



## The skin sensitization adverse outcome pathway: exploring the role of mechanistic understanding for higher tier risk assessment

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REVIEW ARTICLE



# The skin sensitization adverse outcome pathway: exploring the role of mechanistic understanding for higher tier risk assessment

Maja Aleksic, Ramya Rajagopal, Renato de-Ávila, Sandrine Spriggs and Nicola Gilmour

Safety and Environmental Assurance Centre, Unilever, Sharnbrook, UK

## ABSTRACT

For over a decade, the skin sensitization Adverse Outcome Pathway (AOP) has served as a useful framework for development of novel *in chemico* and *in vitro* assays for use in skin sensitization hazard and risk assessment. Since its establishment, the AOP framework further fueled the existing efforts in new assay development and stimulated a plethora of activities with particular focus on validation, reproducibility and interpretation of individual assays and combination of assay outputs for use in hazard/risk assessment. In parallel, research efforts have also accelerated in pace, providing new molecular and dynamic insight into key events leading to sensitization. In light of novel hypotheses emerging from over a decade of focused research effort, mechanistic evidence relating to the key events in the skin sensitization AOP may complement the tools currently used in risk assessment. We reviewed the recent advances unraveling the complexity of molecular events in sensitization and signpost the most promising avenues for further exploration and development of useful assays.

**Abbreviations:** AOP: Adverse Outcome Pathway; OECD: The Organisation for Economic Co-operation and Development; DA: defined approach; SARA model: Skin Allergy Risk Assessment model; MIE: molecular initiating event; KE: key event; ACD: allergic contact dermatitis; NAM: new approach methodology; TG: test guideline; DPRA: Direct Peptide Reactivity Assay; ADRA: Amino acid Derivative Reactivity Assay; kDPRA: kinetic Direct Peptide Reactivity Assay; h-CLAT: Human Cell Line Activation Test; GARD: genomic allergen rapid detection; UN GHS: United Nations Globally Harmonized System; NICEATM: National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; ITS: integrated testing strategy; NGRA: Next Generation Risk Assessment; DNCB: 2,4-dinitro-1-chlorobenzene; MA: Michael acceptor; SB: Schiff Base; K5: K14: K10: keratin 5: keratin 14: keratin 1: keratin 10; Cys: cysteine; TRITC: tetramethylrhodamine isothiocyanate; MIF: migration inhibitory factor; NFκB: nuclear factor-kappa B; 4-HNE: 4-hydroxy-2-nonenal; RCS: reactive carbonyl species; IKK: IκB kinase; SN2: bimolecular nucleophilic substitution; SNAr: nucleophilic substitution on an aromatic center; HR-MAS NMR: high-resolution magic angle spinning nuclear magnetic resonance; HSA: human serum albumin; RHE: reconstituted human epidermis; His: histidine; Met: methionine; MI: 2-methylisothiazolin-3-one; MCI: 5-chloro-2-methylisothiazolin-3-one; GSH: glutathione; GST: glutathione-s-transferase; ROS: reactive oxygen species; LP: lipid peroxidation; PPRA: Peroxide-Peroxidase Reactivity Assay; CYP: cytochrome p450; AO/ALDH: aldehyde oxidases/dehydrogenase; NQR: NAD(P)H quinone reductase; EH: epoxide hydrolase; UGTs: UDP-glucuronosyl transferases; SULTs: sulfotransferases; NATs: N-acetyl transferases; MDBGN: 2-bromo-2-(bromomethyl)glutaronitrile (methylidibromoglutaronitrile); PUFA: polyunsaturated fatty acid; Nrf2: nuclear factor erythroid 2-related factor 2; ER: endoplasmic reticulum; UPR: unfolded protein response; DNFB: 2,4-dinitro-1-fluorobenzene; LA: linoleic acid; AA: arachidonic acid; MDA: malondialdehyde; Lys: lysine; TBA: thiobarbituric acid; Trx: thioredoxin; TrxR: thioredoxin reductase; APC: antigen presenting cell; Keap1: Kelch-like ECH-associated protein; EpRE/ARE: electrophile/anti-oxidant response element; IL: interleukin; ICE: interleukin converting enzyme; TNF-α: tumor necrosis factor alpha; DAMPs: damage-associated molecular patterns; LMWHA: low molecular weight hyaluronic acid; TLR: toll-like receptor; DC: dendritic cell; CD: cluster of differentiation; ATP: adenosine triphosphate; MHC: major histocompatibility complex; HSF1: heat shock inducible factor 1; HIF1α: hypoxia-inducible transcription factor 1 alpha; Hsp90: heat shock protein 90; LC: Langerhans cell; HLA-DR: human leukocyte antigen – DR isotype; LPS: lipopolysaccharide; RXR: Retinoid X Receptor; LLNA: Local Lymph Node Assay; Ig: immunoglobulin; PPD: *p*-phenylenediamine.

