Combining *in silico* and *in vitro* Methods to Improve the Accuracy of Skin Sensitization Predictions for Chemicals

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ABSTRACT

Given the complex cascade of events leading to skin sensitization, it is generally thought that an integrated testing approach combining multiple assays and *in silico* tools will be needed to fully replace the animal based methods. Recently, regulatory guidelines were published for two non-animal skin sensitization testing methods, the ARE-Nrf2 Luciferase Test Method (also referred to as the KeratinoSens™ Assay) and the Direct Peptide Rectivity Assay (DPRA). The KeratinoSens™ assay is a cell-based reporter gene method which identifies skin sensitizers by measuring the induction of luciferase under the control of the antioxidant response element (ARE) derived from the human AKR1C2 gene. The DPRA is an *in chemico* assay which identifies skin sensitizers based on their reactivity with synthetic peptides containing either lysine or cysteine. In this study we sought assess the skin sensitization potential of a set of 18 chemicals, including chemicals requiring metabolic activation, using *in silico* tools (OECD Toolbox) in combination with the DPRA method and the KeratinoSens™ assay. First, the chemicals were evaluated using the OECD Toolbox to identify structural alerts for each chemical and metabolites. If pro-haptens, chemicals which may cause skin sensitization following conversion into electrophilic molecules by skin enzymes, were identified by structural alerts then enzymatic steps using human liver microsomes were incorporated into the KeratinoSens™ assay and DPRA test method to improve accuracy as the standard protocols for these assays generally do not detect chemicals requiring metabolic activation. For the six chemicals requiring activation to become sensitizing, including cinnamic alcohol, use of the standard protocol led to false negatives whereas when the enzymatic steps were added the chemicals were correctly classified. The results indicated that testing strategies based on structural alerts and modification of standard protocols when necessary may allow for improved accuracy of skin sensitization predictions.

SKIN SENSITIZATION OVERVIEW

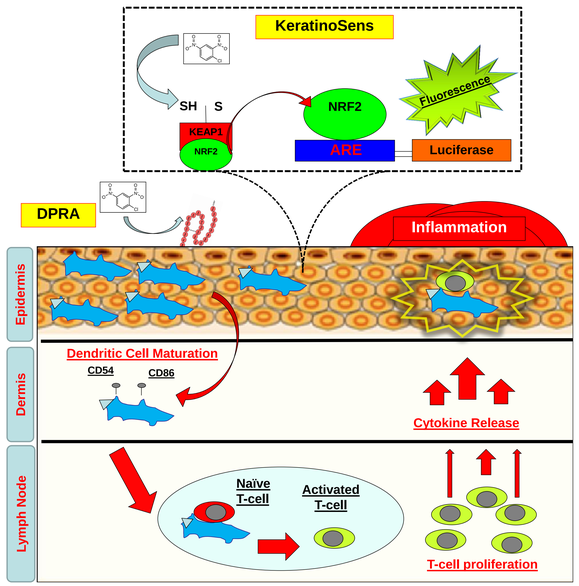

Figure 1. Adverse Outcome Pathway (AOP) for Skin Sensitization and Methods Addressing these key events. The diagram depicts the general pathway by which allergic contact dermatitis is elicited. The DPRA is an *in chemico* assay that identifies dermal sensitizers based on their reactivity with synthetic peptides containing either lysine or cysteine. The KeratinoSens™ assay is a cell-based reporter gene assay which identifies skin sensitizers by measuring the induction of luciferase under the control of the antioxidant response element (ARE) derived from the human AKR1C2 gene. The Organization for Economic Cooperation and Development (OECD) published the following test guidelines for non-animal skin sensitization testing on 5 February 2015: ARE-Nrf2 Luciferase Test Method (also referred to as the KeratinoSens™ Assay) (OECD TG 442D) and Direct Peptide Reactivity Assay (DPRA) (OECD TG 442C).

