Mimicking Metabolism Using Human Microsomes in Two *In Vitro* Assays in Order to Predict the Sensitization Potential of Pro-haptens

Megan Lamm and Kimberly Norman
Institute for In Vitro Sciences Inc., Gaithersburg, MD, USA

Introduction

Skin sensitization is an important endpoint for products ranging from cosmetics to pharmaceuticals. Two *in vitro* assays, KeratinoSens™ and the Direct Peptide Reactivity Assay (DPRA), recently had regulatory guidelines published. The KeratinoSens™ assay uses a keratinocyte cell line to monitor the induction of luciferase under the control of the Antioxidant Response Element. The DPRA is an *in chemico* assay that uses High Performance Liquid Chromatography in order to monitor the reactivity of test chemicals with synthetic peptides containing cysteine or lysine. Given the complexity of the sensitization pathway, there are some limitations associated with each assay. One limitation is the inability of these assays to provide a sufficient source of metabolism for test chemicals. Some chemicals, known as pro-haptens, require enzymatic oxidation in order to act as sensitizers. There are limited metabolic capabilities with the cell line used for KeratinoSens™ and no metabolic capabilities for DPRA, potential leading to false negative results in both assays. In this study we sought to add a preliminary step, using human liver microsomes, to KeratinoSens™ and the DPRA that would provide a source of metabolism. We used initial *in silico* predictions to decide if traditional assay procedures were sufficient or if the preliminary step incorporating the microsomes was needed on an individual chemical basis. A set of 13 chemicals made up of non-sensitizers, sensitizers, and pro-haptens, were evaluated.

Skin Sensitization Pathway

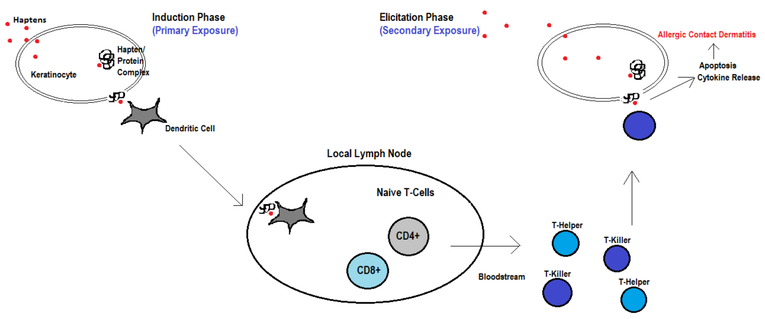

Figure 1. The skin sensitization pathway starts with a hapten freely diffusing through the membrane of a skin cell and then covalently binding to a cellular protein. The complex is displayed on the cell surface and is recognized by a dendritic cell. The dendritic cell travels to a lymph node and displays the complex to naïve CD4+ and CD8+ T-cells. The T-cells mature into T-helper and T-killer cells and clonal expansion occurs. The mature T-cells travel through the blood stream and are available upon a secondary exposure to the hapten. The T-killer cells recognize the complex on the keratinocyte's surface leading to apoptosis and cytokine release.

Developing the Microsomal Pre-step

- We wanted to design an assay step using microsomes that allowed us to perform the rest of the assay according to the validated guidelines and without added complications.
- Various conditions were tested in the DPRA to find optimal exposure conditions.

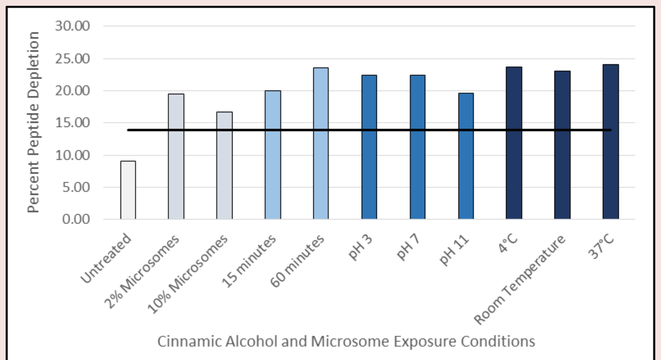

Figure 2. Cinnamic alcohol was mixed with human liver microsomes under the various conditions shown on the x-axis and then exposed to the cysteine peptide for approximately 24 hours. The black line represents 13.89% depletion, which is the cutoff between a non-sensitizer and a sensitizer using the cysteine prediction model.

- The modified versions of the assays were performed by preparing the test article dilutions at 2X the top stock concentration used for the assays (200 mM for DPRA and 400 mM for KeratinoSens™).
- The dilution was prepared in water supplemented with 2% microsomes. The test article/microsome reaction mixture was incubated at 37°C for 2 or 4 hours with periodic vortexing.
- After the exposure, acetonitrile for DPRA or DMSO for KeratinoSens™ were added to the reaction mixtures so as to bring the final concentration of the test article to the concentrations used in the assays.
- The reaction mixtures were centrifuged at 300 x g for 10 minutes to collect the microsomes. The supernatant was used to continue the assays in the normal fashion.

