Inter-Laboratory Validation of an *In Vitro* Method to Classify Skin Sensitizers

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Introduction

Allergic contact dermatitis presents a concern for developers of personal care, chemical, pharmaceutical, and medical device products. The development of non-animal methods to assess skin sensitization is a priority due to the EU cosmetics testing ban, the 2018 REACH deadline, and the goal of reducing animal use. Currently approved methods use either guinea pigs (GPMT) or mice (LLNA) to assess skin sensitization after the test substance has been injected or applied to their skin. Parameters such as redness, itchiness, scaling, and inflammation or increased cell count in lymph nodes are used to rate the skin sensitizing hazard or potency of the chemical. The SenCeeTox® assay represents a method to assess skin sensitizing potential and potency of chemicals in a tiered approach without the use of animals.

This study builds upon previous studies (McKim *et al*, 2010; McKim *et al*, 2012) showing that the *in vitro* SenCeeTox® assay can correctly identify and categorize chemical sensitizers when used in-house. The aim of this project was to further validate the SenCeeTox® assay by conducting an inter-laboratory validation at the Flemish Institute for Technological Research (VITO).

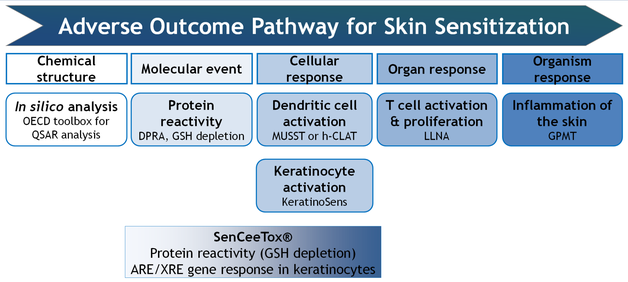


Fig. 1: Schematic representation of the adverse outcome pathway for skin sensitization. Adapted from [3, 4]

Methods

-MatTek's three-dimensional human skin model, EpiDerm™ was treated in triplicate with six concentrations of each blinded test article. The test articles evaluated were: metol, isoeugenol, 2,3-butanedione, 2-mercaptobenzothiozol, eugenol, 1-chloro-2,4-dinitrobenzene, glycerol, 2-hydroxyethylmethacrylate, 2-hydroxyethylacrylate, and lactic acid.

-Following 24 hr exposure to the test articles, the following endpoints were measured:

1) Cytotoxicity was assessed by measuring lactate dehydrogenase (LDH) in tissue supernatant.
2) The ability of each chemical to directly react with glutathione (GSH).
3) Expression of seven genes controlled by the Nrf2/Keap1/ARE or AhR/ARNT/XRE signaling pathways:

- NADPH-quinone oxidoreductase 1 (NQO1)
- Aldoketoreductase 1C2 (AKR1C2)
- Interleukin 8 (IL8)
- Cytochrome P450 1A1 (CYP1A1)
- Aldehyde dehydrogenase 3A1 (ALDH3A)
- Heme-oxygenase 1 (HMOX1)
- Glutamate cysteine ligase catalytic subunit C (GCLC)

-The cytotoxicity, GSH depletion, and potency of gene expression (lowest concentration that produces a significant increase, number of genes responding and the magnitude of induction) results were analyzed with a proprietary algorithm to generate an *In Vitro* Toxicity Index (IVTI) for each test article and predict each chemical's likelihood of causing a human sensitization reaction.