EXPERIMENTAL PLAN

ADDITION OF HUMAN LIVER MICROSOMES TO DPRA AND KERATINOSENS™

- An incubation of test chemical with microsomes was included prior to proceeding with each assay according to standard procedures.
- Various conditions were tested in the DPRA to determine optimal exposure conditions. Modified KeratinoSens™ assay procedures were based on Natsch *et al*.
- A set of 13 chemicals with known sensitizers, non-sensitizers, and pro-haptens were tested in both the traditional assays and the modified versions of the assays.
- The modified versions of the assays were performed by preparing the test chemical dilutions at 2X the top stock concentration used for the assays (200 mM for DPRA and 400 mM for KeratinoSens™). Each 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 (4 hours required for the test article, 3-Aminophenol only) 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 100 mM for DPRA and 200 mM for KeratinoSens™. The reaction mixtures were centrifuged at 300 x g for 10 minutes to collect the microsomes. The supernatant was used to continue the assays according to the standard protocols.

USING OECD TOOLBOX TO ASSESS PROTEIN BINDING

- OECD Toolbox was used to assess the protein binding potential of each test chemical and a metabolic simulator was used as a protein binding check for the metabolites. Chemicals were grouped as non-sensitizers, sensitizers, and pro-haptens.
- As a proof-of-concept, all test chemicals were evaluated using the standard DPRA and KeratinoSens™ assay methods and the modified versions (pre-incubation with microsomes) of each assay.

RESULTS

Table 1. OECD Toolbox and Traditional and Modified Assay 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

EXPERIMENTAL PLAN

EXAMINING ANTIOXIDANTS IN KERATINOSENS™

- We assessed the sensitization potential of four chemicals with antioxidant properties using the KeratinoSens™ assay.
- Direct antioxidants reduce reactive oxygen species (ROS) through their redox properties (ex. Tocopherols). Indirect antioxidants function by activating the Keap1-Nrf2-ARE pathway in order to express cytoprotective genes.
- Chemicals with indirect antioxidant properties may result in a false positive in the KeratinoSens™ assay.

RESULTS

Table 2. *In vivo* and KeratinoSens™ Assay Results for Chemicals with Antioxidant Properties.

Test Article	Classification *in vivo* data	KeratinoSens™ Results
Resveratrol	Non-Sensitizer	Sensitizer
Genistein	No Data	Sensitizer
L-Ascorbic Acid	Non-Sensitizer	Sensitizer
α-Tocopherol	Non-Sensitizer	Non-Sensitizer

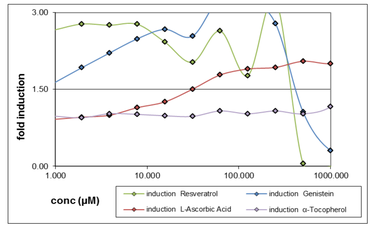

Figure 2. Dose response curves in the KeratinoSens™ Assay. Induction of the luciferase gene ≥1.5 fold above the solvent controls is indication of a skin sensitizer.

CONCLUSIONS & FUTURE DIRECTIONS

- In our dataset, 3 chemicals were non-sensitizers according to LLNA data. Due to a lack of protein reactivity, each chemical was identified as a non-sensitizer by OECD toolbox and were non-sensitizers when using the standard assay procedures and modified assay procedures for both assays. It is important to note that the pre-incubation step with microsomes did not lead to any false positive predictions in either assay.
- Five chemicals were sensitizers according to LLNA data and the OECD toolbox. Of those, all were identified as sensitizers using all tools/assays, with the exception of Limonene. Limonene is a pre-hapten, requiring oxidation by air to elicit a positive response, and is therefore subject to variable responses.
- Five chemicals were positive per LLNA, and identified as pro-haptens by OECD toolbox. Using standard DPRA and KeratinoSens™ assay methods, in many cases false negative predictions occurred as expected. However, when the modified assay procedures were used the accuracy of the predictions improved. Using a cautious approach where a positive in either assay leads to a positive prediction, all pro-haptens would be correctly classified as sensitizers.
- In the future, we aim to further develop this strategy: First, determining from structural alerts whether or not the chemical is a pro-hapten. Then, for chemicals which are not pro-haptens, performing the DPRA and KeratinoSens™ assay according to standard procedures. For chemicals identified as pro-haptens, a pre-incubation with microsomes will be included. Then, a positive in either assay would led to the classification of a sensitizer, or a third assay (such as h-CLAT) may be performed in the case of mixed results.
- For our investigation of antioxidants, some may activate the ARE-Nrf2 pathway due to their antioxidant properties resulting in false positive predictions in the KeratinoSens™ assay. In these cases, other assays may need to be included in a tiered testing strategy. Further investigation is required on the evaluation of antioxidants in the KeratinoSens™ assay.

REFERENCES

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