In Vitro Methodologies for Assessing Cutaneous Irritation, Sensitization and Photosensitivity

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Conflict of Interests

- None – but in the spirit of full disclosure
  - I am a member of the Cosmetic Ingredient Review's Expert Panel
  - I am a member and chair of the Expert Panel for Fragrance Safety
  - I am reimbursed for my travel & time for both from the Personal Care Products Council (Washington, DC) and the Research Institute for Fragrance Materials (Woodcliff Lake, NJ), respectively

IRRITANT CONTACT DERMATITIS

1) Acute primary irritation

2) Cumulative irritation

3) Corrosion

4) Phototoxicity

CHEMICAL IRRITANCY

- Inherent chemical properties
- Concentration
- Amount
- Duration / frequency

PHOTOIRRITATION/ PHOTOTOXICITY

- Photoexcitable chemical which absorbs
  - UVB (290-320 nm) - less frequently
  - UVA (320-400 nm) - most frequently
  - Visible (400-775 nm) - rare/endogenous

IN VITRO METHODS FOR CORROSION

- **rat skin transcutaneous electrical resistance (OECD TG 430)**
  - liquids (150 ul) & solids ("sufficient to cover surface") x 24h
  - TER > 5 kΩ, non-corrosive; ≤ 5 kΩ, corrosive
  - not applicable to surfactants or neutral organics
    - need to use dye (sulforhodamine B) penetration
  - http://www.oecd-ilibrary.org/environment/test-no-430-in-vitro-skin-corrosion-transcutaneous-electrical-resistance-test-method-ter_9789264203808-en.
- **Corrositex (reconstituted collagen matrix; OECD TG431)**
  - liquids (500 ul) & solids (500 mg)
  - breakthrough time correlated to corrosivity
  - applicable only to materials that produce a color or physical change in the "chemical detection system" w/in 5 mins
  - http://www.oecd-ilibrary.org/environment/test-no-431-in-vitro-skin-corrosion-human-skin-model-test_9789264071148-en

IN VITRO METHODS FOR CORROSION

(OECD TG 435)

- http://www.oecd-ilibrary.org/environment/test-no-435-in-vitro-membrane-barrier-test-method-for-skin-corrosion_9789264242791-en
- Episkin (multilayered epidermis on collagen matrix)
  - liquids & solids (sufficient material “to cover the the skin”)
  - degree of corrosivity defined as ≤ 35% cell viability at defined times (3, 60, & 240 mins)
- EpiDerm (multilayered epidermis on cell culture inserts)
  - liquids (50 ul) & solids (25 mg)
  - degree of corrosivity defined as ≤ 50% cell viability @ 3 mins or ≤ 15% viability at 60 mins at defined times

IN VITRO METHODS FOR ACUTE IRRITATION

Acute: (OECD TG 439)

- **http://www.oecd-ilibrary.org/environment/test-no-439-in-vitro-skin-irritation-reconstructed-human-epidermis-test-method_9789264242845-en**
- EpiSkin™
- EpiDerm™ SIT (EPI-200)
- Modified EpiDerm (EPI-200)
- SkinEthic™ RHE

IN VITRO: ACUTE IRRITATION

- Dose: minimum of 25 µL/cm^2 (liquid) or 25 mg/cm^2 (solid)
- Incubation: 3 hrs
- Viability: mitochondrial dehydrogenases to reduce the vital dye MTT
- The test substance is considered to be:
  - irritant to skin if the tissue viability after exposure and post-treatment incubation is ≤ to 50 %.
  - no category if the tissue viability after exposure and post-treatment incubation is > 50 %.

IN VITRO: CHRONIC CUMULATIVE IRRITATION

- Chronic cumulative irritation
  - no ECVAM / OECD acceptable in vitro method
  - Under development:
    - Reconstructed organotypic skin model (ROSM) w/ keratinocytes, basement membrane & fibroblasts
    - Cultured for varying lengths of time w/ chemical in ?
    - Assay for ↑ heat shock protein (HSP)-27
    - Methodology assessed w/ SLS and acute irritants (acids & bases)
      - Chen, et al. Toxicology Letters. 226: 124 = 131, 2014

