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The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins Part 1: Scientific Evidence

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NO. 168

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

PART 1: SCIENTIFIC EVIDENCE

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The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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EXECUTIVE SUMMARY

An adverse outcome pathway (AOP) is the sequence of events from the chemical structure of a target chemical or group of similar chemicals through the molecular initiating event to an *in vivo* outcome of interest. Each AOP represents the existing knowledge concerning the linkage(s) between a molecular initiating event, intermediate events and an adverse outcome at the individual or population level. Knowledge of the 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. The first key event is the molecular interaction with skin proteins, the site of action. Specifically, the target chemical or a metabolite or abiotic transformation product of the target chemical covalently binds to cysteine and/or lysine residues. The second key event takes place in the keratinocyte. This includes inflammatory responses as well as gene expression associated with particular cell signalling pathways (e.g. antioxidant/electrophile response element-dependent pathways). The third key event is activation of dendritic cells which is typically assessed by expression of specific cell surface markers, chemokines and cytokines. The final key intermediate event is T-cell proliferation, which is indirectly measured in the murine Local Lymph Node Assay. The purpose of this document is to describe the state of knowledge of the AOP for skin sensitisation initiated by covalent binding to proteins, assess the weight-of-evidence supporting the AOP, identify the key events, and identify databases containing test results related to those key events. AOPs can be incorporated into chemical categories-based assessments or integrated approaches for testing and assessment.

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

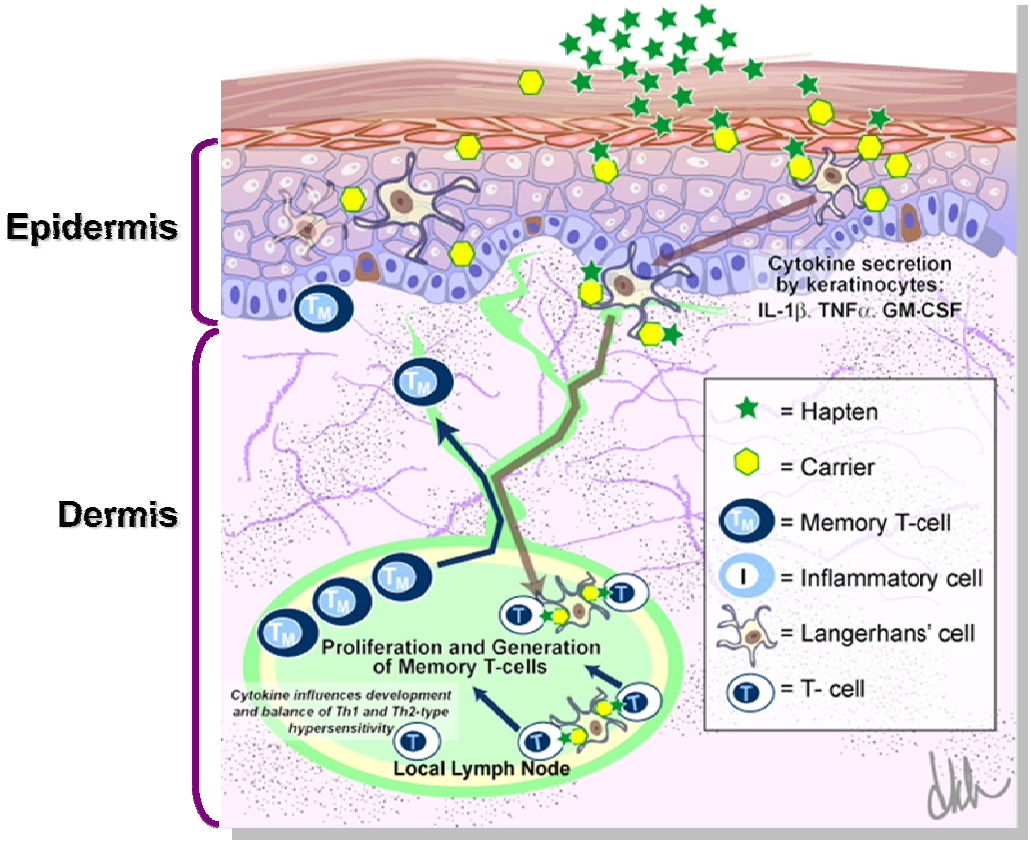

Figure 1. The Induction Phase of Skin Sensitisation.

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.

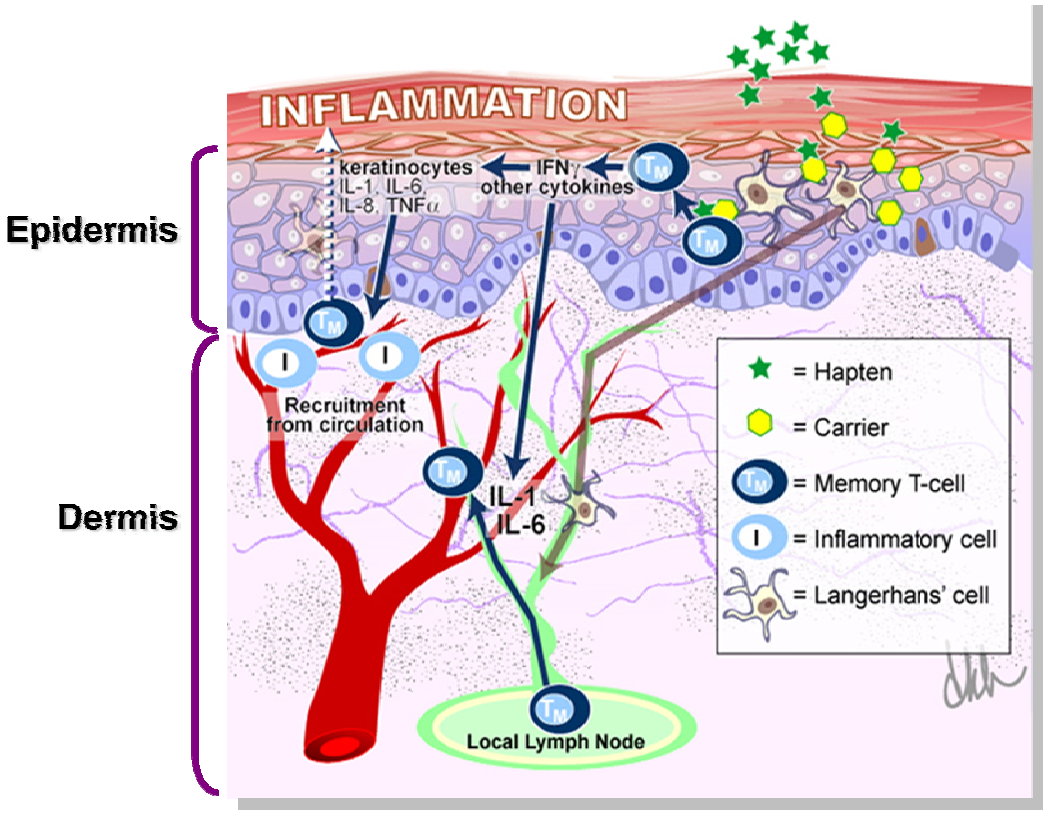

Figure 2. The Elicitation Phase of Skin Sensitisation.