## ARTICLE HISTORY

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Skin sensitization; allergy; hapten; reactivity; antigen; risk assessment; new approach methodologies; mechanistic insights

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**CONTACT** Maja Aleksic  [Maja.aleksic@unilever.com](mailto:Maja.aleksic@unilever.com)  Safety and Environmental Assurance Centre, Unilever, Sharnbrook, UK

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## 1. Introduction

Efforts to speed up and facilitate the replacement of animal experiments with human relevant, mechanistic, non-animal data in assuring chemical safety have led to remarkable changes in recent decades. This process has allowed a careful examination of the existing knowledge about the adverse outcomes resulting from the exposure to chemicals and consolidation of that knowledge into useful tools including Adverse Outcome Pathways (AOPs). Over a decade ago, the Organisation for Economic Co-operation and Development (OECD) launched a program to develop AOPs with the purpose of utilizing them for development of novel approaches and tests, the data from which can be combined and considered together to reach a decision about chemical safety (<https://www.oecd.org/chemicalsafety/testing/projects-adverse-outcome-pathways.htm#SectionA>). The efforts underpinned by the skin sensitization AOP have resulted in development and validation of numerous assays that, when combined, can confidently assign sensitizing potential of chemicals (or lack thereof). For those chemicals identified as sensitizers, potency information can be obtained using defined approaches (DAs).

For example, a derivation of human relevant point of departure and quantitation of uncertainty is achievable using SARA model (Reynolds et al. 2019). Skin sensitization risk assessment remains a tiered approach (Gilmour et al. 2022) and for those chemicals identified as sensitizers, additional mechanistic information will be useful in the higher tiers of that approach (Reynolds et al. 2021). Here, we have reviewed the current and emerging mechanistic knowledge of the induction of skin sensitization which may add value to risk assessment.

### 1.1. Adverse outcome pathway

An adverse outcome pathway (AOP) is a concept describing a sequence of events starting from exposure of an organism (or specific tissue) to a chemical *via* a series of critical molecular and cellular events, culminating in an adverse outcome for the exposed organism/tissue. Originally described by Ankley et al. (2010) AOPs are structured to represent the available knowledge which causally links the molecular initiating event (MIE) and an adverse outcome *via* one or more series of key events (KE).

The skin sensitization AOP, describing induction and elicitation phases required for adverse outcome to occur (manifested as allergic contact dermatitis (ACD) in humans), is one of the earliest developed AOPs (OECD 2014). It is summarized in 11 steps, including 4 which are recognized as “Key Events” (KEs1-4, Figure 1).

The skin sensitization AOP is focused solely on organic chemicals which are inherently electrophilic or can be transformed into electrophilic species *via* phase I skin metabolizing enzymes or on contact with air oxygen (i.e. pro- and pre-haptens). This AOP does not currently include the type of ACD which develops as a result of exposure to metals or chemicals activated by UV light exposure (reviewed recently by de Ávila et al. (2023a)).

