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**The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins.
Part 2: Use of the AOP to Develop Chemical Categories and Integrated Assessment and Testing
Approaches**

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**THE ADVERSE OUTCOME PATHWAY FOR SKIN SENSITISATION
INITIATED BY COVALENT BINDING TO PROTEINS**

**PART 2: USE OF THE AOP TO DEVELOP CHEMICAL CATEGORIES
AND INTEGRATED ASSESSMENT AND TESTING APPROACHES**



INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

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EXECUTIVE SUMMARY

Knowledge of the adverse outcome pathway (AOP) for skin sensitisation elicited by covalent binding of substances to proteins has evolved rapidly over the past decade and may be summarised as eleven steps which include four events that are recognised as key ones. This document outlines a summary of the AOP (a more in-depth description can be found in part I) and focuses on how to use the AOP in the context of forming chemical categories or for developing Integrated Approaches for Testing and Assessment.

The principle of building chemical categories based on the categorisation mechanisms implemented in the OECD QSAR Toolbox is well advanced. It can be expanded to a chemical category approach where the applicability domain of the category based on AOP (e.g. for skin sensitisation) is created by the overlapping of chemical categories each based on a chemical and toxicological/biochemical endpoints reflecting a particular key event of an AOP.

The document also addresses the efforts to build Integrated Approaches to Testing and Assessment for skin sensitisation in line with the present AOP. However no concerted efforts at OECD have yet been initiated to build internationally agreed upon IATA for skin sensitisation.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

BACKGROUND

In December 2010, the OECD held the *Workshop on Using Mechanistic Information in Forming Chemical Categories*. The purpose of the Workshop was to acquire scientific input which would guide further development and use of the concept of adverse outcome pathways (AOPs). One aim of the Workshop was to propose how scientific information on mechanism or mode of toxic action could be organised into key events and processes within an adverse outcome pathway to aid the formation of chemical categories (OECD, 2011a). For the purposes of the Workshop an AOP was defined as a narrative which delineates the documented, plausible, and testable processes by which a chemical induces molecular perturbations and the associated biological responses which describe how the molecular perturbations cause effects at the subcellular, cellular, tissue, organ, whole animal and (if required) population levels of observation (OECD, 2011a).

As part of the Workshop, several case studies were presented and formed the basis of the discussions. Based on the strengths and weaknesses of the case studies, a series of best principles were proposed for the development of an AOP for use in grouping chemicals (OECD, 2011a). These principles included that an AOP should be based on a single, defined molecular initiating event and linked to a stated *in vivo* hazard outcome(s). Any template used for AOP development should include a summary of the experimental support for the AOP, as well as a statement of: 1) the level of qualitative understanding of the AOP; 2) consistency of the experimental data; 3) confidence in the AOP, and 4) level of quantitative understanding of the AOP (OECD, 2011a).

Moreover, it was agreed that the assessment of the qualitative understanding should include documented identification of: 1) the molecular initiating event and molecular site of action; 2) key cellular responses; 3) target tissue/organ(s) and key tissue or organ responses; 4) key organism responses; both physiological and anatomical, and 5) (if required) key population responses (OECD, 2011a).

It was further noted that the assessment of the evidence in support of an AOP should include criteria based on the IPCS mode of action framework (Boobis et al., 2008).

Confidence in an AOP would be ascertained by addressing the following questions:

1. How well characterized is the AOP?
2. How well are the initiating and other key events causally linked to the outcome?
3. What are the limitations in the evidence in support of the AOP?
4. Is the AOP specific to certain tissues, life stages / age classes?
5. Are the initiating and key events expected to be conserved across taxa?

An assessment of the quantitative understanding should include documented identification of: 1) the molecular initiating event; 2) other key events; 3) response-to-response relationships required to scale *in vitro* effect(s) to *in vivo* outcomes (OECD 2011a).

Lastly, it was agreed that identifying the chemical space was critical for the formation of categories and this is ascertained by addressing the following questions:

1. What chemicals trigger and do not trigger the molecular initiating event in the AOP?
6. What chemical features increase / decrease the probability of a chemical being associated with an AOP?
7. Are there similar key events caused by the chemicals that could tie them to a common AOP?
8. Are their differences among the chemicals that could lead to sub-categorization?

The Workshop participants agreed on a series of recommendations on how to advance the use of the AOP concept (OECD, 2011a). These recommendations included engaging toxicologists and other scientists in discussions of AOPs in an effort to foster interactions by developing AOPs for well-established effects, such as skin sensitisation.

Briefly, an adverse outcome pathway (AOP) is the sequence of events from chemical structure through the molecular initiating event to the *in vivo* outcome of interest. AOPs are representations of existing knowledge concerning the linkage(s) between a molecular initiating event and an adverse outcome at the individual or population level. While AOPs may be initially depicted as linear procedures, the amount of detail and linear character of the pathway between a molecular initiating event and adverse outcome can vary significantly, especially for human health endpoints where effects are the result of multiple organ interactions (e.g. skin sensitisation), multiple events (e.g., repeat dose toxicity), which accumulate over time (e.g. neural toxicity), or are particular to a life stage of an organism (e.g. developmental toxicity).

AOPs include the fact that chemical interactions are at the molecular level and not at the whole animal level. Thus, adverse effects observed *in vivo* are the result of many biological responses, as well as the chemical structure of the toxicant. Hence, AOPs are designed to avoid mixing information from multiple mechanisms (i.e. different molecular initiating events which can cause the same *in vivo* outcome through different AOPs). When the molecular initiating event is closely linked to an observed *in vivo* response, one can easily develop a chemical category or derive a traditional quantitative structure-activity relationship (QSAR) between the *in vivo* endpoint and chemical structures (e.g. acute fish toxicity). Within the OECD QSAR Toolbox, hereafter called the Toolbox, (www.oecd.org/env/hazard/qsar) this is accomplished by profiling (i.e. formation of a chemical category) using mechanistic profilers and subsequently filling data gaps through read-across or trend analysis (i.e. a simple QSAR) from *in vivo* databases. However, such direct linkages are not common among human health effects. Moreover, without a transparent description of a plausible progression of adverse effects at the different levels of biological organization, it is difficult to reliably form chemical categories based on 2-dimensional chemical structures and subcategories based on similarity in toxicological behaviour, two crucial aspects of the Toolbox. AOPs aid in resolving these problems by grouping chemicals based on both up-stream chemical and down-stream biological processes. AOPs shift the emphasis for category formations based on just intrinsic chemical activity to chemical activity plus the key events that occur across the different levels of biological organization. In this way, AOPs form a solid mechanistic reasoning to support the use of read-across and categories, thus waiving actual toxicity testing of a substance and can be exploited to improve the Toolbox by basing more toxicologically relevant profiles on established AOPs.

INTRODUCTION

Skin sensitisation is a term used to denote the regulatory hazards known as human allergic contact dermatitis or rodent contact hypersensitivity, an important health endpoint taken into consideration in hazard and risk assessments of chemicals. Skin sensitisation is a well-studied adverse outcome (see Aeby et al., 2010; Basketter and Kimber, 2010; Adler et al., 2011 for recent reviews), of which aspects have been the subject of hundreds of scientific articles over the past decade. While non-covalent reactions with metals and Redox cycling have been linked to skin sensitisation, the majority of the research has focused on chemicals which can form covalent bonds with thiol and/or primary amino groups present in skin proteins. While the details of the AOP will vary with the preferred target substituent and the chemical reaction particular to the chemical under evaluation, much of the downstream biological responses are similar.

Skin sensitisation is an immunological process that is described in two phases the induction of sensitisation and the subsequent elicitation of the immune reaction (Kimber et al., 2002a). The first phase includes a sequential set of events which are described in this AOP. While there is general agreement regarding the events, understanding of the underlying biology of many of the key events remains incomplete. However, unlike the principles and concepts in the IPCS MOA Framework (Boobis et al., 2008), complete understanding of all events are not required for utilizing an AOP for forming a chemical category. Due to the biological complexity (e.g. multiple organs and multiple cell types), skin sensitisation has historically been and continues to be evaluated with *in vivo* tests although alternative methods are under development and pre-validation (see Aeby et al., 2010; Adler et al., 2011).

Whereas some of the processes outlined in this document also play a role as part of the skin's immune response towards metals and allergens of biological origin, this AOP focuses on organic chemical agents, in particular, ones that react with thiol (i.e. cysteine) and primary amines (i.e. lysine). Thus, the crucial role of the Toll-like receptor 4 in sensitisation to nickel (Schmid et al., 2010) would be described under a separate AOP, which then also may explain why nickel is not well-classified in the currently most applied *in vivo* test for skin sensitisation, the murine Local Lymph Node Assay (LLNA).

In the induction or acquisition phase, the chemical or allergen penetrates the outer epidermis of the skin. During this passage, chemicals are potentially subject to biotransformation processes which can both increase or decrease the allergenic potential. The parent chemical or a metabolite then forms a stable conjugate with carrier proteins located within the skin. This stable conjugate, or hapten-protein complex, is then processed by the epidermal dendritic cells (i.e. Langerhans cells) and dermal dendritic cells, which subsequently mature and migrate out of the epidermis to the local lymph nodes. The hapten-protein complexes can also react with and activate response in keratinocytes, which in turn may interact with dendritic cells. In the lymph nodes, the dendritic cells display major histocompatibility complex molecules, which include part of the hapten-protein complex to naive T-lymphocytes (T-cells). This induces differentiation and proliferation of allergen chemical-specific memory T-cells, some of which re-circulate throughout the body (Figure 1).