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; Aeby 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 IL1β 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).

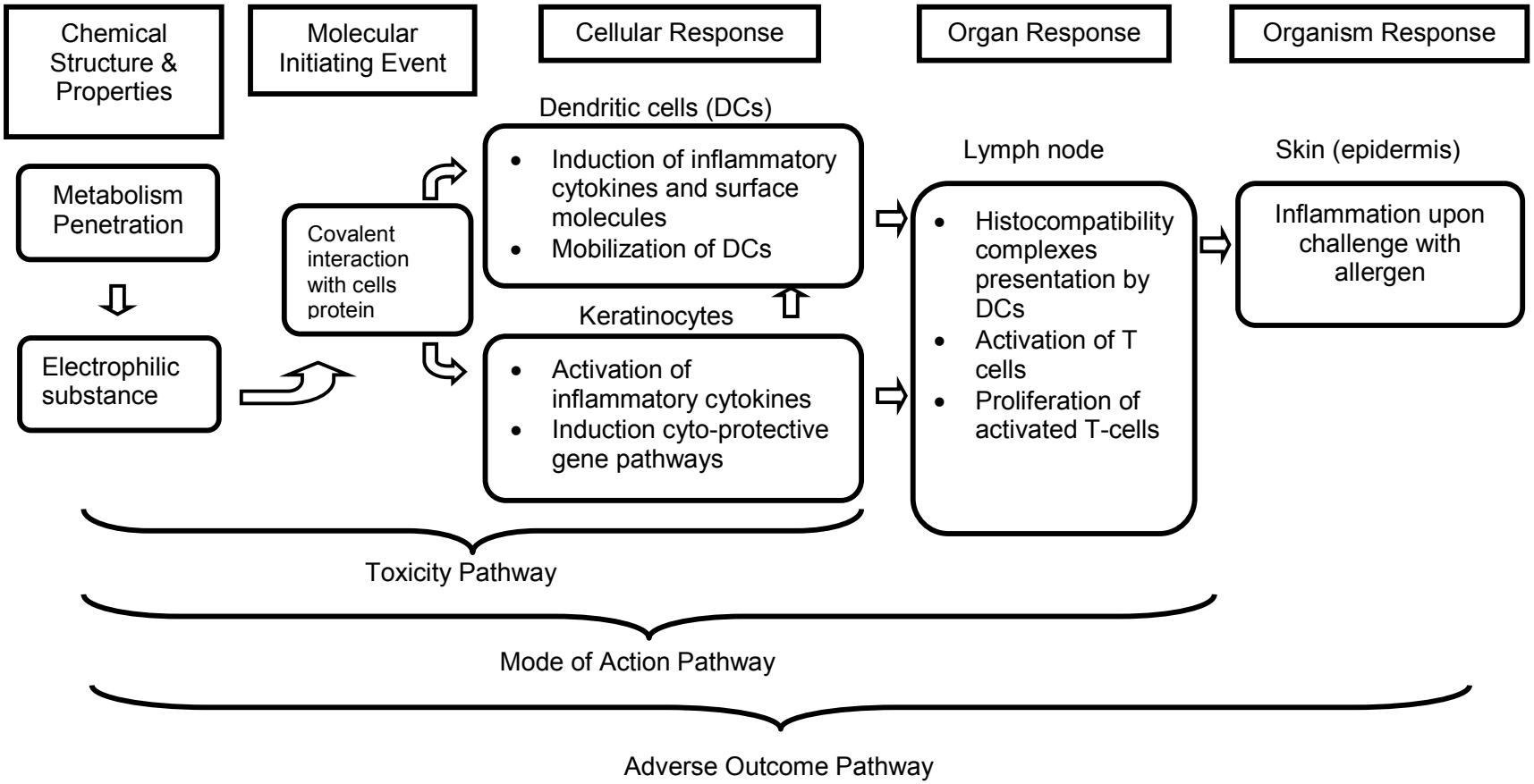

Figure 3. **Flow diagram of the pathways associated with skin sensitisation.**

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.

SCIENTIFIC EVIDENCE SUPPORTING THE AOP

Summary of the Scientific Evidence Supporting the AOP

An AOP may be considered either plausible or probable, depending upon the extent (i.e. depth and breadth) of the available scientific evidence supporting the AOP, and the extent to which the key events have been experimentally tested and found to be consistent with data for other key events. Accordingly, an AOP may be considered a dynamic entity. An evaluation of the scientific evidence supporting a proposed AOP can be conducted by answering a pre-determined set of questions. With regards to this AOP for skin sensitisation, the following questions are asked and answered:

- ***How well characterized is the AOP?*** The skin sensitisation AOP is at least qualitatively well-characterized, as the seminal events are generally accepted by the scientific community.

- ***How well are the initiating and other key events causally linked to the outcome?*** The molecular initiating event (protein binding reactions) is based on long-standing, well-studied organic chemical mechanisms and reactions. Sensitisation is causally linked to keratinocyte activity and T-cell proliferation and, to a lesser extent, dendritic cell activation/maturation.

- ***What are the limitations in the evidence in support of the AOP?*** While there is general agreement that the AOP for skin sensitisation outlined above is appropriate for qualitative hazard identification, there is no agreement on what measurements, other than reactivity, are necessary to reflect potency.

- Is the AOP specific to certain life stages / age classes (i.e., are there critical life stages where exposure must occur to result in the adverse effect)? Or, is the AOP known to be initiated regardless of life stage but key events along the pathway are different dependent on life stage? The proposed skin sensitisation AOP is not associated with life stage-, sex- or age-dependency.

- ***Are the initiating and key events expected to be conserved across taxa?*** While *in vivo* testing focuses on selected mammals including man, the key events for this AOP appear to be conserved across mammals.

All these aspects are demonstrated in detail further down in this document.

Assessment of the Weight-of-Evidence supporting the AOP

An additional aspect of evaluating a proposed AOP is to implement the Hill criteria (Hill, 1965) to assess the Weight-of-Evidence supporting the AOP. With regards to the proposed AOP for skin sensitisation, the following issues are addressed:

- ***Concordance of dose-response relationships;*** While no specific citations were found, an examination of the experimental data for selected compounds (e.g. 1-chloro-2,4-dinitrobenzene) reveals general agreement among the dose-response relationships both within and between intermediate endpoints (see Annex 1). With exceptions, there is agreement between sensitizers initiated by covalent binding to proteins and non-sensitizers tested in mice, guinea-pigs, and

humans; this is especially the case for extreme and strong sensitizers but lesser so for weak and non-sensitizers. One problem is that earlier results, especially with the guinea-pig, were not dose-response experiments. Chemical reactivity data show very good concordance of dose-response relationships regardless of the method. In general, available data from *in vitro* assays are fragmentary and often qualitative (i.e., yes/no).