Nrf2/ARE Signaling Pathway

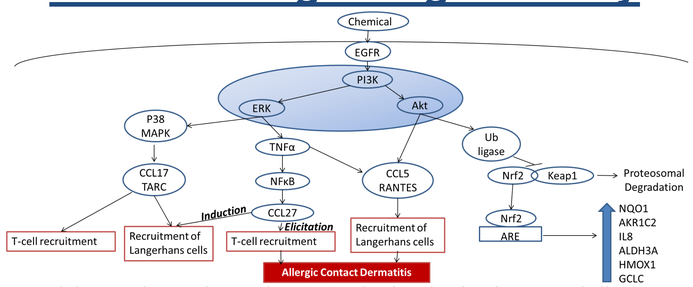
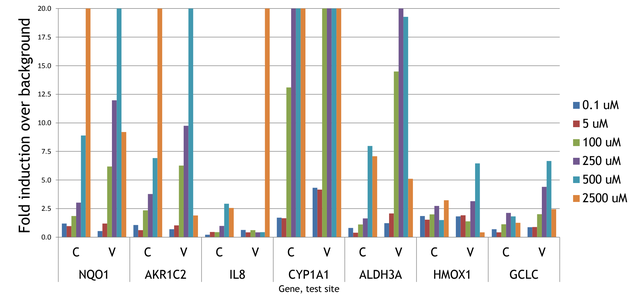


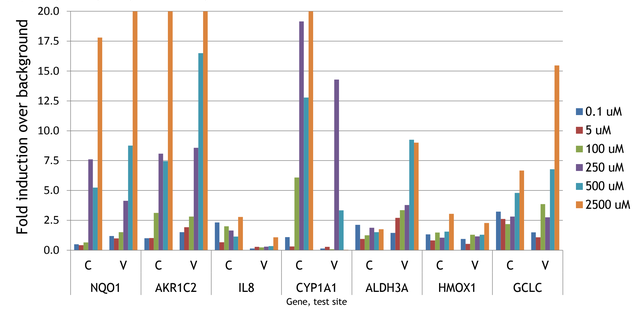
Fig. 2: Overview of the signaling pathway of genes involved in the development of allergic contact dermatitis under the control of the transcription factor Nrf-2 and the antioxidant response element (ARE). Modified from McKim et al, 2012.[2]

Results

(A) p-benzoquinone – extreme sensitizer



(B) 2-hydroxyethylmethacrylate – moderate sensitizer



(C) Benzoic acid – non-sensitizer

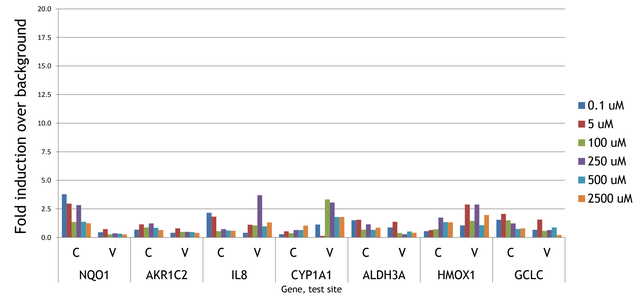


Fig. 3: Gene induction caused by increasing concentrations of (A) p-benzoquinone, (B) 2-hydroxyethylmethacrylate and (C) benzoic acid. Seven genes were examined at two testing facilities: CeeTox/Cyprotex (C) or VITO (V).

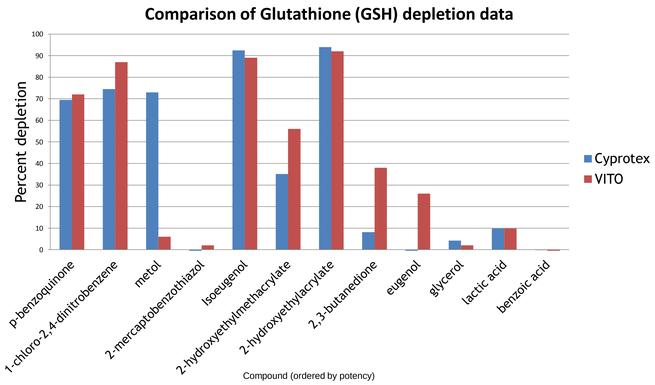


Fig. 4: Comparison of GSH depletion for each compound tested at two facilities: CeeTox/ Cyprotex (blue) or VITO (red). Compounds are ordered from extreme to non-sensitizers.