PHYSICOCHEMICAL METHODS FOR ASSESSING PHOTOTOXICITY

- Physicochemical:
  - Is it a photoabsorber?
    - Henry, et al. J Photchem Photobiol B. 2009; 96: 57 – 62.
- Reactive Oxygen Species (ROS) assay
  - Excitation of molecules by light → ROS
    - superoxide anion (SA) and singlet oxygen (SO)
  - does not measure phototoxicity directly
  - 100% sensitivity; some false positives
  - Not validated by ECVAM / OECD
    - Haranosono, et al. J Toxicol Sci. 2014; 39: 655-64.
- Both systems fail to account for biotransformation & autoxidation

ROS (Japanese Center for the Validation of Alternative Methods): PHOTOTOXICITY

Solvents: DMSO or 20 mM of NaPB
Controls: Quinine hydrochloride (+); Sulisobenzone (-)
Irradiation: Solar stimulator x 1h @ controlled temperature

Judgment	**Conc.**	**SO (mean X 3) (% ↑, irradiated / non-irradiated)**		**SA (mean X3)**
Photoreactive	200 µM	≥25	and	≥70
		<25	and	≥70
		≥25	and	<70
Weakly photoR	200 µM	<25	and	≥20, <70
Non-photoR	200 µM	<25	and	<20

Inconclusive: The results do not meet the above-mentioned criterion.

IN VITRO METHODS FOR ASSESSING PHOTOTOXICITY

- Phototoxicity
  - 3T3 Neutral Red phototoxicity test (OECD TG 432)
  - http://www.oecd-ilibrary.org/content/book/9789264071162-en
    - 3T3 Balb/c fibroblasts incubated for 1 hr w/ test substance and irradiated w/ 5 J/cm^2
    - Photoirritation factor (PIF) = ratio of cytotoxicity with and without UV light (prefer solar simulator)
      - PIF < 2: no potential for phototoxicity
      - PIF >5: phototoxic
      - PIF 2- 5: inconclusive

DRAWBACKS TO 3T3

- false negatives
  - toxicological hazard Primarily due to a mechanism of action not captured by the cell line used
  - the cell lines lack of metabolic capacity, compounds metabolized in vivo to biologically active forms may be missed
- false positive
  - limited bioavailability in vivo due to poor absorption and distribution, or rapid biotransformation and excretion

IN VITRO METHODS FOR ASSESSING PHOTOTOXIC METABOLITES

- Enhanced Phototoxicity Assay in Reconstituted Skin (EPARS) -- Portes, et al. Toxicol In Vitro. 2002; 16: 765-70.

- Enzymatic reactive oxygen species assay (eROS) -- Kato, et al. Enzymatic reactive oxygen species assay to evaluate phototoxic risk of metabolites. Toxicol Lett. 2017; 278: 59-65.

Enhanced Phototoxicity Assay in Reconstituted Skin (EPARS)

- 100 µl test substance directly to tissue surface; incubated at 37ºC x 18-24 h
- UV Irradiation (solar simulator): 6 J/cm^2; control = non-irradiated
- Tissues and incubated x 18-24 h at 37ºC
- Tissue viability measured using the MatTek MTT Viability Assay protocol
  - Currently uses cell viability ($\leq$ 30%) and PIF $\geq$ 2
  - PGE_2 levels as alternative to viability
- Prevalidation suggests under-prediction and a precautionary factor of 10 for extrapolation to man

eROS

- 10 mM chemical in DMSO incubated at 37 °C in 20 mM NaPB containing human liver S9 fractions (0.2 mg-protein/mL) and “typical cofactors” x 5 min
- 100 µM of “activated” substrate in DMSO irradiated w/ solar stimulator x 1h @ controlled temperature
- Controls: Fenofibrate w/ S9 (+); Fenofibrate w/ denatured S9 (-)
- Readings [chemical w/ S9 / chemical w/ denatured S9] as per ROS f
- Favorable intra-/inter-day reproducibility
- eROS assay provided false predictions for some compounds
- Material must be water soluble

ALLERGIC CONTACT DERMATITIS

- Allergic
  - Pre-haptens
  - Pro-haptens
- Photoallergic
  - Pre-haptens
  - Pro-haptens

IN VITRO METHODS FOR ASSESSING ALLERGY: AOP

1: Molecular Initiation
- Direct Peptide Reactivity Assay (DPRA)

2: Keratinocyte Activation
- KeratinoSens™ (ARE-Nrf2 Luciferase Test Method)
- IL-8 Luc Assay (not ECVAM; +JaCVAM)