- ***Temporal concordance among the key events and adverse outcome;*** There is good agreement between the sequences of biochemical and physiological events leading to skin sensitisation (see Gerberick et al., 2008; Karlberg et al., 2008; Vocanson et al., 2009; Aeby et al., 2010; Basketter and Kimber, 2010; Adler et al., 2011).

- ***Strength, consistency, and specificity of association of adverse outcome and initiating event;*** There is excellent strength, as well as good consistency and high specificity, of the association between *in vivo* skin sensitisation and *in chemico* protein binding. This is especially true for reactions that have thiol as the preferred molecular target (Schwöbel et al., 2011). Based on linear regression analyses, there is excellent interlaboratory/protocol correlations within and between nucleophile depletion and adduct formation methods (Schwöbel et al., 2011).

- ***Biological plausibility, coherence, and consistency of the experimental evidence;*** The *in chemico*, *in vitro,* and *in vivo* experimental evidence is logical and consistent with the mechanistic plausibility proposed by covalent reactions based on the protein binding theory (Gerberick et al., 2008; Karlberg et al., 2008; Adler et al., 2011). In selected cases, (e.g. 1-chloro-2,4-dinitrobenzenes) where the same compound has been examined in a variety of assays (see Annex 1), the coherence and consistency of the experimental data is excellent.

  Alternative mechanism that logically present themselves and the extent to which they may distract from the postulated AOP. It should be noted that alternative mechanisms of action, if supported, require a separate AOP. While covalent reactions with thiol groups and to a lesser extent amino groups, are clearly supported by the proposed AOP, reactions targeting other nucleophiles may or may not be supported by the proposed AOP. Limited data on chemical reactivity shows that two competing reactions are possible, the faster reaction dominates. However, this has yet to be proven in vitro or in vivo.

- ***Uncertainties, inconsistencies and data gaps;*** Uncertainties include the structural and physico-chemical cut-offs between theoretical and measured reactivity (Schwöbel et al., 2011), the significance of the preferred amino acid target (e.g., cysteine versus lysine) (OECD, 2011b), the significance of Th1 or type 1 (IFN-γ) versus Th2 or type 2 (IL-2, IL-4, IL-13) cytokine secretion profiles (Hopkins et al., 2005), and sensitisation measurements in different *in vivo* models.

  Inconsistencies within the reported data are seen. There are differences between *in vitro* responses for highly similar chemicals (see Natsch and Emter, 2008; McKim et al., 2010). There are differences within and between *in vivo* test results for highly similar chemicals (see Annex C of the European Centre for Ecotoxicological and Toxicological Chemicals, 2010). Highly hydrophobic chemicals, which are *in vivo* sensitizers, are not active in aquatic-based *in chemico* or *in vitro* assays. The specific nature of the relationship between irritation and sensitisation has yet to be elucidated.

  Data gaps: Based on the more than 50 chemical reactions associated with covalent binding to thiol or primary amine moieties (OECD, 2011b) *in vitro* data for keratinocyte, dendritic cell, and T-cell assays, as well as *in vivo* sensitisation data, is incomplete in that it does not cover the

chemical spaces associated with many of these chemical reactions; *in chemico* data is also incomplete, especially for reactions that favour amino acid targets other than cysteine.

The final aspect of the OECD approach to using the AOP concept is an assessment of the quantitative understanding of an AOP. This includes the evaluation of the experimental data and models used to quantify the molecular initiating event and other key events. It also includes transparent determination of thresholds and response-to-response relationships used to scale *in chemico* and *in vitro* effects to *in vivo* outcomes.

For skin sensitisation, a major hurdle is moving from a qualitative AOP to a quantitative AOP. While the assessment of the experimental evidence, empirical data and confidence in the AOP expressed by the Weight-of-Evidence clearly supports the qualitative AOP as a means to identify and characterize the potential for a chemical to be a sensitizer, these same assessments clearly reveal the current lack of ability to consistently predict relative potency. The European Centre for Validation of Alternative Methods currently uses Weight-of-Evidence criteria for validation activities.

One aspect to be resolved is that of the *in vivo* data with which to scale the response-to-response ratios. Because the LLNA can directly quantify the adverse outcome (Basketter et al., 2009), public databases have recently been made available (Gerberick et al., 2005; Kern et al., 2010). LLNA results are often compared with results from alternative methods (e.g. Ashikaga et al., 2010; McKim et al., 2010). Such one-to-one comparisons may not be the best approach. As noted by Basketter et al. (2009), the LLNA is not without limitations, including variability between EC3 values or any other value (i.e. ECx) within mechanistic classes with equal or near equal chemical reactivity. The specific nature of the *in vivo* relationship between irritation and sensitisation has yet to be elucidated.

Specific Information Supporting the AOP

Step 1) Dermal Bioavailability:

Although toxicological issues are perhaps the most important factors, it is also clear that skin absorption, and to a lesser extent penetration, may be important in the assessment of chemicals for sensitisation potential. It is self-evident that a compound cannot exert sensitisation-related reactivity in deeper layers of the epidermis unless it is absorbed and penetrates the upper layers first (Basketter et al., 2007). The epidermis, in particular the stratum corneum (i.e. the dead keratinized cells of the epidermis), represents the most important barrier in dermal uptake. Hence, bioavailability in skin sensitisation is often thought of in the context of penetration of the stratum corneum. Considerable effort has been directed toward quantifying penetration of the stratum corneum of the skin (see Mitragotri, 2011) and in estimating the steady-state adsorption of organic materials applied to the skin as aqueous solutions (see Rivere, 2006). Skin transport occurs via passive diffusion in response to the concentration gradient between the supplication surface and the epidermal-dermal interface. While both *in vivo* and *in vitro* test guidelines have been adopted (OECD 2004a, 2004b, respectively), experimental data is not sufficient to resolve all issues effecting epidermal transport (Buist et al., 2009), with the issue of the relationship between relative absorption and dermal loading (i.e. amount of chemical per unit area) being particularly difficult. In addition, it is evident from an examination of experimental data that octanol/water partition coefficients, molecular size, as well as volatility or evaporation, may also affect dermal bioavailability (Bos and Meinardi, 2000; Guy, 2009; Roberts and Natsch, 2009).

In silico models, including physiological-based pharmacokinetic models and traditional structure-activity ones, as well as *in vitro* and *in vivo* experimental approaches exist. Most approaches try to balance evaporation rates with skin absorption rates. *In silico* structure–activity models often include vapour pressure, 1-octanol/water partitioning, water solubility, and/or molecular weight as molecular descriptors.