The elicitation or challenge phase occurs following a subsequent contact with the same allergen. Again, the hapten-protein conjugate is formed and subsequently taken up by epidermal dendritic cells, as well as other

antigen-presenting cells. The circulating allergen-specific, activated memory T-cells are triggered to secrete specific cytokines, which induce the release of inflammatory cytokines and mobilization of cytotoxic T-cells, as well as other inflammatory cells

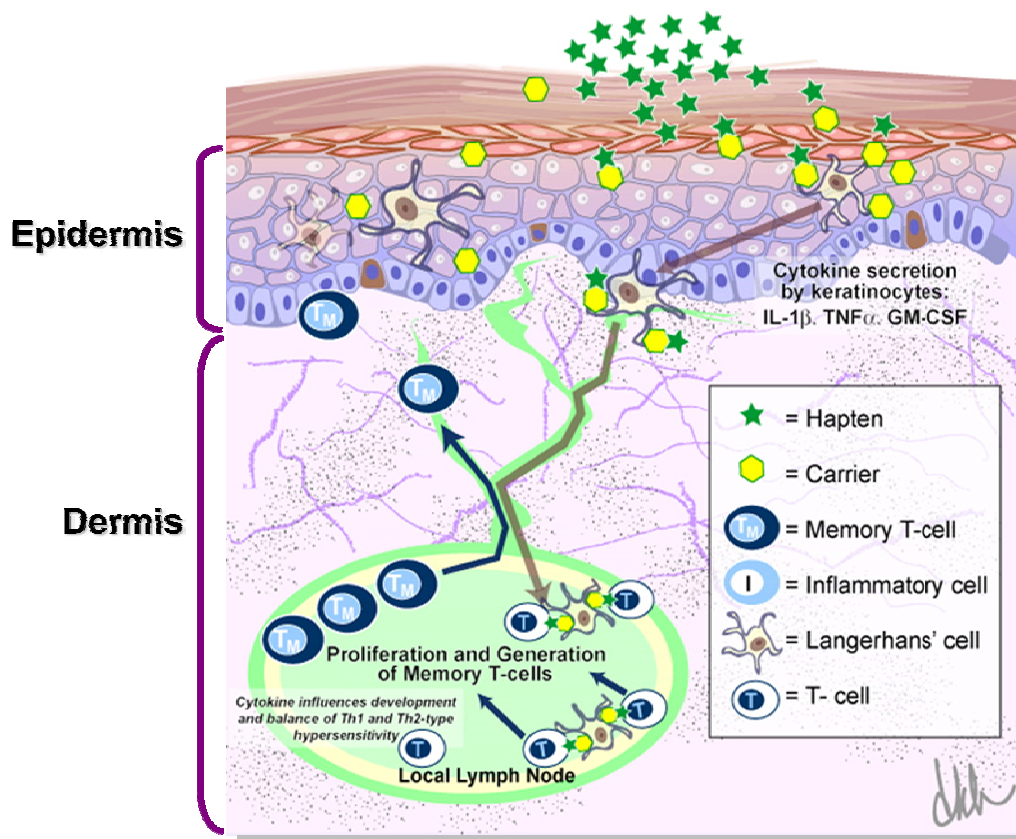


Figure 1. The Induction Phase of Skin Sensitisation.

from the circulating blood. These cells migrate to the epidermis of the skin and induce the distinguishing local inflammatory response of red rash, blisters and welts, and itchy and burning skin (Figure 2).

With exceptions, the uncertainty in identification of positive and negative responses with whole animal assays has been met with acceptance by most regulators and other stakeholders. While earlier efforts developed potency categories by optical inspection of guinea-pigs, it is with the introduction of the murine LLNA (see Basketter et al., 1996; Kimber et al., 2002b) and the EC3 value (the effective concentration of test substance needed to induce a stimulation index of three) (see Basketter et al., 1999), that there was a greater emphasis on placing chemicals into potency groups (e.g., extreme, strong, moderate, weak, and nonsensitizers).

Today the LLNA is often used as a benchmark against which new approaches are compared. While the LLNA may help to show the validity of new approaches, knowing the accuracy of the LLNA is incomplete (Basketter et al., 2009) has sparked interest in a Weight of Evidence evaluation for skin sensitisation (Ball,

2011). In either case, efforts to replace *in vivo* testing with a single or combination of many alternative methods are on-going (see Adler et al., 2011). Unlike *in vivo* test systems, which are intact dynamic systems, alternative approaches, including *in chemico* and *in vitro* methods, are relatively static and focus on characterizing or quantifying discrete chemical, biochemical and/or cellular events for subsequent use in an AOP-directed integrated approach to testing and/or assessment.

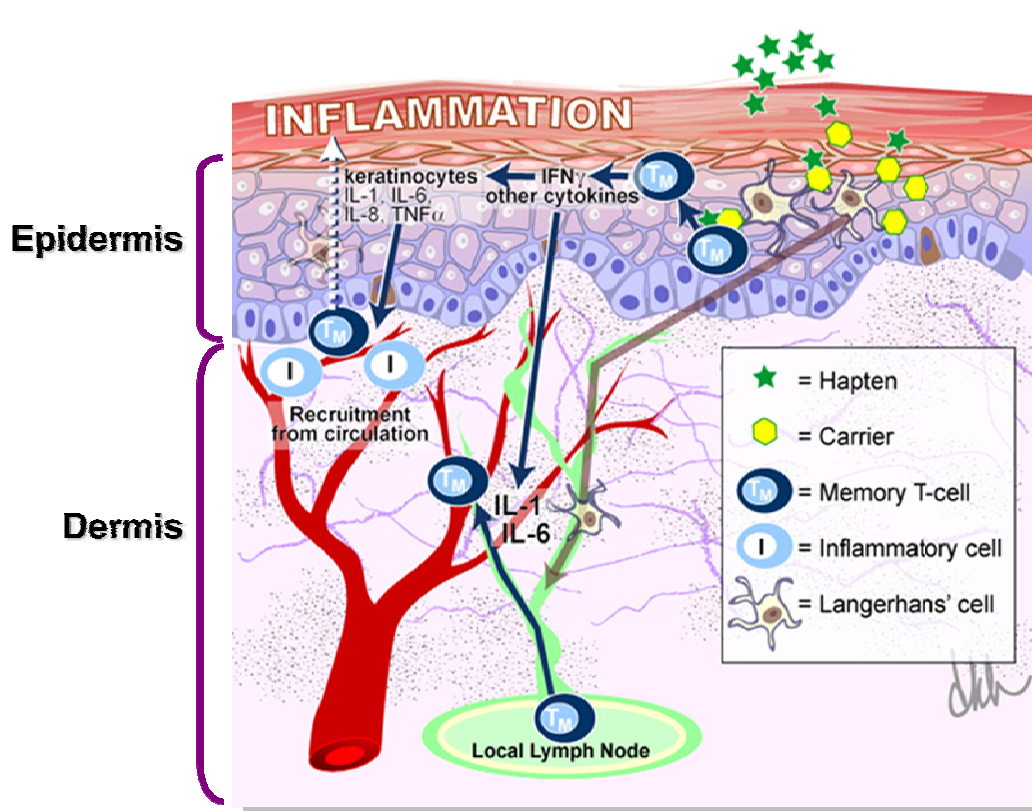


Figure 2. The Elicitation Phase of Skin Sensitisation.

Earlier work on the molecular basis of skin sensitisation was reviewed by Lepoittevin et al. (1998), since then our knowledge of skin sensitisation has continued to expand. Recent reviews (see Gerberick et al., 2008; Karlberg et al. 2008; Vocanson et al., 2009; Aebly et al., 2010; Basketter and Kimber, 2010; Adler et al., 2011) repeatedly stress the same key steps leading to sensitisation. These events include skin bioavailability and metabolism, hapten formation (i.e., the ability of a chemical to react with skin proteins), epidermal inflammation via keratinocyte and/or dendritic cell signalling, dendritic cell activation, maturation and migration, and T-lymphocyte (T-cell) proliferation.

SUMMARY OF THE AOP

Knowledge on the AOP for skin sensitisation elicited by covalent binding of substances to proteins has evolved rapidly over the past decade and may be summarized as:

- Step 1)** The target substance must be bioavailable (i.e. it must penetrate the stratum corneum of the skin).
- Step 2)** The target substance must be a direct-acting electrophile, be converted from a non-reactive substance (pro-electrophile) to a reactive metabolite via metabolism, or be converted from a non-reactive substance (pre-electrophile) to a reactive derivative via an abiotic process, typically oxidation.
- Step 3)** The molecular sites of action are targeted nucleophilic sites in proteins (e.g. cysteine and lysine residues) in the epidermis.
- Step 4)** The molecular initiating event is the covalent perturbation of dermal proteins, which is irreversible (i.e. formation of the hapten-protein complex or complete antigen). *In vivo*, this event is associated with the production of a specific memory T-cell response.
- Step 5)** Biochemical pathways affected by the definitive electrophile's action on the molecular targets are incompletely known but often include inflammation-related pathways, including the mitogen-activated protein kinase signalling pathway and the oxidative stress response pathway, especially in keratinocytes and dendritic cells.
- Step 6)** The cellular/tissue-level outcomes are incompletely known but include epidermal responses such as: 1) immune recognition of chemical allergens by keratinocytes, specialized epidermal dendritic cells (i.e. Langerhans cells) and dermal dendritic cell; 2) responses in the form of expression of specific cell surface markers, such as adhesion molecules, chemokines, and cytokines such as IL1 β or IL-12p70 are typically taken as evidence of dendritic cell maturation.
- Step 7)** The organ-level responses include:
 - a) Dendritic cell migration to the lymph node, where they present major histocompatibility complex (MHC) molecules to naive T-lymphocytes (T-cells), and
 - b) T-cell differentiation and proliferation as allergen-specific memory T-cells.
- Step 8)** The target organ(s) are the skin and local lymph nodes; the target cell populations are the immune cells, especially effector T-cells.
- Step 9)** The key physiological response is acquisition of sensitivity.
- Step 10)** The key organism response is dermal inflammation upon receiving the substance challenge in the elicitation phase. This response is associated with stimulation of specific memory T-cell produced in the induction phase.
- Step 11)** The overall effect on mammals is allergic contact dermatitis in humans, or its rodent equivalent contact hypersensitivity.