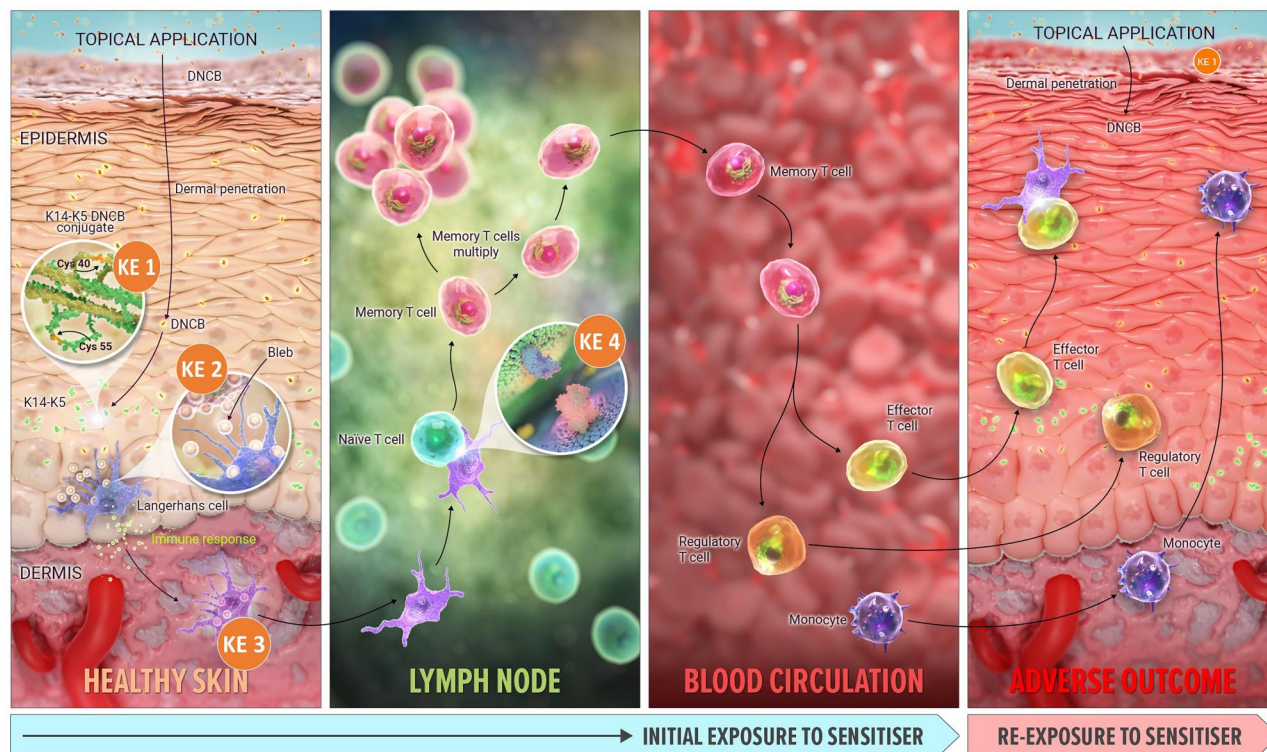
### 1.2. Current predictive assays

Prior to the development of the skin sensitization AOP, efforts were already underway to develop predictive non-animal assays for skin sensitization based on events that were well understood to occur during the induction of sensitization. Establishment of the AOP has, however, provided a framework together with a mechanistic knowledge base, which has further facilitated *in chemico* and *in vitro* assay development as well as their integration into “defined approaches” (DAs) for risk assessment of skin sensitization (OECD 2023a). This has also facilitated development and further refinement of several *in silico* tools, providing useful information on chemistry and mechanistic domain of sensitizers (e.g. DEREK Nexus, OECD QSAR Toolbox, TIMES, ToxTree).