In silico models are often based on a series of assumptions that include: 1) the total chemical dose to the skin falls within a small dose limit in which first-order absorption is observed; the upper ranges is typically taken as 100 µg cm^{-2}, 2) evaporation and absorption are independent of one another, 3) the compound in question does not bind irreversibly to the skin, 4) the absorptive flux is a fraction of the maximum flux and the latter is proportional to the lipid solubility and inversely related to molecular weight, and 5) evaporative flux is related to Henry's Law with epidermal lipid being the relative solvent adjusted for air flow. *In vitro* and *in vivo* models typically take into consideration that: 1) skin permeability and evaporative flux are temperature dependent (the range typically being 20 – 40 °C), ionization affects dermal absorption (a minimum of 10% of the chemical should be in the unionized form), and 2) the solvent system must not compromise (more than water does) the barrier of the skin (Rivere, 2006).

Dermal absorption is concentration-dependent; however, the exact relationship between concentration and absorbed percentage is not known (Buist et al., 2009). As no accepted model currently exists, a conservative but widely used approach is to assume 100% absorbption (Robinson et al., 2000). While bioavailability may be part of any assessment, it is not integral to forming chemical categories and its use should be undertaken with knowledge of the uncertainty associated with available data.

Steps 2 – 4; Key Event 1) Protein-Binding Reactions, Reactivity and Metabolism:

Since the 1930's, there has been growing evidence that the main potency-determining step in skin sensitisation of industrial organic compounds is the formation of a stable hapten-protein conjugate (see Roberts and Aptula, 2008; Gerberick et al., 2008; Karlberg et al., 2008). Consequently, the molecular initiating event leading to skin sensitisation is postulated in this AOP to be covalent binding of electrophilic chemical species with selected nucleophilic molecular sites of action in skin proteins (Gerberick et al., 2008; Karlberg et al., 2008). Protein binding reactions are a means of identifying different chemical structures associated with skin sensitisation, which may or may not lead to different expressions in other key events along the AOP.

In contrast to receptor-mediated chemical interactions (e.g. oestrogen-receptor binding), electrophiles are not specific with regard to their molecular target. For example, Wong and Liebler (2008) in their examination of mitochondrial proteins from cells treated with two different electrophiles, observed that adducts were formed with more than 800 proteins. Moreover, some chemicals, such as acrolein and isothiocyanates, are able to react with several different nucleophilic chemical substituents. Therefore, the identification of the specific target protein is not considered in this AOP to be critical to accurately predict skin sensitisation. Moreover, it is recognized that reactivity measured with a particular nucleophilic target or model nucleophile does not necessarily reflect a specific chemical reaction, as many reactions target the same chemical substituent (Schwöbel et al., 2011).

Reactivity in general, and measurements and estimations of reactivity, have recently been reviewed (see Gerberick et al., 2008; Roberts et al., 2008; Enoch et al., 2011; OECD, 2011b; Schwöbel et al., 2011). For skin sensitisation and other toxicological endpoints for which protein binding is important, the biological nucleophile is assumed to be selected amino acids. The exact extent of adduct formation to each amino acid is dependent on the relative hardness / softness of the electrophile and nucleophile (see Schwöbel et al., 2011). Nucleophilic sites related to skin sensitisation are, in order of increasing hardness: 1) thiol group of cysteine and glutathione, 2) sulphur atoms of methionine, 3) primary amino groups of lysine and arginine, and 4) secondary amino groups of histidine (Schwöbel et al., 2011). The inability to identify the exact biological nucleophile is deemed less important than information regarding the electrophile. As noted in the hard-soft acid base theory, a soft electrophile will have a relative preference for a soft nucleophile; while a hard electrophile will have a relative preference for a hard nucleophile. As a consequence, for a series of electrophiles assigned to the same mechanistic cluster within a particular domain, the relative rates of reactivity between each electrophile and any nucleophile will remain the same.

In other words, while absolute reactivity may vary with protocols, relative reactivity will usually not vary significantly (see Schwöbel et al., 2011).

Binding experiments with small model nucleophiles reveal that, within a particular reaction within a mechanism, the rate of reactivity varies markedly. Moreover, while some compounds appear to bind exclusively with thiol or amine, others bind to a variety of nucleophiles. However, an electrophile is most likely to exhibit a preference for a particular nucleophile. For example, isothiocyanates have been shown to preferentially bind to lysine residues (Banks and Paquette, 1995), yet isothiocyanates strongly and rapidly react with cysteine (Schultz et al., 2005). Isothiocyanates are also genotoxic (Kassie and Knasmuller, 2000), which indicates interaction with nucleic acids. This is often explained on the basis of quantum chemical analyses of amino and nucleic acid reactivity (Mekenyan et al., 2010).

In more complex systems, nucleophilic target preferences may be masked by other factors. It is self-evident that the number of cysteine and lysine residues within a protein will impact target probability. For example, for serum albumin, a major serum protein, 10% of the amino acid residues are lysine but albumin has very few free cysteine residues. Also, it is self-evident that a target site (e.g. cysteine or lysine) which is located on an exposed surface of a protein is more likely to react with an electrophile than one that is located within a grove or fold of a protein. Such steric constraints are imposed by the primary structure (i.e. amino acid sequence) of the peptide or protein, as well as the secondary and tertiary structure of proteins imposed by disulfide bridges, and folding and coiling. Similarly, the microenvironment of the reaction site (e.g. hydrophilic versus hydrophobic) may affect the probability of a particular reaction. Free cysteine residues are more abundant in proteins in the aqueous cytosol than in the nonaqueous biomembranes (Hopkins et al., 2005).

An ancillary event in identifying protein-binding is metabolism and/or abiotic transformation (e.g. autoxidation) (Lepoittevin, 2006). *In vivo*, the keratinocyte is the primary site of metabolism in the skin (see Smith and Hotchkiss, 2001). *In silico* methods for identifying reactive metabolites exist (Dimitrov et al., 2005; Patlewicz et al., 2007; Roberts et al., 2007), however, their current predictivity varies depending on the reaction being simulated. *In vitro* metabolism with a skin-based cytochrome P450 mixture has been proposed (Bergström et al., 2007). Recently, Emter et al. (2010) published a new *in vitro* keratinocyte-based reporter cell line assay, which has metabolic potential. Gerberick et al. (2009) developed an *in vitro* simulation through forced oxidation with the peroxidase-peroxide system to mimic oxidative transformations. The latter method (Gerberick et al., 2009) currently appears more feasible. The rationale is that the test protocol should be severe enough to eliminate false negatives, but the opposite, false positives are more probable.

Oxidation of simple compounds, such as polyphenols and unsaturated alcohols, is well documented and well modelled *in silico* (Bajot et al., 2011). However, metabolism of more structurally complex chemicals, especially ones with the potential for multiple reactions, is less well understood and predictivity is hampered by not knowing which metabolites are the more likely ones to be formed (see Dimitrov et al., 2005; Patlewicz et al., 2007; Roberts et al., 2007).

Step 5; Key Event 2) Biochemical Pathways Related to Skin Sensitisation:

Biochemical or intracellular pathways affected by the action of reactive chemicals on molecular targets are incompletely known. However, there is evidence that during the sensitisation response, hapten-protein conjugates (hereafter noted as haptens) can react with cell surface proteins and activate mitogen-activated protein kinase signalling pathways. In particular, the biochemical pathways involving extracellular signal-regulating kinases- the c-Jun N-terminal kinases and the p38 kinases have been shown to be activated upon exposure to protein-binding chemicals (Trompezinski et al., 2008). These pathways are of particular importance in keratinocytes and dendritic cell response to skin sensitizers.