A summary of the qualitative understanding of the AOP is presented in Table 1, which lists the key events, documentation of the experimental support for each event, and an evaluation of the strength of scientific evidence for that event.

Flow diagram of the Intermediate Events Associated with the AOP

A flow diagram of the pathways and intermediate steps associated with skin sensitisation is presented in Figure 3. The ‘pathway’ explanations are taken from OECD (2011a).

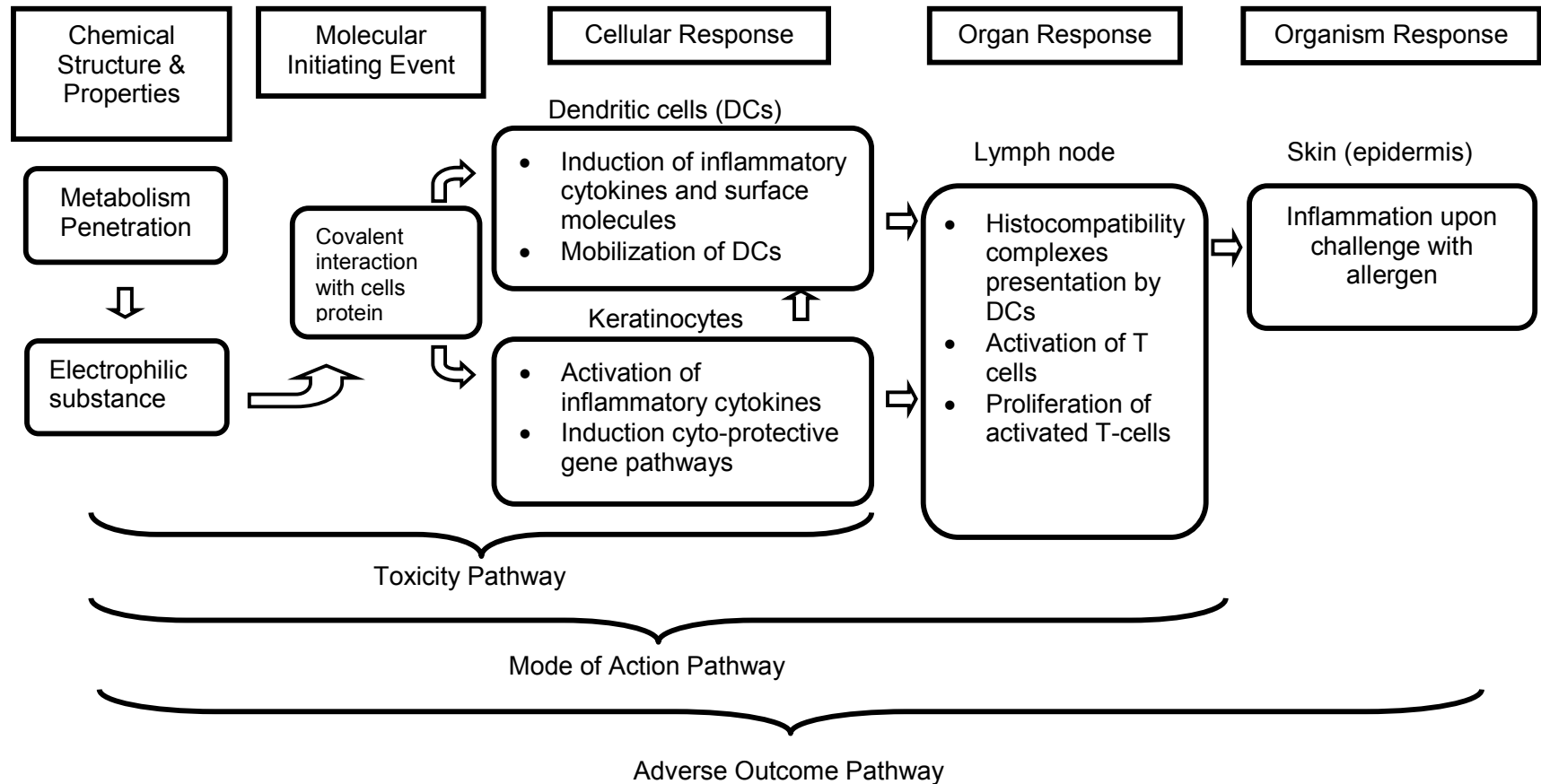


Figure 3. Flow diagram of the pathways associated with skin sensitisation.

Summary of the Key Events of the AOP

A summary of the qualitative understanding of the AOP is presented in Table 1 which lists the key events, documentation of the experimental support for each event, and a subjective evaluation of the strength of the scientific evidence for that event.

Table 1. Summary of the Key Events in the AOP.

Key Events	Experimental Support	Strength of Evidence
Key Event 1 (initial event)	Site of action proteins (see Karlberg et al., 2008; Wong and Liebler, 2008). Covalent binding at cysteine and/or lysine (see Roberts and Natsch, 2009; Schwöbel et al., 2011).	Strong; well-accepted mode of toxic action associated with skin sensitisation with 100s of chemicals evaluated for binding in quantitative endpoints.
Key Event 2	Keratinocyte inflammatory responses (Van Och et al., 2005; Corsini et al., 2009). Gene expression of antioxidant response element in keratinocytes (see Natsch and Emter, 2008; Emter et al., 2010; McKim et al., 2010; Vandebriel et al., 2010).	Adequate; well-accepted cytokine IL-18 associated with skin sensitisation. Strong; well-accepted cell signalling pathway antioxidant/electrophile response element ARE/EpRE-dependent pathways with 10's of compounds evaluated in a quantitative endpoint.
Key Event 3	Activation of dendritic cells (see Ryan, 2007; dos Santos et al., 2009; Vandebriel and Van Loveren, 2010; Ashikaga et al., 2010; Kimber et al., 2011).	Adequate; well-accepted expressions of cell adhesion and co-stimulatory molecules, and cytokines associated with skin sensitisation; various endpoints; 10s of compounds evaluated for endpoints which tend to be qualitative rather than quantitative.
Key Event 4	T-cell proliferation (see (Gerberick et al., 2005; Kern et al., 2010)	Strong; two decades of development and testing with the Local Lymph Node Assay (LLNA); 100s of chemicals evaluated in a quantitative endpoint.
Adverse Outcome	Allergic contact dermatitis in humans or its rodent equivalent contact hypersensitivity.	Well-Established; test guidelines and data for guinea-pig, as well as data for human.

FORMING CHEMICAL CATEGORIES FOR SKIN SENSITISERS

There are many national and international efforts to assess the hazards or risks of chemical substances, in particular, industrial organic compounds to humans and the environment. It is generally agreed that establishing the adequacy of information for each endpoint to be evaluated is the first step in the assessment process. If adequate information is not available, which is often the case, then additional data is required to complete the assessment. For a number of reasons including time, resources, and animal welfare, testing, especially *in vivo* testing is not always the first option in securing additional data. One approach to filling data gaps in an assessment, which is gaining favour, is to consider closely related chemicals as a group or “chemical category”, rather than as individual chemicals (OECD, 2007).

In its widest context, a chemical category can be thought of as a group of chemicals whose physicochemical properties, human health and/or environmental toxicological properties, and/or environmental fate properties are likely to be similar or follow a regular pattern. As such, a chemical category can be expressed by a matrix consisting of the chemicals included in the category and corresponding sets of chemical properties and toxicological endpoints data.

The crucial stage in building chemical categories for skin sensitisers or any other type of toxicants is the ability to select ‘similar’ chemicals (Diderich, 2010). Ideally similarity should be based on a common mechanism of mode of action; such mechanism or modes may have a chemical reaction basis or a toxicological basis (OECD, 2007). Central to using a chemical category for predictive purposes is the ability to ascertain whether the target chemical resides in the applicability domain or the appropriate prediction space for the category (i.e. is the chemical in question within the boundaries of the category).

The proposed AOP for skin sensitisation elicited by covalent binding to proteins provides a transparent, chemical and biological mechanistic-based roadmap leading from a molecular initiating event to the *in vivo* outcome and provides a framework for developing or refining chemical categories. Whereas an *in vivo* model gives an integrated output, an *in vitro* system provides information only on what happens at a particular event along the pathway. This fact has led to the argument that to assess endpoints such as skin sensitisation, there is a need to have multiple *in vitro* tests similar to the concept of toxicity for the 21st Century (Andersen and Krewski, 2009) whose data are in some way integrated (see Jowsey et al., 2006; Basketter and Kimber, 2009). It is intuitive that the greater the number of key events that are experimentally consistent within a particular chemical category, the greater the weight-of-evidence and confidence one has in the prediction of the *in vivo* outcome by read-across for chemicals in the category that have not been tested *in vivo*. Similarly, the greater the number of chemicals tested for a key event for a chemical category also increases the weight-of-evidence and confidence one has in the prediction.

Since the formation of the hapten-protein complex is the molecular initiating event for skin sensitisation via chemical binding to proteins, chemical structure information regarding the possibility of a target chemical to such adducts is particularly useful in grouping chemicals into mechanistic categories. Skin sensitisers are generally electrophilic in nature and have the ability to react with nucleophilic amino acid residues. Therefore, being able to discern this from the molecular structure would be a first step in categorisation.