To date, there are nine developed and validated New Approach Methodologies (NAMs, *in chemico* and *in vitro* tests) that have achieved OECD test guidelines (TG) status (Figure 2). Each of the test methods/assays addresses a KE in the skin sensitization AOP. OECD TG 442C describes the direct peptide reactivity assay (DPRA), amino acid derivative



## SKIN SENSITISATION OVERVIEW



**Figure 1.** Overview of the skin sensitization AOP. *KE1*: Covalent binding to skin proteins, the MIE for skin sensitization. Bioavailable, electrophilic chemical must bind covalently to skin proteins, forming a complete antigen recognised by the immune system. *KE2*: Keratinocytes activation, resulting in upregulation of inflammatory cytokines and chemokines, and induction of cytoprotective gene pathways. *KE3*: APCs activation and presentation of haptened proteins to T cells, leading to migration of activated T cells into circulation, completing induction phase of sensitization. *KE4*: T cell proliferation in response to the haptened protein presented by APCs, leading to the activation of memory T cells. *Adverse outcome (ACD)*: the clinical manifestation of skin sensitization (elicitation) occurs on subsequent exposure to the same or a cross-reactive chemical. Each KE described above also occurs during elicitation, with some modification. The sensitizing chemical binds covalently to skin proteins, which are then processed and presented to the memory specific T cells. Memory T cells are attracted to the skin site of the exposure by the increased secretion of inflammatory cytokines by keratinocytes. The elicitation phase culminates in the inflammatory response local to the site of exposure to the same (or cross-reactive) chemical the individual has previously been sensitized to. *Image*: NEXU Science communication.

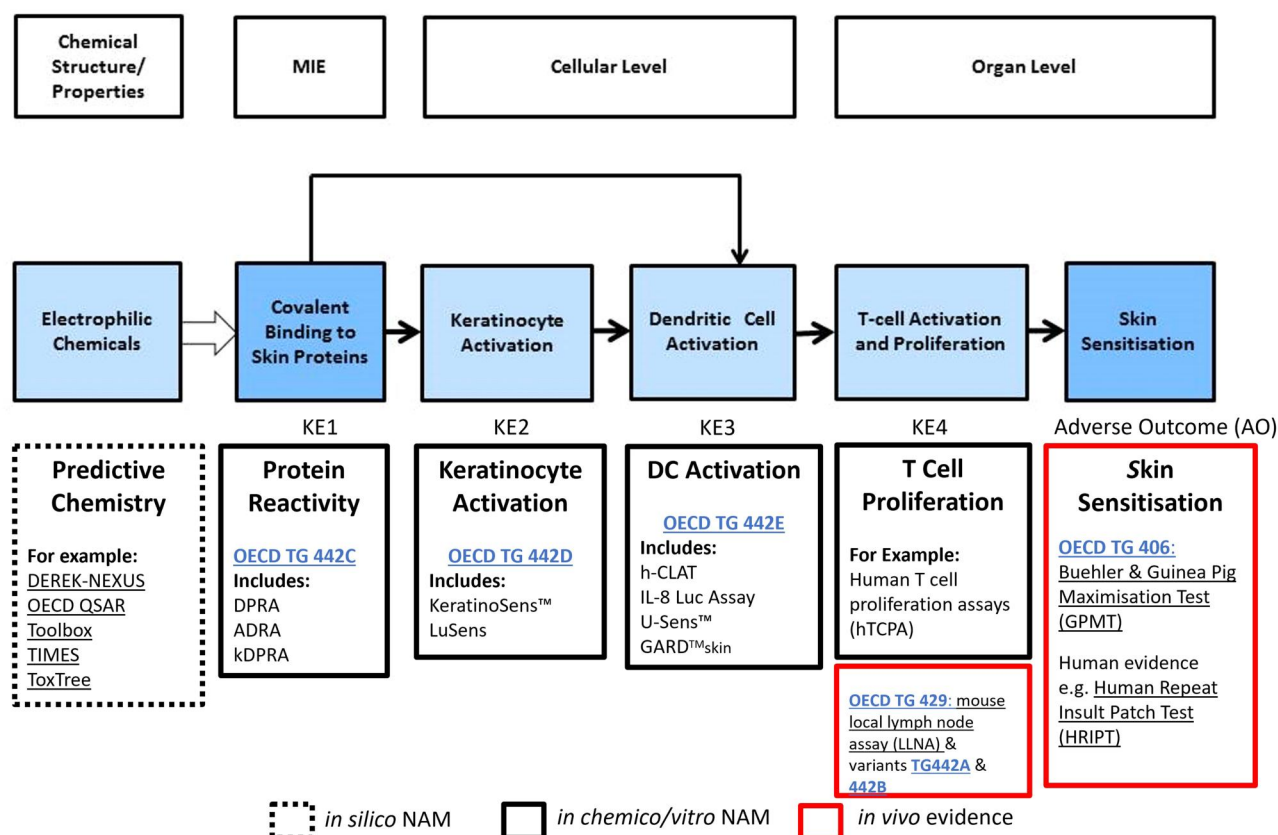
reactivity assay (ADRA) and the kinetic DPRA (kDPRA), which are test methods addressing KE1 (the binding of haptens to proteins in the skin (OECD 2023b)). OECD TG 442D describes the KeratinoSens<sup>TM</sup> and LuSens methods which address KE2 (the activation of keratinocytes (OECD 2022a)). OECD TG 442E describes the Human Cell Line Activation test (hCLAT), U937 cell line activation Test (USens<sup>TM</sup>), Interleukin-8 Reporter Gene Assay (IL 8 Luc assay) and Genomic Allergen Rapid Detection (GARD<sup>TM</sup>) for assessment of skin sensitizers (GARD<sup>TM</sup>skin) which are test methods addressing KE3 (the activation of dendritic cells (DCs)) (OECD 2023c)). With the exception of the GARD<sup>TM</sup>skin assay in certain circumstances (JRC EC 2021), these NAMs should not be used alone for hazard and potency categorization, but the output should be combined with information from other NAMs within a DA.

