Integrated non-animal testing methods for skin sensitization characterization of chemicals

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Abstract

The adverse outcome pathway for skin sensitization describes four key events (KE) involved in development of allergic contact dermatitis: covalent binding of chemicals to host proteins, keratinocyte activation, dendritic cell activation and T cell proliferation. Traditional methods for testing skin sensitization (e.g. animal models) require concomitant triggering of all key events in single experimental system. Successfully replacing animal models in skin sensitization risk assessment requires integrated approaches where each key event is assessed independently in targeted non-animal models. The Direct Peptide Reactivity Assay (DPRA) has been the first *in chemico* method validated for the characterization of the KE1, the haptenation process. Two additional *in chemico* methods (HTS-DCYA and NMR-DCYA) to test KE1 have been developed and validated by the UM-NCNPR research team. Elicitation of inflammatory pathways in keratinocytes (KE2) can be measured by the KeratinoSens assay, while the human Cell Line Activation Test (hCLAT) has been adopted to characterize the induction of KE3 (activation of responses in dendritic cells). The methodologies, applicability, and pitfalls of these non-animal testing approaches for skin sensitization are presented.

Introduction

Skin sensitization is an important toxicological end-point to be characterized for the safety evaluation of chemicals which can come into contact with the skin, such as ingredients used in the formulations of drugs, cosmetics, fragrances, traditional herbal remedies, and other household products. Skin sensitization is a complex form of immuno-toxicity involving numerous molecular pathways highly controlled at intra- and inter-cellular levels. For this reason, the development of stand-alone alternatives to animal tests is challenging.

In 2012, a new program focused on the development of Adverse Outcome Pathways (AOP) was launched by the Organisation for Economic Co-operation and Development (OECD). The AOP approach relies on the use of mode-of-action to understand and predict the potential toxicological effects of chemicals, and on the integration of data from chemical, biological, and computational models for risk assessment. Currently, skin sensitization AOP identifies four major key events leading to allergic contact dermatitis (ACD) as the clinical outcome caused by exposure to skin sensitizers.

Successfully replacing animal models in skin sensitization risk assessment requires integrated approaches where each key event is assessed independently in targeted non-animal models. Numerous *in chemico*, *in vitro*, and *in silico* approaches have been proposed to replace, reduce and refine the use of animals in dermato-toxicology. At the present, two *in chemico* method (DPRA and ADRA) and few *in vitro* methods (KeratinoSens, LuSens, hCLAT, U-SENS and IL-8 Luc Assay) have recommended by OECD guidelines.

In the present work, a brief overview of the main three validated methods (DPRA, KeratinoSens assay and hCLAT) is presented along with two innovative *in chemico* methods (HTS-DCYA and NMR-DCYA).

Methods for Skin sensitization

DPRA

Rationale: Small heptapeptides containing reactive cysteine or lysine are used as models to characterize skin protein reactivity. Test chemicals are incubated with each peptide separately for 24 h, then the degree of reactivity is determined by the rate of peptide depletion, evaluated by HPLC-UV quantification of the peak area corresponding to the unreacted peptide. Skin sensitizers reacting with the peptides will result in higher percentage of peptide depletion.[1]

Outcome: Characterization of haptenation process (KE1, OECD TG 442C).

Advantages: Simple workflow, compatible with water soluble compounds. Validated toward both Lys- and Cys-peptides.

Disadvantages: Low throughput, insensitive, indirect measurement of reactivity, not applicable to mixtures, false positive caused by non-specific peptide losses and by long incubation times.

HTS-DCYA

Rationale: A model fluorescent thiol, dansyl cysteamine (DCYA), is used as a surrogate for skin proteins, similarly to the DPRA peptides. Skin sensitizers react with DCYA resulting in the generation of fluorescent DCYA-adducts, which can be quantified using end-point fluorescence assays. The thiol-binding potential of the test article is thus tested by high throughput fluorescence assays using a microplate reader [2].

Outcome: Characterization of the ability of the test article to covalently bind to skin proteins (haptenation, KE1).

Advantages: Rapid, sensitive, fewer false positives, high-throughput, compatible with mixtures including plant extracts. Direct quantification of reaction adducts.

Disadvantages: Poor solubility of highly polar compounds, no structural information.

NMR-DCYA

Rationale: Thio-reactivity of skin sensitizers is characterized using DCYA as a model thiol. The degree of reactivity is determined through quantification of the electrophile depletion (*DoEs)* using ^{1}H-NMR studies. Electrophilic skin sensitizers will promote changes in proton resonances at the reactive site, or in their proximity. The areas corresponding to the resonance signals of interest is quantified over time. Sensitizers reacting with DCYA will result in higher electrophile depletion (high *DoEs* value).[3]

Outcome: Structural characterization of the haptenation process (KE1).