Step 6; Key Event 2) Events in Keratinocyte:

Haptens can also react with cell surface proteins and activate response pathways in keratinocytes (see Weltzien et al., 2009). Uptake of the hapten by keratinocytes activates multiple events, including the release of pro-inflammatory cytokines and the induction of cyto-protective cellular pathways. Activation of the pro-inflammatory cytokine IL-18 results from cleavage of inactive IL-18 precursor protein by inflammasome-associated caspase-1 (Martinon et al., 2009). Sensitizers can activate the inflammasome (Sutterwala et al., 2006; Watanabe et al., 2007) and in so doing induce IL-18 production. Intracellular Nod-like receptors (NLR) contain sensors for a number of cellular insults. Upon activation (by a currently unknown mechanism), NLRs oligomerise form molecular complexes (i.e. inflammasomes) that are involved in the activation of inflammatory-associated caspases, including caspase-1. Inductions of intracellular levels of IL-18 exhibit responses upon exposure to sensitizers which can be used to establish potency (van Och et al., 2005).

Keratinocyte exposure to sensitizers also results in induction of antioxidant/electrophile response element ARE/EpRE-dependent pathways (Natsch and Emter, 2008). Briefly, reactive chemicals bind to Keap1 (Kelch-like ECH-associates protein 1) that normally inhibit the nuclear erythroid 2-related factor 2 (Nrf2). Released Nrf2 interacts with other nuclear proteins and binds to and activates ARE/EpRE-dependent pathways, including the cytoprotective genes NADPH-quinone oxidoreductase 1 (NQ01) and glutathione S-transferase (GSHST), among others (Natsch and Emter, 2008; Ade et al., 2009). An *in vitro* reporter assay based on activation via the ARE/EpRE response element has been shown to be responsive to known sensitizers in HaCaT keratinocytes (Emter et al., 2010). Expression of ARE/EpRE-dependent genes and other cytoprotective genes (including CYP1A1, MT1 and MT2) in HaCaT cells are part of a proprietary *in vitro* battery approach to determining sensitisation potency (McKim et al., 2010). Both the Natsch and McKim groups have shown that this signalling pathway responds in a quantitative fashion, which is related to LLNA potency (e.g. strong, moderate, and weak).

Step 6; Key Event 3) Events in Dendritic cell:

As noted in the AOP during allergen contact with the skin, immature epidermal dendritic cells, known as Langerhans cells, and dermal dendritic cells serve as antigen-presenting cells (Ryan et al, 2005, 2007; Kimber et al., 2011). In this role, they recognize and internalize the hapten-protein complex formed during covalent binding. Subsequently, the dendritic cell loses its ability to seize new hapten-protein complexes and gains the potential to display the allergen-MHC-complex to naive T-cells; this process is often referred to as dendritic cell maturation. Simultaneously, under the influence of fibroblast- blood endothelial- and lymph endothelial-chemokines (e.g. CCL19, CCL21) and epidermal cytokines (e.g. interleukin (IL), IL-1 α, IL-1β, IL-18, tumour necrosis factor alpha (TNF-α)) maturing dendritic cells migrate from the epidermis to the dermis of the skin and then to the proximal lymph nodes, where they can present the hapten-protein complex to T-cells via a major histocompatibility complex molecule (Antonopoulos et al., 2008; Ouwehand et al., 2008). Dendritic cell activation, upon exposure to sensitizers, also leads to functional changes in the cells. For example, there are changes in chemokine secretion, cytokine secretion and in the expression of chemokine receptors (see dos Santos et al., 2009). Additionally, during dendritic cell maturation major histocompatibility complex (MHC), co-stimulatory and intercellular adhesion molecules (e.g. CD40, CD86, and DC11 and CD54, respectively) are up-regulated (see dos Santos et al., 2009; Vandebriel and Van Loveren, 2010, Kimber et al., 2011).

Signal transduction cascades precede changes in expression of surface proteins markers and chemokine or cytokine secretion. Components of signal transduction pathways are kinases, which phosphorylate and dephosphorylate a variety of substrates in order to elicit a change in the expression or secretion of target molecules. As a result, components of the signal transduction cascade are thought to be biomarkers which can distinguish sensitizers from non-sensitizers (Lambrechts et al., 2010a). Few

investigations have examined this possibility by measuring changes in kinase expression in different signal transduction pathways (e.g. p38 MAPK, ERK, PGK, and NFκB) (Trompezinski et al., 2008; dos Santos et al., 2009). Miyazawa and co-workers (2008a and 2008b) examined P38 MAPK, a mitogen-activated protein kinase, and ERK, an extracellular signal-regulated kinase in THP-1 cells. It is important to note that moderate and weak sensitizers needed 10x and 100x higher exposure concentrations, respectively, than strong sensitizers to activate kinase pathways. Investigations into the possible role of calcium influx as an early event in dendritic cell activation suggest that calcium influx is a second event following reactive oxygen species induction (Migdal et al., 2010). The complexity of dendritic cells role in skin sensitisation is further evident in the investigations of crosstalk between kinase activities, calcium influx and oxidative stress (Aeby et al., 2010).

Genomic and proteomic studies also have the potential to reveal biomarkers in dendritic cell-based assays. Custom designed arrays or quantitative polymerase chain reaction (PCR) of selected genes have been used to distinguish sensitizers from non-sensitizers (see dos Santos et al. 2009). VITOSENS, an assay that uses human CD34+ progenitor-derived dendritic cells (CD34-DC), is based on the differential expression of the cAMP-responsive element modulator (CREM) and monocyte chemotactic protein-1 receptor (CCR2) able to discriminate between skin sensitizers and non-sensitizers (Hooyberghs et al., 2008). A biomarker signature, the Genomic Allergen Rapid Detection test (GARD), was also developed for the identification of human sensitizing chemicals (Borrebaeck et al., 2009). It is based on the human myelomonocytic cell line MUTZ-3. Briefly, the mRNA expression levels induced by chemicals are determined by using DNA microarrays of 200 genes identified involved in relevant biological pathways, such as oxidative stress and xenobiotic induced responses. In addition, another genomic platform, SENS-IS, which consists of measuring, by real-time PCR, the over expression of 3 sets of genes may allow the *in vitro* assessment of the sensitizing potential of a compound (Groux et al., 2010).

Step 7; Key Event 4) Events in Lymphocytes:

T-cells are typically affected by protein-hapten complexes presented by dendritic cells on MHC molecules. Molecular understanding of this process has improved in recent years (see Martin et al., 2010). Briefly, MHC molecules are membrane proteins which present the small peptide antigens placed in a "groove" of the MHC molecule during its intracellular synthesis and transport to the cell surface. In the context of the MHC molecular on the cell surface, the small peptide antigen is recognized via the T-cell receptors as self or non-self (e.g. foreign). If this peptide is a foreign peptide, such as part of a protein-hapten complex, the T-cell will be activated to form a memory T-cell, which subsequently proliferates. If reactivated upon hapten presentation by skin dendritic cells, these memory T-cells will induce allergic contact dermatitis (Vocanson et al., 2009).