Three DAs are currently included in the OECD guideline 497 (OECD 2023a) which allow data from the individual assays to be integrated for chemical classification (either binary (sensitizer/non-sensitizer) or the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) hazard subcategories 1A (strong sensitizer) or 1B (moderate or weak sensitizer) (UN 2021)); reviewed by (Ezendam et al. 2016; Kleinstreuer et al. 2018)). The outputs from the majority of predictive assays do contain a quantitative component, however, use of quantitative

information within the DAs currently described in OECD 497 is somewhat limited. The most recent effort is utilization of quantitative output from reactivity assays to redefine thresholds for improved UN GHS classification (Alépée et al. 2023).

Better utilization of the quantitative outputs and more refined predictions of the skin sensitization potency has been achieved in recently developed DAs. The SARA model allows derivation of a human relevant point of departure with quantifiable uncertainty and a risk metric for use in quantitative risk assessment (Reynolds et al. 2019) and has recently been adapted in collaboration with NICEATM to address UN GHS classification (Reynolds et al. 2023, manuscript in preparation). Additional DAs include Bayesian Integrated Testing Strategy (ITS) (Jaworska et al. 2013), Artificial Neural Network (Tsujiata-Inoue et al. 2015) and a series of linear regression models (Natsch and Gerberick 2022a, 2022b; Natsch 2023). The value of these DAs within a risk assessment for skin sensitization has been recently explored using case studies (Gilmour et al. 2020, 2022, 2023; Reynolds et al. 2021; OECD 2022c).

Currently, there are no NAMs addressing KE4 (the activation and proliferation of a T cell response), which are sufficiently progressed for implementation in OECD TG or for use in a Next Generation Risk Assessment (NGRA) (van Vliet et al. 2018).