Conventional organic chemistry (see Jacobs, 1997) divides reaction between electrophilic and nucleophilic chemicals into mechanistic domains; acylation, aromatic nucleophilic substitution (S_NAr), bimolecular nucleophilic substitution (S_N2), Michael addition, Schiff base formation, and unimolecular nucleophilic substitution (S_N1) (Aptula and Roberts, 2006; Enoch, 2011). Realising the importance of chemical reactivity, efforts were made to divide the LLNA data into mechanistic domains (Roberts et al.,

2007). However, even after separating the LLNA data into chemical mechanisms, it is apparent that *in vivo* sensitisation can be observed with some but not all members of a particular mechanistic domain. For example, even within a common reactive mechanism (Michael addition) compounds do not display a regular pattern in regards to *in vivo* sensitising potency (Roberts and Natsch, 2009). This has led to proposing that it can be helpful to divide the chemical category into sub-categories based on more restrictive analogous chemical sets (Schultz et al., 2009).

Much information related to the chemical basis for electrophilic reactions has been documented (Enoch et al., 2011) and converted to Simplified Molecular Information Line Entry Structures (SMILES)-based structural alerts (OECD, 2011) which have been computationally coded into computer-assisted tools within version 2.2 of the OECD QSAR Toolbox (www.oecd.org/env/hazard/qsar).

Since the formation of the hapten-protein complex is the molecular initiating event for skin sensitisation via chemical binding to proteins, information regarding the rate of formation for such adducts (obtained either experimentally or by calculations) is also extremely useful in grouping chemicals into mechanistic categories. A number of *in chemico* databases reporting measurements of reactive currently exist. The direct peptide reactivity assay of Gerberick et al. (2004) reports experimental % binding data measured with cysteine and lysine as model nucleophiles. One of the largest databases report RC50s values for GSH binding (Yarbrough and Schultz, 2007). Specifically, a quantitative reactivity database of nearly 250 chemicals meeting the SMILES-based structural criteria for Michael Addition and covering nearly 4 orders of magnitude is mapped to version 2.2 of the OECD QSAR Toolbox. A similar database with more than 150 chemicals meeting the 2-dimensional structural criteria for S_N2 substitution, especially the halo-sp³C S_N2 reaction (Roberts et al., 2010), and covering more than 3 orders of magnitude is also mapped to the Toolbox. Recently, a GSH reactivity database for S_NAr reaction has been donated to the Toolbox.

Using these data, the chemical within one of the above-noted mechanistic domains can be further grouped into sub-domains or mechanistic clusters. These mechanistic clusters can be formed on the basis of a common reactive centre (i.e., the site of nucleophilic attack which may be activated by a variety of substituents). For example, an alkene (C=C) acting as a Michael acceptor due to the influence of a polarising moiety, forms a mechanistic cluster, while a separate cluster are the alkynes (C#C) polarised by the same set of substituents. As important, a number of mechanistic groups may make up a mechanistic cluster. Mechanistic groups consist of very closely related chemicals. For example, benzoquinones and quinone-methides belong to the same mechanistic cluster but different mechanistic classes. The ability to identify clusters and classes for Michael addition in the form of a protein binding potency profiler is also found within version 2.2 of the OECD QSAR Toolbox (www.oecd.org/env/hazard/qsar).

Further efforts to build chemical categories based on bioactivity signature will be possible once *in vitro* databases of sufficient depth and breadth are made available. For example, having experimental Keap1/Nrf2/ARE/EpRE gene expression data (e.g. KeratinoSen) and dendritic cell activation data (e.g. experimental flow cytometry results for CD86 and CD54 expression in THP-1 cells (h-CLAT) or CD86 expression in the myeloid U937 skin sensitisation test (MUSST)), would allow for further refinement of a chemical category or at least add a greater weight-of-evidence to and a greater confidence in a category. One can envision a chemical category approach to skin sensitisation assessment, where the applicability domain of the chemical category used in data gap filling was created by the overlapping of chemical categories based on different chemical and toxicological similarities, each based on an endpoint reflecting a particular key event of an AOP (Figure 2).

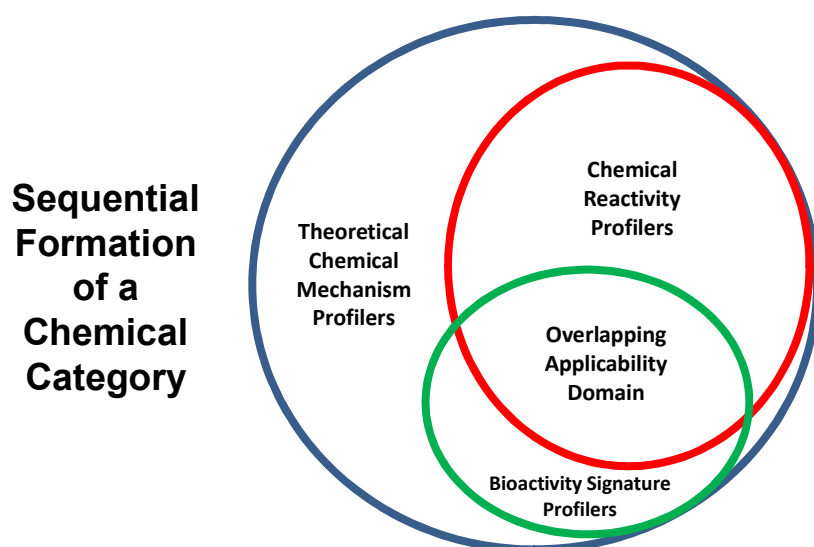


Figure 2. Sequential profiling to form a single chemical category meeting several chemical and toxicological criteria.

INTEGRATED APPROACHES TO TESTING AND ASSESSMENT (IATA) FOR SKIN SENSITISERS

IATA is a concept of toxicity testing, and hazard and risk assessment that incorporates different types of chemical and toxicological data into the decision-making process. This reduces the number of chemicals that are subjected to regulatory tests. IATAs are typically progressive and may be tiered- or non-tiered-evaluations. They start with hazard-based hypotheses about the plausible toxicological potential of a target chemical or a chemical category. Existing information is then combined with computer modelling and data from diagnostic assays (e.g. *in vitro* and omics) to identify further information needs specific to the target chemical or chemical category. The concept of the IATA raises the issue of how the particular *in silico*, *in chemico*, and/or *in vitro* tests or data used in the IATA are justified? The AOP is a means of providing that relevance.

Among the first effort to integrate data for key events along the AOP for skin sensitisation elicited by covalent binding to proteins was undertaken by Natsch et al. (2009). Using data for 116 chemicals for which there is LLNA data, they examined several ways of evaluating data on: 1) 1-octanol/water partitioning, 2) thiol reactivity (i.e. cysteine depletion), 3) Tissue Metabolism Simulator predictions, and 4) *in vitro* induction of ARE signalling linked to a luciferase reporter. LLNA data was scaled to five levels (extreme, strong, moderate, weak, and nonsensitisers). Similarly, results for each alternative method were scaled from 0 to 4. While different ways of evaluating these data were examined, the optimised model had a classification accuracy based on the LLNA data, of approximately 88%, with a sensitivity of 86% and a specificity of 94%. Cysteine depletion and ARE up regulations (data representing two different key events along the pathway) were the more significant contributors to the integrated model (Natsch et al., 2009).

A more recent attempt to integrate data for key events along the AOP for skin sensitisation elicited by covalent binding to proteins was undertaken by McKim and co-workers (2010) who used chemical reactivity and gene expression profiling in the HaCaT human skin cell line to identify positive and negative responses; these data were then used to place chemicals into sensitising potency classes of extreme/strong (ES), moderate (M), weak (W), and nonsensitisers (N), and to provide an estimate of corresponding LLNA values. Briefly, chemical reactivity was determined by measuring glutathione (GSH) % depletion *in chemico*. Three cell signalling pathways (Keap1/Nrf 2/ARE/EpRE, ARNT/AhR/XRE, and Nrf1/MTF/MRE), which are known to be activated by sensitising agents, were monitored by measuring the relative abundance of eleven genes whose expression is controlled by one of these pathways. The Nrf2/ARE/EpRE-controlled genes are: 1) NQO1, 2) AKR1C2, 3) thioredoxin (TXN), 4) interleukin 8 (IL8), 5) aldehyde dehydrogenase 3A (ALDH3A), 6) heme-oxygenase 1 (HMOX1), 7) musculoaponeurotic fibrosarcoma (MafF), and 8) GCLC. The XRE-controlled gene is 9) CYP1A1. The MRE-controlled genes are 10) metallothionein 1 (MT1), and 11) metallothionein 2 (MT2). Gene expression was reported on a scale of 4+, 3+, 2+, + and NC). The Predicted Toxicity Index (PTI) is an algorithmically determined score on a scale of 8 to 0. While the algorithm is proprietary, back calculation suggest that about 2/3 of the score is based on reactivity and 1/3 on gene expression.

Data for 39 chemicals was used for model development and data for an additional 58 compounds was used in validation. Compounds placed into ES and M classes were considered sensitisers, while compounds placed in the W and N classes were considered non-sensitisers. Classification accuracy based on results in the LLNA was approximately 84%, with a sensitivity of 81% and a specificity of 92%.

Glutathione depletion alone did not provide the accuracy necessary to differentiate potency classes. However, chemical reactivity combined with gene expression profiles (i.e. using data for two key events along the pathway), significantly improved predictivity.

Bauch et al. (2011) showed that while experimental *in vitro* data from: 1) direct peptide reactivity assay, 2) KeratinoSen, 3) h-CLAT and 4) MUSST were singly good predictors of sensitisation in both humans and the LLNA, combining the data in a simple test battery improved predictivity, especially for nickel. Having concordance for these four data provide a greater Weight-of-Evidence and a greater confidence in the prediction.