Using OECD Toolbox to Identify Pro-haptens

- OECD Toolbox makes predictions by comparing the structure of the chemical of interest to chemicals in the database with known results.
- The software can identify sensitizers, non-sensitizers, and pro-haptens based on suspected protein binding capabilities.
- To identify pro-haptens the software first decides potential metabolites for the chemical and looks at the protein reactivity of the metabolites.

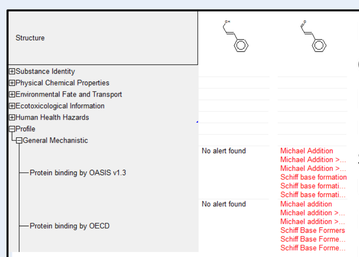

Image taken from OECD Toolbox version 3.3

Figure 3. This test chemical, cinnamic alcohol, was originally predicted to have no protein reactivity, and therefore be a non-sensitizer (chemical on the left). Using the skin metabolism predictor in the software, a protein-reactive metabolite was found for the chemical, thereby classifying the chemical as a pro-hapten.

Results

Test Article	LLNA Data	OECD Toolbox Prediction	DPRA Results	Modified DPRA Results	KeratinoSens™ Results	Modified KeratinoSens™ Results
1-Butanol	Non-Sensitizer	Non-Sensitizer	Non-Sensitizer	Non-Sensitizer	Non-Sensitizer	Non-Sensitizer
Glycerol	Non-Sensitizer	Non-Sensitizer	Non-Sensitizer	Non-Sensitizer	Non-Sensitizer	Non-Sensitizer
Diethyl Phthalate	Non-Sensitizer	Non-Sensitizer	Non-Sensitizer	Non-Sensitizer	Non-Sensitizer	Non-Sensitizer
Farnesal	Sensitizer	Sensitizer	Sensitizer	Sensitizer	Sensitizer	Sensitizer
Cinnamic Aldehyde	Sensitizer	Sensitizer	Sensitizer	Sensitizer	Sensitizer	Sensitizer
1-Chloro-2,4-dinitrobenzene	Sensitizer	Sensitizer	Sensitizer	Sensitizer	Sensitizer	Sensitizer
Diethyl Maleate	Sensitizer	Sensitizer	Sensitizer	Sensitizer	Sensitizer	Sensitizer
Limonene	Sensitizer	Non-Sensitizer	Sensitizer	Sensitizer	Non-Sensitizer	Non-Sensitizer
Cinnamic Alcohol	Sensitizer	Pro-Hapten	Non-Sensitizer	Sensitizer	Sensitizer	Sensitizer
3-Aminophenol	Sensitizer	Pro-Hapten	Non-Sensitizer	Sensitizer	Non-Sensitizer	Sensitizer
2-Methoxy-4-methylphenol	Sensitizer	Pro-Hapten	Non-Sensitizer	Non-Sensitizer	Non-Sensitizer	Sensitizer
Eugenol	Sensitizer	Pro-Hapten	Non-Sensitizer	Sensitizer	Sensitizer	Non-Sensitizer
Isoeugenol	Sensitizer	Pro-Hapten	Sensitizer	Sensitizer	Sensitizer	Sensitizer
	13 out of 13 chemicals correctly predicted	12 out of 13 chemicals correctly predicted	1 out of 5 pro-haptens correctly predicted	4 out of 5 pro-haptens correctly predicted	3 out of 5 pro-haptens correctly predicted	4 out of 5 pro-haptens correctly predicted

Table 1. The results from the OECD Toolbox predictions along with the traditional and microsome-modified assay results for the set of 13 chemicals

Conclusions

- There were three predicted non-sensitizers in the chemical set. These chemicals were predicted correctly in the traditional assays, and still predicted correctly after the addition of the microsomal pre-step. It is important to note that the microsomal pre-step did not lead to false positive predictions for the non-sensitizers.
- Of the five chemicals predicted to be pro-haptens, only one was correctly predicted in the traditional DPRA, but four of the pro-haptens were correctly predicted after the addition of the microsomal pre-step.
- Of the five chemicals predicted to be pro-haptens, three were correctly predicted in the traditional KeratinoSens™ assay, but four were correctly predicted after the addition of the microsomal pre-step.
- OECD Toolbox correctly predicted most non-sensitizers, sensitizers, and pro-haptens in the chemical set. OECD Toolbox appears to be a useful tool for making initial predictions and determining which assay procedures to perform in order to obtain accurate results.
- Future studies should incorporate mass spectroscopy to analyze the effectiveness of the microsome removal step with the organic solvents and centrifugation since residual microsomes in the mixtures may have adverse effects in both assays.
- Future studies should look at a larger chemical set consisting of many chemical families to test the prediction capabilities of OECD Toolbox and further refine the conditions of the microsomal pre-step.