**Figure 2.** Predictive tools for risk assessment aligned to skin sensitization AOP. Current set of non-animal methods (NAMs) developed for key events in skin sensitization, underpinned by the AOP framework include: the direct peptide reactivity assay (DPRA) (Gerberick et al. 2004; 2007), amino acid derivative assay (ADRA) (Fujita et al. 2014; Yamamoto et al. 2015; Fujita et al. 2019) as well as kinetic DPRA (kDPRA) (Roberts and Natsch 2009; Wareing et al. 2017; Natsch et al. 2020), all included in the OECD TG 442C (OECD 2023b); KeratinoSens™ (Emter et al. 2010; Natsch and Emter 2016) and LuSens (Ramirez et al. 2014) included in the OECD TG 442D (OECD 2022a); human cell line activation test (hCLAT) (Ashikaga et al. 2006), U937 cell line activation Test (U-SENS™) (Piroird et al. 2015), Interleukin 8 Reporter gene assay (IL 8 Luc assay) (Takahashi et al. 2011) and Genomic allergen rapid detection for assessment of skin sensitizers (GARD™skin) (Johansson et al. 2011, 2013) included in the OECD TG 442E (OECD 2023c). In addition to the above tools, numerous predictive chemistry (*in silico*) tools are also available (dotted box). *In vivo* evidence (red box) comes from mouse local lymph node assay (LLNA) (OECD 2010a) and its variants (OECD 2010b, 2018) and Buehler and Guinea pig maximization Test (GPMT) (OECD 2022b). The LLNA, in fact, addresses KE4, measuring T cell proliferation in treated mice rather than established sensitization.

SENS-IS and GARDskin™ dose response assays (Cottrez et al. 2015, 2016; Gradin et al. 2021), which cover multiple KEs, show some promise in providing more quantitative skin sensitizing potency assessment by predicting a skin sensitization class (strong/moderate/weak/non-sensitizing). However, it remains to be seen how these can be utilized in risk assessment.

### 1.3. Current mechanistic research and future possibilities

The current AOP, which serves as a backdrop for the development of predictive assays for sensitization, is somewhat linear in nature, implying that events occur one after the other in an orderly sequence. However, as most of the events described above directly result from the electrophilicity of a topically applied chemical, it is more likely that they happen concomitantly or that they significantly overlap. As evident from investigations of haptenation and metabolism of 2,4-dinitro-1-chlorobenzene (DNCB) in HaCaT cells, interactions with cell surface proteins, relevant detoxification mechanisms as well as cell defense mechanisms otherwise capable of undoing or ameliorating the damage from protein haptenation most likely occur concomitantly to antigen formation

(Jacquilleot et al. 2015; Parkinson et al. 2020b). This represents an opportunity to “branch” the AOP in certain areas to gain better mechanistic understanding of each KE and their potential overlap. It is possible that this could facilitate more meaningful data integration in the future and increase the biological relevance of sensitization potency predictions.

Currently, quantitative reactivity data appears to be the single most valuable parameter when predicting the potency of skin sensitizers (Natsch and Emter 2017). Increasing our understanding of how chemical reactivity “plays out” in the skin tissue may be of additional value in making further progress to improve the current risk assessment of a variety of chemicals with different chemical mechanisms. Determining the optimal ways to measure reactivity related to antigen generation as well as additional events associated with reactivity and deeper quantitative understanding of cell stress pathways involved represent research avenues that could be explored further. This would facilitate further discussion on each of the KEs and strengthen the evidence on the relevance of each parameter that may be useful in decision making. The value of reviewing and furthering the detailed understanding of KEs in skin sensitization AOP lies in keeping the information up to date and motivating discussion to use new scientific insights to constantly look to improve our

safety assessments, rather than as a critique of the existing or emerging test methods or the DAs currently used or under development. While predictive assays need not be complicated and exactly replicate the entirety of events occurring in the skin, to be informative in the risk assessment, they should enable a reliable and confident assessment of the risk posed to individuals exposed to electrophilic chemicals and be used as a part of a tiered approach (Gilmour et al. 2022). Currently, *in chemico/in vitro* predictive assays used within DAs do not incorporate NAMs based on some of the additional mechanistic insights into the MIE in skin sensitization and this information might be valuable in risk assessment decisions.

We consider the current understanding of the KEs in the skin sensitization AOP, discuss the challenges posed by the linear interpretation of the AOP and introduce novel reasoning of how new and emerging hypotheses on individual KEs and their potential overlap could be investigated, measured, and potentially utilized in design of future predictive assays and DAs for potential use in future skin sensitization risk assessment.