Recognizing the importance of the process of antigen presentation (i.e. T-cell priming), *in vitro* T-cell priming assays have been developed (see Martin et al., 2010). While first generation assays could only detect strong or extreme sensitizers, more recent development using normal human peripheral blood depleted of regulatory cells that normally prevent the sensitisation phase increased the probability that T-cell proliferation would be detected (Vocanson et al., 2008). A related approach is based on the hypothesis that there is a correlation between the potency of contact allergens and T-cell frequency and T-cell receptor repertoire (Kotturi et al., 2008). It is plausible that sensitisation potency may correlate with the size of the contact allergen-specific effector and regulatory T-cell pools and their diversity, and this could form the basis of a new generation of *in vitro* T-cell priming assays. It should be remembered that lymph node cell proliferation is the basis for the LLNA.

Perhaps the most interesting finding about lymphoid tissue, as related to the sensitisation is the selectivity of cytokine secretion. Hopkins et al. (2005), building on earlier work of Dearman and co-workers, reported that lymphoid tissue of mice exposed to classic electrophiles with conjugate proteins via

nucleophilic substitution as halo-nitro-aromatic compounds (i.e. 1-chloro-2,4-dinitrobenzene and 1-fluoro-2,4-dinitrobenzene) expresses high levels of the Th1 cytokine IFN-γ and low levels of the Th2 cytokines IL-5 and IL-10. Conversely, lymphoid tissue for mice exposed to 2,4-dinitrobenzene sulphonyl chloride, which conjugates with proteins via nucleophilic substitution as a sulphonyl halides, the acylating agent trimellitic anhydride, or fluorescein isothiocyanate, which conjugates with proteins via nucleophilic addition to the carbon atom of the isothiocyanate (-N=C=S) moiety, express high levels of the Th2 cytokines IL-5 and IL-10 and low levels of the Th1 cytokine IFN-γ (Hopkins et al., 2005). Based on differential binding to cellular and serum proteins Hopkins et al. (2005) showed that chemicals that stimulate a Th1 cytokine response bind selectively to cellular proteins, while chemicals that stimulate a Th2 cytokine response bind selectively to serum proteins. While it would be tempting to say electrophiles which preferentially bind to cysteine express a Th1 cytokine profile and electrophiles which preferentially bind to lysine express a Th2 cytokine profile, it is most likely not that simple.

Steps 9-11; In Vivo Skin Sensitisation:

In vivo studies, including ones with human subjects, have been critical in the evolution of the science of skin sensitisation. Human sensitisation testing is conducted with the human repeat insult patch test (HRIPT), as described by McNamee et al. (2008). The experimental endpoint of this type of study can be considered the adverse outcome described as allergic contact dermatitis. Dermal sensitizers are assumed to elicit an adverse effect only after a threshold dose is reached. Above this threshold, the severity of the adverse effect is assumed to increase proportionally to the dose, so the total dose per area of skin (e.g. $\mu g/cm^2$) is the critical exposure determinant. In this regard, animal data is consistent with human clinical data (Api et al., 2008). These observations are consistent with the immunological mechanism presented with this AOP, where it is assumed that for an adverse outcome to commence, a certain number of dendritic cells is required to be activated and to migrate to the nearest lymph node in order to instigate the further cascade of biological events (see Api et al., 2008).

Today the generation of sensitisation data in animal models remains the basis of assessing the sensitisation potential of chemicals. Adler et al. (2011) have reviewed animal test methods for skin sensitisation. Briefly, among these *in vivo* assays are the guinea-pig occluded patch test (Buehler, 1965; OECD, 1992), the Magnusson-Kligman guinea-pig maximization test (Magnusson and Kligman, 1970; OECD, 1992; Maurer et al., 1994), and the murine LLNA (Basketter et al., 1996; Kimber et al., 2002a; OECD, 2010a, 2010b, 2010c). Positive results in the occluded patch test or the guinea-pig maximization test can be considered the adverse outcome described as contact hypersensitivity.

The similarities and differences between results, with the guinea-pig test, the LLNA and where available human evidence, is important in the IPCS MOA Framework (Boobis et al., 2008) and addressed through the Bradford Hill criteria. While it will have implication in human risk assessment and classification and labelling under existing standards, .it has less of an impact on the acceptance of an AOP and its use in forming chemical categories and integrated assessment and testing approaches.

Methods and Databases for the Key Events of the AOP

There are a number of hurdles in moving from a qualitative AOP to a quantitative AOP. One question is what is the best *in vivo* adverse outcome data with which to scale the AOP? Because it can directly quantify the adverse outcome (Basketter et al., 2009), and public databases have recently been made available (Gerberick et al., 2005; Kern et al., 2010) LLNA results are often compared with results from alternative methods (e.g. Aptula et al., 2006; Natsch and Emter, 2008; Ashikaga et al., 2010; McKim et al., 2010).

The chief limitation in shifting from a qualitative AOP to a quantitative AOP for skin sensitisation elicited by covalent binding to proteins is the lack of databases with results from assays representing key events along the pathway. As an interim approach to continuous scale potency, efforts are being made to group chemicals by relative potency (e.g. extreme, strong, moderate, weak, and non-active).

Steps 2 – 4; Key Event 1) Reactions, Reactivity and Metabolism:

Methods and databases for the quantification of protein-binding are further in development than for other key events (Schwöbel et al., 2011). Among the other key events, keratinocyte responses, especially via the Keap1/Nrf2/ARE/EpRE cell signalling path (i.e. KeratinoSens) and dendritic cell activation (i.e. Myleoid U-937 Skin Sensitisation Test and human Cell Line Activation Test) have the most robust databases (Bauch et al., 2011).

In silico Methods

It is generally recognized that reaction-based methods, as opposed to other means of defining chemical similarity, allow for easier interpretation and provide greater confidence in their use (Freidig and Hermens, 2001). Chemical reactions related to covalent protein binding have recently been reviewed (Roberts et al., 2008; Enoch et al., 2011; OECD, 2011b). Measurements and estimations of reactivity have also recently been reviewed (Gerberick et al., 2008; Schwöbel et al., 2011). Computational or *in silico* techniques to predict chemical reactivity have been developed; they vary in complexity from the relatively simple approach of forming chemical categories from 2D structural alerts (i.e. SARs for qualitative identification of chemical sub-structures with the potential of being reactive), such as used in the OECD QSAR Toolbox (www.oecd.org/env/hazard/qsar), to QSAR models (i.e. quantitative prediction of relative reactivity) as described by Schwöbel et al. (2010).