No IATA for skin sensitisation has yet been developed at OECD.

REFERENCES

- Andersen, M.E. and Krewski, D. 2009. Toxicity testing in the 21st century: bringing the vision to life. *Toxicol. Sci.* 107: 324-330.
- Aptula, A.O. and Roberts, D.W. 2006. Mechanistic applicability domain for non-animal based predictions of toxicological endpoints. *Chem. Res. Toxicol.* 18: 1420-1426.
- Ashikaga, T., Sakaguchi, H., Sono, S., Kosaka, N., Ishikawa, M., Nukada, Y., Miyazawa, M., Ito, Y., Nishiyama, N. and Itagaki, H. 2010. A comparative evaluation of *in vitro* skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). *Altern. Lab. Anim.* 38:275-84.
- Basketter, D.A. and Kimber, I. 2009. Updating the skin sensitisation *in vitro* data assessment paradigm in 2009. *J. Appl. Toxicol.* 29: 545-550.
- Basketter, D.A. and Kimber, I. 2010. Contact hypersensitivity. In: McQueen, C.A. (ed) *Comparative Toxicology* Vol. 5, 2nd Ed. Elsevier, Kidlington, UK, pp. 397-411.
- Bauch, C., Kolle, S.N., Fabian, E., Pachel, C., Ramirez, T., Wiench, B., Wruck, C.J., van Ravenzwaay, B. and Landsiedal, R. 2011. Intralaboratory validation of four *in vitro* assays for prediction of skin sensitizing potential of chemicals. *Toxicol. In Vitro* 25: 1162-1168.
- Corsini, E., Mitjans, M., Galbiati, V., Lucchi, L., Galli, C.L. and Marinovich, M. 2009. Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens. *Toxicol. In Vitro* 23: 769-796.
- Diderich, R. 2010. Tools for category formation and read-across. Overview of the OECD (Q)SAR Application Toolbox. In; Cronin, M.T.D. and Madden, J.C. (eds.) *In Silico Toxicology: Principles and Applications*. RSC Publishing, Cambridge, United Kingdom. pp. 385-407.
- dos Santos, G.G., Reinders, J., Ouwhand, K., Rustemeyer, T., Scheper, R.J. and Gibbs, S. 2009. Progress on the development of human *in vitro* dendritic cell based assays for assessment of skin sensitizing potential of compounds. *Toxicol. Appl. Pharmacol.* 236: 372-382.
- Emter R., Ellis, G. and Natsch, A. 2010. Performance of a novel keratinocyte-based reporter cell line in screen skin sensitizers *in vitro*. *Toxicol. Appl. Pharmacol.* 245: 281-290.
- Enoch, S.J., Ellison, C.M., Schultz, T.W. and Cronin, M.T.D. 2011. A review of the electrophilic reaction chemistry involved on covalent protein binding relevant to toxicity. *Crit. Rev. Toxicol.* 41: 783–802.
- Gerberick, G.F., Vassallo, J.D., Bailey, R.E., Chaney, J.G., Morrall, S.W. and Lepoittevin, J.-P. 2004. Development of a peptide reactivity assay for screening contact allergens. *Toxicol. Sci.* 81: 332-343.

- Gerberick, G.F., Ryan, C.A., Kern, P.S., Schlatter, H., Dearman, R.J., Kimber, I., Patlewicz, G.Y. and Basketter, D.A. 2005. Compilation of historical lymph node data for evaluation of skin sensitisation alternative methods. *Dermatitis* 16: 157-202.
- Jacobs, A. 1997. *Understanding Organic Reaction Mechanisms*, Cambridge University Press: Cambridge, United Kingdom. p 304.
- Jowsey, I.R., Basketter, D.A., Westmoreland, C., Kimber, I., 2006. A future approach to measuring relative skin sensitising potency: A proposal. *J. Appl. Toxicol.* 26: 341-350.
- Karlberg, A.-T., Bergström, M.A. Börje, A. Luthman, K. and Nilsson, J.L. 2008. Allergic contact dermatitis- formation, structural requirements, and reactivity of skin sensitizers. *Chem. Res. Toxicol.* 21: 53-69.
- Kern, P.S., Gerberick, G.F., Ryan, C.A., Kimber, I., Aptula, A. and Basketter, B.A. 2010. Local lymph node data for the evaluation of skin sensitisation alternatives: A second compilation. *Dermatitis* 21: 8-32.
- Kimber, I., Basketter, D.A., Gerberick, G.F., Ryan, C.A. and Dearman, R.J. 2011. Chemical allergy: Translating biology into hazard characterization. *Toxicol. Sci.* 120(S1): S238-S268.
- McKim, J.M. Jr, Keller, D.J. III and Gorski, J.R. 2010. 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.* 29: 171-192.
- Natsch, A. and Emter, R. 2008. Skin sensitizers induce antioxidant response element dependent genes: Application to the in vitro testing of the sensitisation potential of chemicals. *Toxicol. Sci.* 102: 110-119.
- Natsch, A., Emter, R. and Ellis, G. 2009. 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 sensitisation testing. *Toxicol. Sci.* 107: 106-121.
- Organization for Economic Co-operation and Development OECD 2007. OECD Guidance on grouping chemicals. OECD Environment, Health and Safety Publications Series on Testing and Assessment No. 80. [ENV/JM/MONO\(2007\)28](#).
- OECD 2011. Report of the Expert Consultation on Scientific and Regulatory Evaluation of Organic Chemistry-based Structural Alerts for the Identification of Protein-binding Chemicals. OECD Environment, Health and Safety Publications Series on Testing and Assessment No. 139. [ENV/JM/MONO\(2011\)9](#).
- Roberts, D.W., Patlewicz, G., Kern, P.S., Gerberick, F., Kimber, I., Dearman, R.J., Ryan, C.A., Basketter, D.A. and Aptula, A.O. 2007. Mechanistic applicability domain classification of a local lymph node assay dataset for skin sensitisation. *Chem. Res. Toxicol.* 20: 1019-1030.
- Roberts, D.W. Schultz, T.W., Wolf, E.M, and Aptula, A.O. 2010. Experimental reactivity parameters for toxicity modeling: Application to aquatic toxicity of Sn2 electrophiles to *Tetrahymena pyriformis*. *Chem. Res. Toxicol.* 23:228-234.

- Roberts, D.W. and Natsch, A. 2009. High throughput kinetic profiling approach for covalent binding to peptides: Application to skin sensitisation potency of Michael acceptor electrophiles. *Chem. Res. Toxicol.* 22: 592-603.
- Ryan, C.A., Kimber, I., Basketter, D.A., Pallardy, M., Gildea, L.A. and Gerberick, G.F. 2007. Dendritic cells and skin sensitisation. Biological roles and uses in hazard identification. *Toxicol. Appl. Pharmacol.* 221: 384-394.
- Schultz, T.W., Rogers, K. and Aptula, A.O. 2009. Read-across to rank skin sensitisation potential: Subcategories for the Michael acceptor domain. *Contact Dermatitis* 60: 21-31.
- Schwöbel, J.A.H., Koleva, Y.K., Bajot, F., Enoch, S.J., Hewitt, M., Madden, J.C., Roberts, D.W. Schultz, T.W. and Cronin, M.T.D. 2011. Measurement and estimation of electrophilic reactivity for predictive toxicology. *Chem. Rev.* 111: 2562-2596.
- Vandebriel, R.J. and van Loveren H. 2010. Non-animal sensitisation testing: State-of-the-art. *Crit. Rev. Toxicol.* 40: 389-404.
- Vandebriel, R.J., Pennings, J.L., Baken, K.A., Pronk, T.E., Boorsma, A., Gottschalk, R., and Van Loveren, H. 2010. Keratinocyte gene expression profiles discriminate sensitizing and irritating compounds. *Toxicol. Sci.* 117: 81-89.
- Van Och, F.M.M., Van Loveren, H., Van Wolfswinkel, J.C., Machielsen, A.J.C., Vandebriel, R.J. 2005. Assessment of potency of allergenic activity of low molecular weight compounds based on IL-1 α and IL-18 production by a murine and human keratinocyte cell line. *Toxicology* 210: 95-109.
- Wong, H.L. and Liebler, D.C. 2008. Mitochondrial protein targets of thiol-reactive electrophiles. *Chem. Res. Toxicol.* 21: 796-804.
- Yarbrough, J.W. and Schultz, T.W. 2007. Abiotic sulfhydryl reactivity: A predictor of aquatic toxicity for carbonyl-containing α,β -unsaturated compounds. *Chem. Res. Toxicol.* 20: 558-562.

ANNEX 1: ADVERSE OUTCOME PATHWAY (AOP) -BASED EVIDENCE FOR 1-CHLORO-2,4-DINITROBENZENE (DNCB) BEING A SKIN SENSITISER.