Results (continued)

Table 1: Summarizes results of the validation studies at CeeTox/Cyprotex and VITO compared to the LLNA and human data. LLNA and human data were taken from published sources.[5-8]

	CeeTox/Cyprotex		VITO			
Test Article Name (Unblinded)	**IVTI**	**PPC**	**IVTI**	**PPC**	**LLNA Potency Category**	**human patch test**
p-benzoquinone (+ control)	7	Extreme	6	Strong	Extreme	+
1-chloro-2,4-dinitrobenzene	6	Strong	7	Extreme	Extreme	+
metol	6	Strong	5	Moderate	Strong	
2-mercaptobenzothiozol	6	Strong	5	Moderate	Moderate	+
isoeugenol	6	Strong	6	Strong	Moderate	+
2-hydroxyethylmethacrylate	4	Moderate	4	Moderate	Moderate	
2-hydroxyethylacrylate	6	Strong	6	Strong	Moderate	+
2,3-butanedione	3	Weak	3	Weak	Weak	
eugenol	3	Weak	3	Weak	Weak	+
glycerol	0	Non-sensitizer	1	Non-sensitizer	Non-sensitizer	-
lactic acid	3	Weak	1	Non-sensitizer	Non-sensitizer	+
Benzoic acid (- control)	3	Weak	1	Non-sensitizer	Non-sensitizer	

Legend: IVTI = In Vitro Toxicity Index; PPC = Predicted Potency Category

SenCeeTox® Advantages

• Provides potency categorization (± one potency category).

• Assay is applicable for soluble compounds, insoluble compounds or finished products. MatTek's EpiDerm™, a 3-dimensional human skin model, allows topical application of the test material.

• Mechanistically based: measures key events in the AOP for skin sensitization including protein reactivity (GSH depletion) and increased expression of genes regulated by the ARE and XRE signaling pathways in keratinocytes (see Fig. 1).

• Complies with current European Union, Indian, and Israeli requirements that cosmetics not be tested on animals.

• Completely replaces animal use and reduces time and cost as compared to animal testing.

Conclusions and Future Direction

• Predictivity is as good as or better than animal data for the compounds shown.

• SenCeeTox® accurately predicted the ability to elicit a sensitization reaction for all ten blinded compounds tested at VITO compared to LLNA data.

• SenCeeTox® correctly predicted the sensitization potency category for five/seven out of the ten compounds tested, missing the LLNA category by only one potency category.

• Further validation of this assay is ongoing; following the validation, all results will be submitted to the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM).

References

1. McKim *et al*. A new *in vitro* method for identifying chemical sensitizers combining peptide binding with ARE/EpRE-mediated gene expression in human skin cells. Cutan Ocul Toxicol, 2010. 29(3):171-92
2. McKim *et al*. An *in vitro* method for detecting chemical sensitization using human reconstructed skin models and its applicability to cosmetic, pharmaceutical, and medical device safety testing. Cutan Ocul Toxicol, 2012. 31(4):292-305.
3. OECD ENV/JM/MONO(2012)10/PART1. 04-May-2012.
4. Bauch *et al*. Putting the parts together: Combining *in vitro* methods to test for skin sensitizing potentials. Reg Tox and Pharm, 2012. 63:489-504.
5. Basketter *et al*. Threshold for Classification as a Skin Sensitizer in the Local Lymph Node Assay: a Statistical Evaluation. Food Chem, 1999. Toxicol. 37: 1167-1174.
6. Natsch *et al*. Filling the concept with data: integrating data from different *in vitro* and *in silico* assays on skin sensitizers to explore the battery approach for animal-free skin sensitization testing. Tox Sci, 2009. 107(1):106-121.
7. Gerberick *et al*. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. Dermatitis, 2005. 16(4):157-202.
8. Kimber *et al*. Classification of contact allergens according to potency: proposals. Food and Chem Tox, 2003. 41:1799–1809.