asp n 14	Asp n 14	35.20	*Aspergillus niger*	Non food

5. Exact match for 8 Contiguous amino acids
No exact matches for 8 contiguous amino acids have been found between the query sequence and all ALLERGEN ONLINE allergens.

Table 5: Hit info exact match for 8 aa

Query sequence	Allergen	Source link	Allergen host	Allergen type
Beta-glucosidase	No			

6. Available information of detected allergens (hits)
(Link to the source, allergen host, type of allergen (food, nonfood)

Asp n 14	Biochemical name	Source link	Organism	Allergen type
Asp n 14	Beta-xylosidase	Asp n 14	*Aspergillus niger*	Non food

Bioinformatics testing for putative allergenicity of *A. niger ARO*1 - DS06047 beta-glucosidase

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Conclusion

The potential allergenicity of the enzyme Beta-glucosidase from *Aspergillus niger ARO*1 DS06047 was evaluated by comparing the amino acid sequence of the enzyme with known (food) allergens. For the comparison we made use of the database AllergenOnline.

Two stretches of 80 amino acids could be identified with an identity of more than 35% to 1 protein sequence in the AllergenOnline database.

1. Asp n 14 from Aspergillus niger is a non-food allergen

No identical stretches of 8 amino acids or more could be detected in the enzyme sequence as compared to the protein sequences in the AllergenOnline database.