Advantages: Direct measurement of reactivity, suitable for kinetic studies and testing of mixtures. Useful structural information can be gathered.

Disadvantages: Low throughput, relatively insensitive, long reaction times (for slow or non-reacting compounds).

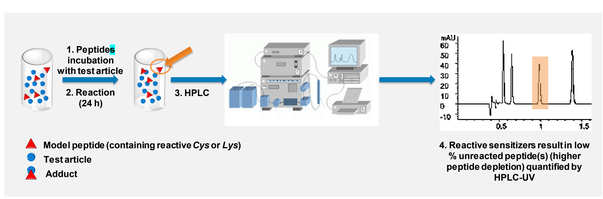

Figure 1. Direct Peptide Reactivity Assay.

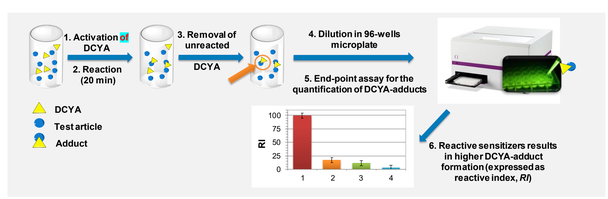

Figure 2. HTS-DCYA method

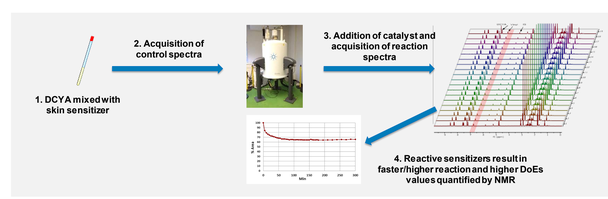

Figure 3. NMR-DCYA method

KeratinoSens™

Rationale: Skin sensitizers can induce expression of genes regulated by the antioxidant response element (ARE). The activation of Keap1-Nrf2-ARE cell signaling pathways is associated with inflammatory responses and in the skin, occurs in keratinocytes. An immortalized adherent cell line derived from HaCaT human keratinocytes is used. The cell line is stably transfected with a selectable plasmid that contains a luciferase gene under the transcriptional control of a constitutive promoter fused with an ARE element from a gene that is known to be up-regulated by contact sensitizers.[4]

Outcome: Characterization of the activation of early inflammatory responses in keratinocytes (KE 2, OECD TG 442D).

Advantages: Characterization of early inflammation responses on whole cell models.

Disadvantages: Limited applicability with cytotoxic compounds/mixtures, low sensitivity to lysine-selective sensitizers.

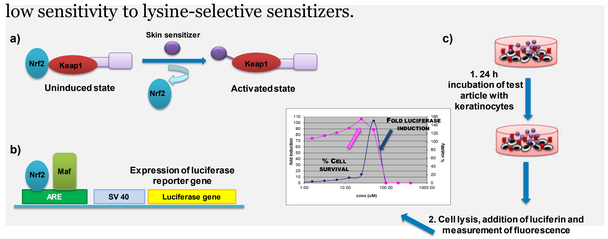

Figure 4. **a**) Keap1 activation mediated by Nrf2, **b**) construct of the luciferase gene containing a constitutive promoter, and **c**) methodology.

hCLAT

Rationale: This assay tests activation of dendritic cells (DC) following exposure to the test article. The test is performed using a human monocytic leukaemia cell line (THP-1). Changes in the expression of cell surface markers associated with the process of activation of monocytes and DC (CD86 and CD54) are quantified by cytofluorimetric assays that measure changes in relative fluorescence intensity (RFI).

Outcome: Characterization of the potential of the test article to promote activation of inflammatory responses in dendritic cells (KE 3, OECD TG 442E).

Advantages: Characterization of later cellular events *in vitro*, some pre- or pro-hapten can be detected.[5]

Disadvantages: Low throughput, long experimental procedure, limited metabolic capacity and applicability to cytotoxic compounds/mixtures.

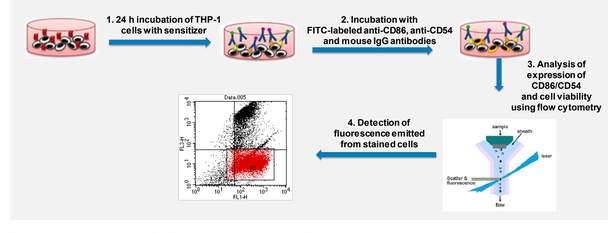

Figure 5. Human Cell Line Activation Test

Conclusion

The three OECD methods and two novel methods developed in collaboration with the University of Mississippi – NCNPR discussed above may be useful for integrated testing approaches of skin sensitization using non-animal alternative methods.

REFERENCES

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