In Chemico Protocols and Databases

While methionine, histidine, and serine all possess nucleophilic groups that are found in skin proteins, the –SH group of cysteine and the ε-NH_2 group of lysine are the most often studied. Soft electrophilic interactions involving the thiol group can be modelled with small molecules, such as mercaptopropionate, propanethiolate or nitrobenzenethiol (see Schwöbel et al., 2011), as well as cysteine, acetylcysteine, or peptides with a cysteine residue (Natsch, et al., 2007; Natsch and Gfeller, 2008; Gerberick et al., 2008; Aleksic et al., 2009). Glutathione (GSH; *L*-γ-glutamyl-*L*-cysteinyl-glycine) is the most widely used model nucleophile in soft electrophilic reactivity assays (see Kato et al., 2003; Schultz et al., 2005; Böhme et al., 2009). It is the most prevalent cellular thiol and the most abundant low molecular weight peptide in eukaryotic cells (Aptula et al., 2006).

While a variety of *in chemico* protocols have been developed (see Roberts et al., 2008; Schwöbel et al., 2011), many are based on GSH as the model nucleophile, which is usually dissolved in an aqueous phosphate buffer solution. Aqueous-based protocols are consistent with the theory that target thiols are largely confined to the cytosol, especially the intramitochondrial space (Wong and Liebler, 2008). Typically, after a defined reaction time, the concentration of free thiol groups is measured. Such depletion-based assays assume adduct formation, which is typically not confirmed. Good relationships between GSH reactivity and toxicity have been demonstrated; for example, although structurally very similar, the different aquatic toxicity of methacrylate, crotonate, and acrylate can be explained by their difference in the GSH reactivity. Methacrylates are the most slowly reactive, while crotonates are more moderately reactive, and acrylates are highly reactive (Yarbrough and Schultz, 2007).

The importance of reaction chemistry for sensitisation indicates that identification of the reaction-limited chemical spaces is critical for using the proposed AOP. Systematic databases for reaction-specific

chemical spaces are being developed. For example, *in chemico* databases reporting measurements of reactive potency currently exist for Michael acceptors (Yarbrough and Schultz, 2007, Böhme et al., 2009; Roberts and Natsch, 2009). The use of model nucleophiles containing primary amino ($–NH_2$) groups, such as in the amino acids lysine are less well-documented, with the principle of measuring relative reactivity being the same as for thiol (Gerberick et al., 2008). For example, *n*-butylamine and pyridoxylamine are used as a surrogate for primary amines, such as lysine. Currently, these amine-based databases are not systematically developed. It is widely known that acyl halides and carboxylic acid anhydrides, and to a lesser extent sulphonyl halides, hydrolyse when placed in an aqueous environment. Therefore, acyl halides and carboxylic acid anhydrides, and to a lesser extent sulphonyl halides often are non-reactive in aquatic protocols.

Step 5; Key Event 2) Biochemical Pathways Related to Skin Sensitisation:

Biochemical or intracellular pathways affected by the action of reactive chemicals are in general thought to be related to skin sensitisation; however, current knowledge is incomplete. The c-Jun N-terminal kinases and the p38 kinases have been shown to be activated upon exposure to protein-binding chemicals (Trompezinski et al., 2008). However, these studies are based on only a few compounds, in particular, 1-chloro-2,4-dinitrobenzene. A few investigations have examined this possibility by measuring changes in kinase expression in different signal transduction pathways (e.g. p38 MAPK, ERK, PGK, and NFκB) (see dos Santos et al., 2009). Miyazawa and co-workers (2008a and 2008b) examined P38 MAPK (mitogen-activated protein kinase) and ERK, (extracellular signal-regulated kinase) in THP-1 cells and showed that 1-chloro-2,4-dinitrobenzene, an extreme sensitizer, activates both cellular pathways and stimulates TNF-α release and subsequent phenotypic changes in the cells. As noted, the Keap1/Nrf2/ARE/EpRE cell signalling assay is a potential cellular marker for sensitisation because Keap1 is a thiol-rich sensor protein which has been shown to be covalently modified by electrophiles that leads to activation of ARE-dependent genes (Dinkova-Kostova et al., 2005).

Step 6; Key Event 2) Events in Keratinocyte:

In recent years, investigations have focused on the DNA antioxidant-response element (ARE), also known as electrophile response element (see Natsch, 2010). The KeratinoSens system of Natsch's group uses a luciferase reporter gene under control of a single copy of the ARE element of the human *AKR1C2* gene stably inserted into immortalized human keratinocytes (HaCaT cells) (Emter et al., 2010). The experimental design is robust with chemicals routinely tested at twelve concentrations in triplicate before evaluating for significant induction of gene activity (Natsch et al., 2011). One advantage of this assay is that it appears to address the issue of metabolism (Emter et al., 2010). While several variants of the luciferase-based ARE assay have been developed based on data derived from the assay described by Natsch and Emter (2008), Natsch et al. (2009) evaluated the predictive performances of the ARE assay evaluated against LLNA data for more than 100 chemicals. They report a 79%, concordance, 79% sensitivity and 81% specificity (Natsch et al., 2009). An *in vitro* assay based on IL-18 induction in human keratinocytes (cell line NCTC 2544) can also distinguish between sensitizers and irritants (Corsini et al., 2009; Mitjans et al., 2010). Other studies have described chemokines (e.g. CCL2, CCL4) and receptor (e.g. CCR7) (see dos Santos et al., 2009).

The Keap1/Nrf2/ARE/EpRE cell signalling assay is also the mechanistic basis for the work on skin sensitisation chemicals at CeeTox Inc. (McKim et al., 2010). Briefly, this work includes quantitative real-time polymerase chain reaction measurements of the relative abundance of mRNA for eleven selected genes. While most of the data is proprietary, the reported results are highly promising. Interestingly, both Emter et al. (2010) and McKim et al. (2010) combine their cell signalling results with chemical reactivity data in algorithms, which can be viewed as a first step in using the AOP in quantitative assessment.

Step 6; Key Event 3) Events in Dendritic cell:

Omic Studies

As noted by the work of McKim et al. (2010), genomic and proteomic studies also have the potential to reveal biomarkers associated with sensitisation. VITROSENS is an *in vitro* assay that uses human $CD34^+$ progenitor-derived dendritic cells (CD34-DC) isolated from human cord blood and, based on the differential expression of the cAMP-responsive element modulator (CREM) and monocyte chemotactic protein-1 receptor (CCR2), the assay discriminates between chemical skin sensitizers and non-sensitizers after six hours of exposure (Hooyberghs et al., 2008). Hooyberghs et al. (2008) describes 13 target genes which were first pinpointed by microarray techniques and then screened with the aid of quantitative PCR. Results with VITROSENS were able to distinguished 10 sensitizers from 11 non-sensitizers with a 89% concordance, a specificity of 97% and a 83% sensitivity (Lambrechts et al., 2010b).

In Vitro Assays for Cell Surface Markers, Cytokines, and Chemokines

Alterations in intercellular adhesion molecules, cytokines, and chemokines are part of the immunology response associated with skin sensitisation which can serve as biomarkers for skin sensitisation. *In vitro* expressions of these markers have been measured in endothelial cell-, keratinocyte- and especially dendritic cell-based cell lines.