3: Dendritic Cell Activation
- Human Cell line Activation Test (h-CLAT)
- Myeloid U937 skin sensitization test (MUSST; ECVAM, not OECD)

4: T cell activation
- none

DPRA (OECD TG 442C)

- peptide to chemical ratio used is 1:10 (cysteine) and 1:50 (lysine) [controls: cinnamic aldehyde (+); vehicle (-)]
  - **Cys & Lys: % depletion (mean)**
- Minimal Reactivity < 6.38%
- Low Reactivity: ≥ 6.38 - < 22.62%
- Moderate Reactivity: ≥ 22.62 - < 42.47%
- High Reactivity: ≥ 42.47%
  - **http://www.oecd-ilibrary.org/environment/test-no-442c-in-chemico-skin-sensitisation_9789264229709-en**

LIMITATIONS of DPRA

DPRA test method does not contain a metabolic/bioactivation system, therefore pro-haptens & pre-haptens are not detected

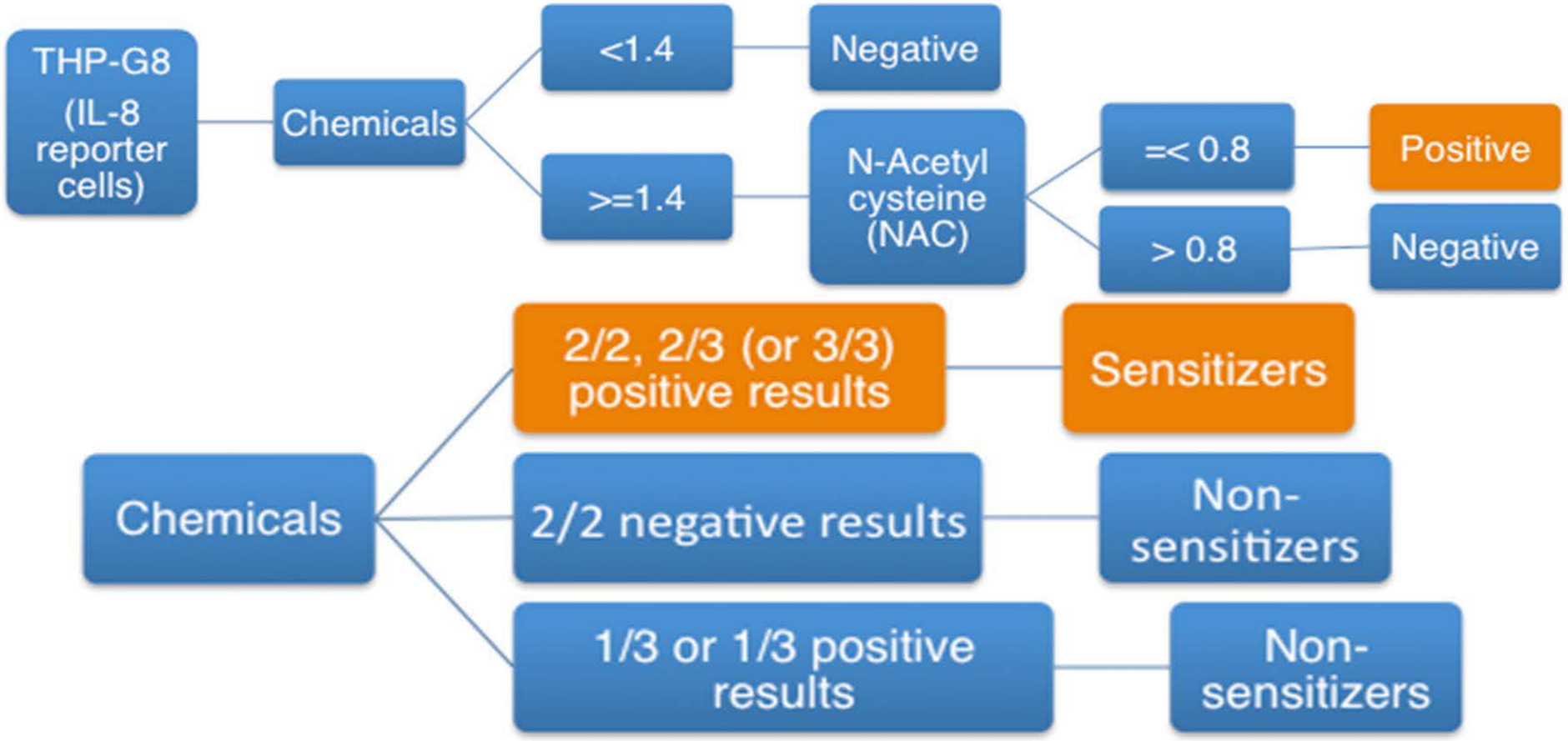
- pre-haptens (i.e. simple chemical transformation, for example oxidation by air or photo-activation in the presence of UV light)
- pro-haptens (i.e. chemicals requiring enzymatic activation)
  - peroxidase peptide reactivity assay “PPRA” identifies pro-haptens via the use of peroxidase in the DPRA
    - Gerberick. Altern Lab Anim. 2016; 44: 437-442.