Knowledge on the AOP for skin sensitisation elicited by covalent binding of substances to proteins has evolved rapidly over the past decade and may be summarized as:

- Step 1)** The target substance must be bioavailable (i.e. it must penetrate the stratum corneum of the skin).
- Step 2)** The target substance must be a direct-acting electrophile, be converted from a non-reactive substance (pro-electrophile) to a reactive metabolite via metabolism, or be converted from a non-reactive substance (pre-electrophile) to a reactive derivative via an abiotic process, typically oxidation.
- Step 3)** The molecular sites of action are targeted nucleophilic sites in proteins (e.g. cysteine and lysine residues) in the epidermis.
- Step 4)** The molecular initiating event is the covalent perturbation of dermal proteins, which is irreversible (i.e. formation of the hapten-protein complex or complete antigen). *In vivo*, this event is associated with the production of a specific memory T-cell response.
- Step 5)** Biochemical pathways affected by the definitive electrophile's action on the molecular targets are incompletely known but often include inflammation-related pathways, including the mitogen-activated protein signalling pathway and the oxidative stress response pathway, especially in keratinocytes and dendritic cells.
- Step 6)** The cellular/tissue-level outcomes are incompletely known but include epidermal responses such as: 1) immune recognition of chemical allergens by keratinocytes, specialized epidermal dendritic cells (i.e. Langerhans cells), and 2) dermal dendritic cells. Responses in the form of expression of specific cell surface markers, chemokines, and cytokines are typically taken as evidence of dendritic cell maturation.
- Step 7)** The organ-level responses include:
 - a) dendritic cell migration to the lymph node where they present major histocompatibility complex (MHC) molecules to naive T-lymphocytes (T-cells), and
 - b) T-cell differentiation and proliferation as allergen-specific memory T-cells.
- Step 8)** The target organ(s) are the skin and local lymph nodes; the target cell populations are the immune cells, especially T-cells.
- Step 9)** The key physiological response is acquisition of sensitivity.
- Step 10)** The key organism response is dermal inflammation upon receiving the substance challenge in the elicitation phase. This response is associated with stimulation of specific memory T-cell produced in the induction phase.
- Step 11)** The overall effect on mammals is allergic contact dermatitis in humans or its rodent equivalent contact hypersensitivity.

Specific evidence for DNCB presented by AOP steps:

Step 1) With a log Kow value of 2.40, modest molecular size and moderate volatility, there is nothing in the literature to suggest that skin penetration or bioavailability is a mitigating factor in the sensitisation of DNCB.

Step 2) While DNCB enhances expression of cytochrome P450's (McKim et al., 2010), there is no experimental evidence that metabolism is a mitigating factor in skin sensitisation of DNCB.

DNCB is a classic aromatic nucleophilic substitution (SnAr) electrophile and resides in the SnAr domain (Enoch et al., 2011). As noted by Schwobel et al. (2011), DNCB has been reported to be highly reactive with a variety of *in chemico* methods (Table 1).

Steps 3 & 4) Hopkins et al. (2005) found DNCB preferentially binds (five-fold higher) to cellular proteins versus serum proteins in a non-protein-specific fashion. Cellular proteins are considered to be thiol-rich proteins while serum proteins are considered to be thiol-poor. McKim et al. (2010) reports a 24-hr depletion-based (i.e. loss of free thiol) % binding to glutathione of 75% for DNCB.

As noted by Bruchhausen et al. (2003) and Trompezinski et al. (2008), DNCB interact with dendritic cells (DCs) by way of glutathione depletion.

Step 5) Cell signalling pathways such as MAPKs and NF-kappaB (κ B), are thought to be involved in DC maturation. The JNK and p38 MAPK pathways are primarily active by various environmental stressors, including oxidative stress and inflammatory cytokines. The ERK1/2 pathway is linked to the regulations of cell proliferation. In human DCs cultured from CD14+ peripheral monocytes, exposure to DNCB activated the p38 and JNK signalling pathways (Aiba et al., 2003). Miyazawa et al. (2008) demonstrated the role of the p38 MAPK pathway in DNCB activation of DC-like human monocytic leukaemia cell line HTP-1; this was later confirmed by Mitjans et al. (2010). The p38 MAPK and ERK1/2 pathways also are activated by trinitrochlorobenzene (TNCB) (a compound very similar to DNCB) this activation could be inhibited by the thiol antioxidant N-acetyl-L-cysteine which prevents haptenization (Bruchhausen et al., 2003).

Reaction Oxygen species are secondary messengers that control a wide range of systems effects. In particular, they control DC maturation by way of phosphorylation of proteins subsequent to thiol (i.e. cysteine) oxidation. Trompezinski et al. (2008) showed dose-response activation via phosphorylation of the p38 MAPK and JNK pathways and inhibition of the ERK1/2 pathway by DNCB. The relationship between oxidative stress and p38 MARK activation and of the ERK1/2 pathway inhibition was demonstrated by pre-treatment with the antioxidant N-acetyl-L-cysteine.

Step 5) Keratinocytes are the major cell type of the epidermis of the skin. They are known to be the primary site of skin metabolism and play an important role in epithelial DC activation.

Using human keratinocytes (HaCaT cells), McKim et al. (2010) evaluated selected genes associated with three cell signalling pathways (Keap1/Nrf 2/ARE/EpRE, ARNT/AhR/XRE, and Nrf1/MTF/MRE) which are known to be activated by sensitizing agents. Briefly, the relative abundance of eleven genes whose expression is controlled by one of these pathways, was measured. The Nrf2/ARE/EpRE-controlled genes are: 1) *NQO1*, 2) *AKR1C2*, 3) thioredoxin (*TXN*), 4) interleukin 8 (IL8), 5) aldehyde dehydrogenase 3A (*ALDH3A*), 6) heme-oxygenase 1 (*HMOX1*), 7) musculoaponeurotic fibrosarcoma (*MafF*), and 8) *GCLC*. The XRE-controlled gene is 9) *CYP1A1*. The MRE-controlled genes are 10) metallothionein 1 (*MT1*), and 11) metallothionein 2 (*MT2*). Gene expression was reported on a scale of 4+, 3+, 2+, + and NC). For DNCB McKim et al, report: *NQO1* expression = 4+; *AKR1C2* = 2+; *TXN* = NC; IL8 = +; *CYP1A1* = +; *ALDH3A* = NC; *HMOX* = NC; *MafF* = +; *GCLC* = NC; *MT1A* = NC,

and *MT2A* = 2+. Microarray analysis of DNCB-treated HaCaT cells by Vanderbriel et al. (2010) show similar results.

The KeratinoSens assay (Emter et al., 2010) examines dose-responses (routinely twelve concentrations in triplicate) for significant induction of gene activity in an *in vitro* assay with a luciferase reporter gene under control of a single copy of the ARE-element of the human AKR1C2 gene stably inserted into HaCaT keratinocyte cell line. Using a standard operating procedure (Emter et al., 2010), experimental data was generated and the average maximal induction of gene activity (*I*_{max}) and the average concentration inducing gene activity >50% above control values (*EC*_{1.5}) were determined. The latter calculations were performed with linear extrapolation from the values above and below the induction threshold (as for the *EC*₃ value determination in the LLNA. Intra- and inter-laboratory testing of DNCB with the KeratinoSens assay (Natsch et al., 2011) report repeatable and reproducible results (Table 2).

Table 2. Intra- and inter-laboratory testing of DNCB with the KeratinoSens assay.

Laboratory	<i>I</i> _{max} (fold induction)	<i>EC</i> 1.5 (μM)
A (historical)	14.8	2.5
A	12.9	3.3
B	4.3 2.1	
C	12.2	3.0
D	19.5	1.4
E	15.6	2.1

Cultures of human keratinocyte cell line NCTC 2544 were exposed to DNCB and cell-associated IL-18 evaluated 24 hours later (Corsini et al., 2009). Intracellular IL-18 content was assessed by specific sandwich ELISA, with results expressed in pg/mg of total intracellular protein. DNCB induces a significant dose-response increase in IL-18 production; however, production is modest as compared to other sensitizers that were tested. IL-18 production by DNCB-treated HaCaT and HEL-30 cells was reported by Van Och et al. (2005).

Step 6) Dendritic cells can be generated *in vitro* either from human monocyte or from cord blood progenitors. In addition, some immortal cell lines have DC-like characteristics. Immature DCs acquire a mature phenotype characterised by a decrease in E-cadherin expression and an increase in selected cell surface markers (e.g. CD54 and CD86) and increased secretion of inflammatory cytokines (e.g. IL1β, IL-8 and TNFα).

Using long exposure times (24h and 48h) to monocyte derived DC, Aiba et al. (1997) reported DNCB elicited a significant increase in the expression of the surface biomarkers CD54, CD86 and HLA-DR.

Tuschl and Kovac (2001) reported dendritic cells, from peripheral mononuclear blood cells, responded to DNCB by up-regulating the co-stimulatory molecule CD86, the intercellular adhesion molecule CD54 and the HLA-DR antigen.

Ashikaga et al. (2002) reported an up-regulation of CD86 and internalization of Class II major histocompatibility complex (MHC) in DC-like human monocytic leukaemia cell line THP-1 cells treated for 24 hours with DNCB; moreover, upregulation was enhanced when gamma interferon was applied simultaneously with DNCB.

In human CD14⁺ peripheral monocyte-derived DCs, DNCB induced expression of CD86 and production of the cytokine IL-8 (Aiba et al., 2003) in p38 MAPK-dependent manners (Trompezinski et al., 2008).

In human CD34⁺ cord blood cell-derived DCs, DNCB induced CD40 and HLA-DR expression, and IL-8 production all in NF- κ B independent manners (Ade et al., 2007).

Using the DC-like myeloid U937 cell line in the MUSST assay, exposure to DNCB was shown to induce expression of CD86 at a non-cytotoxic dose (Ovigne et al., 2008).

Using the HTP-1 cells in the h-CLAT assay, exposure to DNCB was demonstrated to induce expression of CD86 and the cell surface adhesion molecule CD54 greater than 150% and 200%, respectively (Sakaguchi et al., 2009). Nukada et al. (2011) report CD86 EC150 and CD54 EC200 values of 2.30 μ g/ml and 2.66 μ g/ml, respectively, which is at the more potent end of the more than 70 sensitizers for which such values are reported.