Primary cells including both the $CD34^+$ derived and the CD14+ monocyte-derived dendritic cells, as well as dendritic cell-like cell lines including human monocytic leukaemia cell line (THP-1), human histiocytic lymphoma cell line (U937), $CD34^+$ human acute myeloid leukaemia cell line (MUTZ-3), human bone marrow-derived acute myelogenous leukaemia cell line (KG-1), human acute pro-myeloid leukaemia cell line (HL-60), and human erythroleukaemia cell line (K562), have been used in biomarker studies aiming to distinguish known sensitizers from non-sensitizers (see dos Santos et al., 2009; Vandebriel and Van Loveren, 2010; Aeby et al., 2010; Kimber et al., 2011).

Since dendritic cell maturation upon exposure to a sensitizing agent is accompanied by changes in surface marker expression, these surface markers are perceived as promising candidates as primary biomarkers of dendritic cell activation for the development of cell-based *in vitro* assays. While a variety of surface markers have been described to be up-regulated upon dendritic cell maturation, a review of the literature reveals that CD86 expression, followed by CD54 and CD40, are the most extensively studied intercellular adhesion and co-stimulator molecules to date. The human cell line activation test (h-CLAT) (Sakaguchi et al., 2009; Ashikaga et al., 2010) reported flow cytometry results for CD86 and CD54 expression in THP-1 cells treated with test chemicals. The predictive performances of the h-CLAT have been evaluated with 100 chemicals against the LLNA data, with a concordance of 84%, 88% sensitivity, and 75% specificity (Ashikaga et al., 2010). While h-CLAT was designed to provide a yes/no answer to sensitisation, it also can provide quantitative information in the form of the CD86 EC150 and CD54 EC200 values; both of these endpoints are correlated with LLNA EC3 values (Sakaguchi et al., 2009). Other studies with THP-1 cells include that of An et al. (2009). Another assay, the myeloid U937 skin sensitisation test (MUSST), is based as well on the measurement of CD86 by flow cytometry (Ade et al., 2006; Python et al., 2007; Ovigne et al., 2008). The prediction model is defined as the threshold of viability (CV70), positivity (CD86 EC150) of vehicle control. The predictive performances of the MUSST assay have been evaluated with 50 chemicals against the LLNA data, with a concordance of 83%, 81% sensitivity, and 84% specificity (Aeby et al., 2010). The overall performance of the h-CLAT and MUSST tests in various ring studies is encouraging (Sakaguchi et al., 2010).

Upon dendritic cell maturation, changes in cytokine and chemokine secretion and receptor expression occurs; these changes permit these cells to migrate to the lymph node. A variety of cytokines have been

studied in relationship to skin sensitizers (Kimber et al., 2011). IL-8 is a promising chemokine for distinguishing sensitizers from non-sensitizers. Studies indicate that increased IL-8 secretion or transcription may be just as sensitive a biomarker as CD86 expression; moreover, quantification of IL-8 can be performed by Enzyme Linked Immunosorbent Assay, a technique that is far simpler and amenable to high throughput screening than the flow cytometry technique used to measure CD86 expression (dos Santos et al., 2009).

The expression of other cytokines linked to skin sensitizers include IL-1 α, IL-1β, IL-18, and TNF-α) form the basis for other dendritic cell assays. In general, an increase in cytokine/chemokine secretion or receptor expression is observed when sensitizers were tested but not when non-sensitizers were tested. However, there is currently only a limited number of chemicals evaluated in more than one assay and results are sometimes contradictory.

The THP-1 cell line is the one most extensively used with CD86, IL-8, TNF-α, and p38 MAP kinase being the most examined surface marker; chemokine, cytokine, and kinase, respectively. Since the major goal of these biomarker assays is to correctly distinguish a sensitizer from a non-sensitizer, the type of cell used or the specific biomarker(s) reported in accomplishing this goal is not as important as the repeatability, reproducibility, and specificity of the assay.

Step 7; Key Event 4) Events in Lymphocytes:

Since the final stage in the sensitisation phase is the activation of naive T-lymphocytes in the local lymph node, an *in vitro* assay based on T-cells are useful in confirming the AOP as well as identifying sensitizers. As noted earlier, T-cells are typically activated to form a memory T-cell by protein-hapten complexes presented by dendritic cells on a MHC molecule. Molecular understanding of this process has improved in recent years (Martin et al., 2010). Most protocols recognize the importance of the process of antigen presentation, so *in vitro* T-cell-based assays are typically co-cultures of allergen-treated dendritic cells and modified T-lymphocytes with expression of selected biomarkers (e.g., interferon gamma), or T-cell proliferation being the reported outcome. Much of this work has been recently reviewed by Martin et al. (2010). It should be remembered that lymph node cell proliferation is the basis for the LLNA.

Steps 9-11; In Vivo Skin Sensitisation:

In vivo studies remain the basis of assessing the sensitisation potential of chemicals (see Adler et al., 2011) As previously noted, human sensitisation testing is conducted with the HRIPT (McNamee et al., 2008). Other *in vivo* methods include the guinea-pig occluded patch test (OECD, 1992), the Magnusson-Kligman guinea-pig maximization test (OECD, 1992), and the mouse LLNA (OECD, 2010a, 2010b, 2010c). While quantitative models have been developed with guinea-pig data, the LLNA assay was designed to allow for a direct quantitative assessment of skin sensitisation potency. Briefly, the relative potency of a skin-sensitizing chemical is measured by derivation of an ECx value, which is the concentration of a test chemical necessary to produce a X-fold increase in lymph node cell proliferation compared with concurrent vehicle controls (i.e. a threshold positive response) (Basketter et al., 1999). Since the LLNA does not include the challenge phase of sensitisation, it may be considered an incomplete *in vivo* sensitisation assay. Using LLNA data, sensitizers can be grouped into potency groups (e.g. extreme, strong, moderate, weak, and non-sensitizers). However, as noted by Basketter et al. (2009), the LLNA is not without limitations, including variability between EC3 values or any other value (i.e. ECx) within isoreactive mechanistic classes. There are increased efforts to look at *in vivo* skin sensitisation potential or lack of potential as concordance between LLNA and guinea-pig data, and human evidence.

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

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 (κB), 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 DCNB-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 (Imax) and the average concentration inducing gene activity >50% above control values (EC1.5) were determined. The latter calculations were performed with linear extrapolation from the values above and below the induction threshold (as for the EC3 value determination in the LLNA. Intra- and inter-laboratory testing of DNCB with the KeratioSens 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	Imax (fold induction)	EC 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-κB 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 that 150% and 200%, respectively (Sakaguchi et al., 2009). Nukada et al. (2011) report CD86 EC150 and CD54 EC200 values of 2.30 µg/ml and 2.66 µg/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 the 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-thyimidine 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:ELration	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 El; 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.

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