KeratinoSens ASSAY (OECD TG 442D)

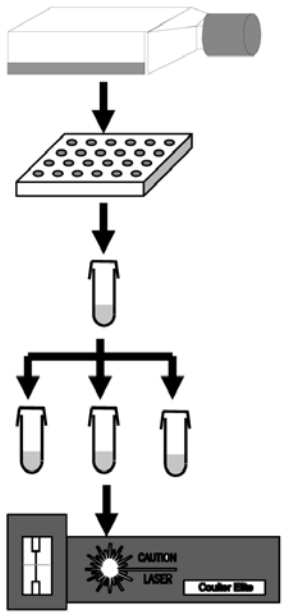
- Based on a stable reporter construct consisting of a luciferase gene under the control of the antioxidant response element (ARE) in a HaCaT (immortalized keratinocyte) cell line.
- The luciferase gene allows for the detection of sensitization potential based on the bioluminescent activity of ATP: light is produced after the breakdown of the protein luciferin by luciferase.
- Inserted genes allow for the exploitation of the signaling pathway of Keap1-Nrf2, which has previously been shown to be activated during skin sensitization events (Natsch & Emter, 2008; Natsch et al., 2009)
- Cell viability also assessed
- ≥ 2/3 trials show 50% increased induction of luciferase gene; viability not a criterion; (-) control, solvent; (+) control, cinnamic aldehyde
  - http://www.oecd-ilibrary.org/environment/test-no-442d-in-vitro-skin-sensitisation_9789264229822-en.

IL-8 Luc Assay

THP-1 cell line is a human monocyte line; not keratinocyte



H-CLAT (OECD TG 442E) / MUSST*



Pre-culture THP-1 cells for 48-72 hours

Plate (1x10^6 cells/well) in 24-well plate, treat with test chemical for 24 hours

Harvest cells, wash and block FcR (0.01% Globulins) for 15 min.

Divide cells into 3 aliquots, stain with FITC-conjugated monoclonal antibodies (isotype control, CD86, CD54) for 30 min.

Analyze by flow cytometry - mean fluorescence intensity of CD86 and CD54, cell viability by propidium iodide exclusion.

http://www.oecd-ilibrary.org/content/book/9789264264359-en

DNCB = (+) control;
Medium = (-) control

CD86 >150% or CD54: >200% = positive;
*MUSST similar; uses Myeloid U937 cell line and only CD86; ECVAM validated; awaiting OECD TG.

IN VITRO METHODS FOR ASSESSING Pro-HAPTEN PHOTOSENSITIZATION

- No accepted methodology; no photo-activated DPRA has been proposed.
- in vitro testing methodologies utilizing KeratinoSens and h-CLAT with the addition of exposure to 5J/cm2 of UVA
  - photo-KeratinoSensTM assay
    - Tsujita-Inoue et al. J Appl Toxicol. 2016; 36: 956-968
  - photo-h-CLAT assay
    - Hoya, et al. Toxicol In Vitro. 2009; 23: 911-918.
- In vitro systems to assess photosensitization are critical
  - EU ban on testing cosmetic ingredients in animals (sunscreens are cosmetic in the EU!)
  - testing for photoallergenicity in humans unethical due to potential for the induction of persistent light reactivity (PLR): extreme sensitivity to UVB in absence of inducing photoallergen

Sensitization Hazard (not risk)

- Integration of results of testing on the first three steps of AOP to predict sensitization hazard not resolved
- Various models proposed
  - Bayesian network
  - Artificial Neural Network
  - Weight of Evidence
    - Weight of Evidence or best “two out of three” to assess sensitization hazard AOP has found the greatest success
- But what about risk??

Thank you!