The VITROSENS assay is based on the differential expression (real-time PCR) of cAMP-responsive element modulator (*CREM*) and monocyte chemotactic protein-1 receptor (*CCR2*) in CD34⁺ DCs after 6-, 11- and 24-hours of exposure (Hooyberghs et al., 2008). Gene expression changes were determined as fold-changes (i.e. ratios of gene expression levels of exposed samples over corresponding solvent control samples), with exposure to DNCB showing significant increases in both *CREM* and *CCR2*.

Exposure of THP-1 cells to DNCB increased expression of two Nrf2-dependent ARE responsive genes, heme oxygenase-1 (*HMOX1*) and NAD(P)H:quinone oxidoreductase 1 (*NQO1*); pre-incubation with N-acetyl-L-cysteine inhibited CD86 expression, as well as *HMOX1* and *NQO1* gene expression (Ade et al., 2009).

Step 7a) The internalization of surface MHC Class II molecules via endocytosis by antigen presenting cells is viewed as an important early step in antigen processing. Lempertz *et al.* (1996) explored the possibility of using endocytosis of contact sensitizers by murine DCs. Briefly, DC present in epidermal cell suspensions were labelled with an anti-MHC Class II monoclonal antibody. Subsequently, by means of flow cytometric using second step reagents labelled with pH-sensitive fluorochromes, they found differences in the mean fluorescence intensity of the internalized label. Endocytosis of the MHC complex into acidic compartments (lysosomes) resulted in a quenching of fluorescence, whereas internalization into less acidic compartments (immunosomes) resulted in a conservation of fluorescence intensity. Exposure to DNCB, 2,4-dinitro-1-fluorobenzene and TNCB resulted in a conservation of fluorescence intensity of 1.7- to 2-fold over DMSO-treated cells.

Cumberbatch et al. (2005), measured DC density in a mouse ear at 4 and 17 hours following exposure to 1% DNCB. Additionally, they measured the number of DC appearing in the lymph node at 24-, 48- and 72-hours following an identical exposure and found significant increase in DCs in all cases.

3D-reconstituted human epidermal (RHE) models consist of human cells grown on a membrane at the air-liquid interface. This method of co-culture induces the cells to grow in multilayers and to form junctions between the cells so that the cultures are similar to pieces of human skin. Using RHE in which CD34⁺ DCs are included, topical application of DNCB induced CD86 expression in DCs (Facy et al., 2004).

A skin model comprised, of keratinocytes, fibroblasts and DCs was exposed to DNCB resulting in CD86 expression and cytokine release (Uchino et al., 2009).

Step 7b) Gerberick et al. (1999) reported a >6-fold increase in lymph node cellularity upon exposure to 0.25% DNCB that was mainly due to proliferation of CD4+ and CD8+ T cells.

Hopkins et al. (2005) reported *in vivo* effects in lymphoid tissue of mice topically exposed to DNCB and the extremely similar DNFB. One percent DNCB resulted in a marked increase in immune activity, including a 5-fold increase in lymph node cellularity with a 6-fold increase in tritiated-thymidine incorporation. A concentration of 0.1% DNFB resulted in comparable levels of activity. Lymph node cells isolated from mice exposed to DNCB and DNFB express high levels of the Th1 cytokine IFN- γ and low levels of the Th2 cytokines IL-5 and IL-10 and mitogen-inducible IL-4.

Step 8) Basketter et al. (1997) and Hopkins et al. (2005) reported DNCB was a powerful sensitizer in the LLNA.

Step 11) Landersteiner and Jacobs (1936) reported that DNCB is an *in vivo* skin sensitizer.

The Guinea Pig Maximisation Test (GPMT) is a highly sensitive method using both intradermal and topical induction treatment and closed challenge. DNCB is positive at 0.1% in the GPMT challenge, which places it at the upper-most end of sensitizers tested in the GPMT.

Buehler Guinea Pig Test uses repeated closed topical applications during induction and closed challenge. DNCB is positive in the Buehler test.

Table 1. Summary of *In Chemico* Reactivity Measurements for DNCB.

DNCB is reactive with a variety of nucleophiles. Its relative rate of reactivity, as compared to other chemicals tested via the same protocol, consistently places it as a highly reactive chemical.

ID #	Nucleophile	Parameter, units	Value	T(C); pH; solvent	React. Time; Nu:EL ratio	Reference	Assay
26	Glutathione	t1/2(GSH) min	200	22 ;7; EDTA buffer	max. 16h; 50:1	Clarke ED et al., Pestic. Sci. 54 (1998), 38	HPLC-UV(210-300 nm).
596	Cor1C-420 (AcNKKCDLF)	RC50(Cor1) mM	0.07	37; 7.5; PBS 0.1 M	150 min; 1:10	Natsch A et al., Toxicol. Sci. 106 (2008), 464	UV (unreacted Cys after reaction with monobromobimane; 385 nm excit., 480 emiss.)
735	Glutathione	krel(GSH): CDNB 1 = CDNB	1.00E+00	25; 7.5; PBS 0.1 M	15 min; 1:1	Hulbert P et al., J. Pharmaceut. Biomed. Anal. 8 (1990), 100	UV (GSH-conjugate). k relative to 1-Chloro-2,4-dinitrobenzene
855	4-Nitrobenzylpyridine	k1(NBP) min-1	-2.71	80; 2-Butanone	60 min; 30:1	Hermens J et al., Toxicol. Environ. Chem. 9 (1985), 219	UV (ca. 560 nm)
961	Piperidine	k(Piperidine) M-1 min-1	1.15E+00 0.06	25;		Bunnet JF et al., Chem. Rev. 49 (1951), 273	
962	Piperidine	k(Piperidine) M-1 min-1	9.20E-01 -0.04	25; Ethanol		Bunnet JF et al., Chem. Rev. 49 (1951), 273	
1043	Methylate. Sodium	k(Methylate) M-1 min-1	1.51E+00 0.18	25; Methanol		Bunnet JF et al., Chem. Rev. 49 (1951), 273	
1044	Methylate. Sodium	k(Methylate) M-1 min-1	1.11E-01 -0.95	0; Methanol		Bunnet JF et al., Chem. Rev. 49 (1951), 273	
1171	Glutathione	k(GSH) M-1 min-1	0.72 -0.14	22; 7; EDTA buffer	max. 16h; 50:1	Clarke ED et al., Pestic. Sci. 54 (1998), 385	
1172	Glutathione	k(GSH) M-1 min-1	0.42 -0.38	25; 6.5; PBS 0.1 M	15 min; 1:1	Hulbert P et al., J. Pharmaceut. Biomed. Anal. 8 (1990), 1009	
1173	Glutathione	k(GSH) M-1 min-1	3.8 0.58	25; 7.5; PBS 0.1 M	15 min; 1:1	Hulbert P et al., J. Pharmaceut. Biomed. Anal. 8 (1990), 1009	UV (GSH-conjugate).
1174	Glutathione	k(GSH) M-1 min-1	0.58	25; 7.5; PBS 0.1 M	15 min; 1:1	Hulbert P et al., J. Pharmaceut. Biomed. Anal. 8 (1990), 1009	est. from pH 6.5: k2(est. pH 7.5) = 10*k2(exp. pH 6.5); UV.

1408	Ethylate. Sodium	k(Ethylate) M-1 min-1	4.95E+00 0.69	25; Ethanol		Bunnet JF et al., Chem. Rev. 49 (1951), 273	
1409	Ethylate. Sodium	k(Ethylate) M-1 min-1	3.90E-01 -0.41	0; Ethanol		Bunnet JF et al., Chem. Rev. 49 (1951), 273	
1410	Ethylate. Sodium	k(Ethylate) M-1 min-1	1.05 0.02	10; Ethanol		Bunnet JF et al., Chem. Rev. 49 (1951), 273	
1411	Ethylate. Sodium	k(Ethylate) M-1 min-1	1.63 0.21	15; Ethanol		Bunnet JF et al., Chem. Rev. 49 (1951), 273	
1412	Ethylate. Sodium	k(Ethylate) M-1 min-1	2.90 0.46	20; Ethanol		Bunnet JF et al., Chem. Rev. 49 (1951), 273	
1413	Ethylate. Sodium	k(Ethylate) M-1 min-1	2.98 0.47	25; Ethanol		Bunnet JF et al., Chem. Rev. 49 (1951), 273	
1414	Ethylate. Sodium	k(Ethylate) M-1 min-1	3.26 0.51	15; Ethanol		Landsteiner et al., J. Exp. Med. 64 (1936), 625	
1597	Aniline	k(Aniline) M-1 min-1	1.79E-02 -1.75	50; Ethanol		Bunnet JF et al., Chem. Rev. 49 (1951), 273	
1598	Aniline	k(Aniline) M-1 min-1	9.75E-03 -2.01	35; Ethanol		Bunnet JF et al., Chem. Rev. 49 (1951), 273 Nu: 3-Aminotoluene.	
1599	Aniline	k(Aniline) M-1 min-1	2.13E-02 -1.67	35; Ethanol		Bunnet JF et al., Chem. Rev. 49 (1951), 273 Nu: 4-Aminotoluene.	
1600	Aniline	k(Aniline) M-1 min-1	1.93E-03 -2.71	50; Ethanol		Bunnet JF et al., Chem. Rev. 49 (1951), 273 Nu: Methylaniline.	
1723	Aniline	DPE(Aniline) % Depletion	>90	100; Ethanol	2 h;	Landsteiner et al., J. Exp. Med. 64 (1936), 625	Assay: Steam bath; >90 = more than 90% liberation of halogen
2155	Glutathione	DP(GSH): Gerberick % Depletion	43.6	25; 7.4; PBS	15 min; 1:100	Gerberick GF et al., Toxicol. Sci. 97 (2007), 417	0.2 GSH : 20 mM EI; RP-HPLC (Derivat. of GSH/GSSH with DNFB; R: [Farris, Reed 1987])
2240	Cys-Peptide (AcRFAACAA)	DP(Cys-P.): Natsch % Depletion	100	30; PBS 20 mM	24 h; 1:100	Natsch A et al., Toxicol. Vitro 21 (2007), 1220	HPLC-DAD 214 nm
2684	Cor1C-420 (AcNKKCDLF)	DP(Cor1) % Depletion	>98	37; 7.5; PBS 0.1 M	31 h; 1:10	Natsch A et al., Toxicol. Sci. 106 (2008), 464	
2766	Arg-Peptide (AcFAARAA)	DP(Arg-P.): Aleksic %	-1.73	37; 10; Carbonate	24 h; 1:100	Aleksic M et al., Toxicol. Sci. 108	LC-Tandem-MS(Electrospray); includes AcFAGAGA as

		Depletion		buffer 50 mM		(2009), 401	internal standard
2948	Cysteine	Adduct(Cys) (yes/no)	yes	Aqueous, NaOH	10 min; 1:1	Saunders BC, Biochem. J. 28 (1934), 1977	room temperature; titration with alcoholic iodine.

References:

- Ade, N., Antonios, D., Kerdine-Romer, S., Boisleve, F., Rousset, F. and Pallardy, M. 2007. NF-kappaB plays a major role in the maturation of human dendritic cells induced by NiSO(4) but not by DNCB. *Toxicol. Sci.* 99: 488–501.
- Ade, N., Leon, F., Pallardy, M., Pfeiffer, J.L., Kerdine-Romer, S., Tissier, M.H., Bonnet, P.A., Fabre, I. and Ourlin, J.C. 2009. HMOX1 and NQO1 genes are upregulated in response to contact sensitizers in dendritic cells and THP-1 cell line: Role of the Keap1/Nrf2 pathway. *Toxicol. Sci.* 107: 451–460.
- Aiba, S., Manome, H., Nakagawa, S., Mollah, Z.U., Mizuashi, M., Ohtani, T., Yoshino, Y., Tagami, H. 2003. p38 Mitogen-activated protein kinase and extracellular signal-regulated kinases play distinct roles in the activation of dendritic cells by two representative haptens, NiCl₂ and 2,4-dinitrochlorobenzene. *J. Invest. Dermatol.* 120: 390–399.
- Aiba, S., Terunuma, A., Manome, H. and Tagami H. 1997. Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. *Euro. J. Immunol.* 27: 3031-3038.
- Ashikaga, T., Hoya, M., Itagaki, H., Katsumura, Y. and Aiba, S. 2002. Evaluation of CD86 expression and MHC class II molecule internalization in THP-1 human monocyte cells as predictive endpoints for contact sensitizers. *Toxicol. In Vitro* 16: 711-716.
- Basketter, D. A., Dearman, R. J., Hilton, J. and Kimber, I. 1997. Dinitrohalobenzenes: evaluation of relative skin sensitisation potential using the local lymph node assay. *Contact Dermatitis* 36, 97-100
- Bruchhausen, S., Zahn, S., Valk, E., Knop, J. and Becker, D. 2003. Thiol antioxidants block the activation of antigen-presenting cells by contact sensitizers. *J. Invest. Dermatol.* 121:1039–1044.
- Corsini, E., Mitjans, M., Galbiati, V., Lucchi, L., Galli, C.L. and Marinovich, M. 2009. Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens. *Toxicol. In Vitro* 23: 769-796.
- Cumberbatch, M., Clelland, K., Dearman, R.J. and Kimber, I. 2005. Impact of cutaneous IL-10 on resident epidermal Langerhans' cells and the development of polarized immune responses. *J. Immunol.* 175: 43-50.
- Emter R., Ellis, G. and Natsch, A. 2010. Performance of a novel keratinocyte-based reporter cell line in screen skin sensitizers in vitro. *Toxicol. Appl. Pharmacol.* 245: 281-290.
- Enoch, S.J., Ellison, C.M., Schultz, T.W. and Cronin, M.T.D. 2011. A review of the electrophilic reaction chemistry involved on covalent protein binding relevant to toxicity. *Crit. Rev. Toxicol.* 41: 783–802.
- Facy, V., Flouret, V., Régnier, M. and Schmidt, R. 2004. Langerhans cells integrated into human reconstructed epidermis respond to known sensitizers and ultraviolet exposure. *J. Invest. Dermatol.* 122: 552–553.

- Gerberick, G.F., Cruse, L.W. and Ryan, C.A. 1999. Local lymph node assay: Differentiating allergic and irritant responses using flow cytometry. *Methods-A Companion to Methods in Enzymology* 19: 48-55.
- Hopkins, J.E., Naisbitt, D.J., Kitteringham, N.R., Dearman, R.J., Kimber, I. and Park, B.K. 2005. Selective haptenation of cellular or extracellular proteins by chemical allergens: Association with cytokine polarization. *Chem. Res. Toxicol.* 18: 375-381.
- Hooyberghs, J., Schoeters, E., Lambrechts, N., Nelissen, I., Witters, H., Schoeters, G. and Van Den Heuvel, R. 2008. A cell-based *in vitro* alternative to identify skin sensitizers by gene expression. *Toxicol. Appl. Pharmacol.* 231: 103-111.
- Landersteiner, K. and Jacobs, J. 1936. Studies on the sensitisation of animals with simple chemical compounds. II. *J. Exp. Med.* 64: 625-639.
- Lempertz, U., Kühn, U., Knop, J. and Becker, D. 1996. An approach to predictive testing of contact sensitizers *in vitro* by monitoring their influence on endocytic mechanisms. *Internat. Arch. Allergy Immunol.* 111: 64-70.
- Mitjans, M., Galbiati, V., Lucchi, L., Viviani, B., Marinovich, M., Galli, C.L. and Corsini, E. 2010. Use of IL-8 release and p38 MAPK activation in THP-1 cells to identify allergens and to assess their potency *in vitro*. *Toxicol. In Vitro* 24: 1803-1809.
- McKim, J.M. Jr, Keller, D.J. III and Gorski, J.R. 2010. 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.* 29: 171-192.
- Miyazawa, M., Ito, Y., Kosaka, N., Nukada, Y., Sakaguchi, H., Suzuki, H. and Nishiyama, N. 2008. Role of MAPK signalling pathway in the activation of dendritic type cell line, THP-1, induced by DNBC and NiSO₄. *Toxicol. Sci.* 33: 51-59.
- Natsch, A., Bauch, C., Foertsch, L., Gerberick, F., Norman, K., Hilberer, A., Inglis, H., Landsiedel, R., Onken, S., Reuter, H., Schepky, A. and Emter, R. 2011. The intra- and inter-laboratory reproducibility and predictivity of the KeratinoSens assay to predict skin sensitizers *in vitro*: results of a ring-study in five laboratories. *Toxicol. In Vitro* 25: 733-44.
- Nukada, Y., Foertsch, L., Ashikaga, T., Sakaguchi, H., Nishiyama, N., Gerberick, F. 2011. Predicting allergy potential by battery evaluation system using two *in vitro* skin sensitisation tests; Direct Peptide Reactivity Assay (DPRA) and human Cell Line Activation Test (h-CLAT). poster 060 Society of Toxicology Meeting.
- Ovigne, J.M., Martinozzi-Teissier, S., Verda, D., Abdou, D., Piroird, C., Ade, N. and Rousset, F. 2008. The MUSST for *in vitro* skin sensitisation prediction: Applicability domains and complementary protocols to adapt to the physico-chemical diversity of chemicals. *Toxicol. Letters*, 180: Supplement 1, 5, S216.
- Sakaguchi, H., Ashikaga, T., Miyazawa, M., Kosaka, N., Ito, Y., Yoneyama, K., Sono, S., Itagaki, H., Toyoda, H. and Suzuki, H. 2009. The relationship between CD86/CD54 expression and THP-1 cell viability in an *in vitro* skin sensitisation test- Human cell line activation test (h-CLAT). *Cell Biol. Toxicol.* 25: 109-126.
- Schwöbel, J.A.H., Koleva, Y.K., Bajot, F., Enoch, S.J., Hewitt, M., Madden, J.C., Roberts, D.W., Schultz, T.W. and Cronin, M.T.D. 2011. Measurement and estimation of electrophilic reactivity for predictive toxicology. *Chem. Rev.* 111: 2562-2596.

- Trompezinski, S., Migdal, C., Tailhardat, M., Le Varlet, B., Courtellemont, P., Haftek, M. and Serres, M. 2008. Characterization of early events involved in human dendritic cell maturation induced by sensitizers: Cross talk between MAPK signalling pathways. *Toxicol. Appl. Pharmacol.* 230: 397–406.
- Tuschl, H. and Kovac, R. 2001. Langerhans cells and immature dendritic cells as model systems for screening of skin sensitizers. *Toxicol. In Vitro* 15: 327-331.
- Uchino, T., Takezawa, T. and Ikarashi, Y. 2009. Reconstruction of three-dimensional human skin model composed of dendritic cells, keratinocytes and fibroblasts utilizing a handy scaffold of collagen vitrigel membrane. *Toxicol. In Vitro* 23: 333–337.
- Vandebriel, R.J., Pennings, J.L., Baken, K.A., Pronk, T.E., Boorsma, A., Gottschalk, R., and Van Loveren, H. 2010. Keratinocyte gene expression profiles discriminate sensitizing and irritating compounds. *Toxicol. Sci.* 117: 81-89.
- Van Och, F.M.M., Van Loveren, H., Van Wolfswinkel, J.C., Machielsen, A.J.C., Vandebriel, R.J. 2005. Assessment of potency of allergenic activity of low molecular weight compounds based on IL-1 α and IL-18 production by a murine and human keratinocyte cell line. *Toxicology* 210: 95-109.