## 2. Methods

An already existing body of literature the authors are familiar with was used as a starting point for this review. In particular, a journal article by Natsch and Emter (2017) served as an inspiring body of work suggesting further mechanistic investigation of chemical reactivity may provide significant and useful insights into underlying factors affecting sensitizer potency of chemicals. An NIH PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) search of peer reviewed journal articles was conducted up to 1 August 2023 using generic terms “skin allergy,” “contact sensitization,” “protein haptenation,” etc., with the intention to source suitable articles for review of the mechanistic research in skin sensitization over the past couple of decades, in particular focusing on the period since the publication of the skin sensitization AOP (OECD 2014). The search terms were widened to include journal articles which deal with phenomena associated with skin sensitization in some mechanistic aspects, in particular including the effects of electrophilic chemicals on biological systems and associated events (chemical metabolism, detoxification, lipid peroxidation etc.). The data and conclusions from the relevant journal articles were critically evaluated by the authors and used to support the discussion on key events of the AOP in sensitization. The focus remained on widening the knowledge of AOP key events and exploring the potential to develop novel and useful insights and assays for higher tier risk assessments.

## 3. Results

### 3.1. Reactivity and consequences

Electrophilic reactivity of a sensitizer, either direct (hapten) or acquired by air oxidation (pre-hapten) or metabolic activation (pro-hapten), has long been understood as a prerequisite for sensitization (Landsteiner and Jacobs 1935). Otherwise

“invisible” to the immune system, the (pro/pre) electrophilic chemical forms a complete antigen by covalently binding to nucleophilic side chains of amino acids in skin proteins. This MIE starts the sequence of events which ultimately lead to sensitization and understanding this has enabled development of *in chemico* reactivity assays and their subsequent refinements. Reactivity assays today are an integral part of non-animal risk assessment of chemicals for skin sensitization potential (e.g. DPRA, ADRA, and kDPRA, see Figure 2).

The majority of reactivity assays (originally developed in 2004 and included in the OECD TG 442C (OECD 2023b)) measure the depletion of the limited number of target nucleophile(s) at a single time point (typically 24 h), except kDPRA, which assesses kinetics of reactivity with Cys peptide at multiple time points. Importantly, nucleophile (peptide) depletion measures the level of haptenation indirectly. As such, it often captures oxidative depletion (of Cys peptide) rather than purely haptenation-induced depletion. The mechanisms of Cys peptide oxidative dimerization are not yet explained (Natsch and Emter 2017), but it is likely that this reaction also occurs in keratinocytes and potentially contributes toward overall oxidative stress. Additional reactivity assays developed since then and not included in the OECD TG 442C can add valuable information for risk assessment. These involve use of multiple nucleophiles, investigating reaction mechanism(s) and/or measurement of the initial rate of reaction (Natsch et al. 2007; Natsch and Gfeller 2008; Aleksic et al. 2009; Chipinda et al. 2010, 2014; Sanderson et al. 2016). Perhaps the most valuable development is classification of sensitizers into mechanistic reactivity domains (Roberts et al. 2007) which can be used for prediction of sensitization potency using reaction kinetics and hydrophobicity parameters (within the constraints of Michael acceptor (MA) and some parts of Schiff base (SB) reactivity mechanistic domains (Roberts and Natsch 2009; Roberts et al. 2017)). However, complex reactivity mechanisms and reactions with multiple nucleophiles, including crosslinking, are not easily captured in reactivity assays and remain difficult to consider in decision making.

Since the original design of the now widely used reactivity assays in 2004, the understanding of the complex nature of protein haptenation and some aspects of how the inherent reactivity of the chemicals results in generation of specific complete antigens when bound to proteins has considerably improved. Relevant mechanistic studies and emerging hypotheses are discussed below.

Utilizing fluorescence of bromobimanes’ adducts, two studies have confidently identified epidermal keratin complexes as major haptenation targets (keratin 5 (K5) in mouse skin (Simonsson et al. 2011) and both K5 and K14 in human epidermal keratinocytes (Bauer et al. 2011)). Keratins are highly abundant heterodimeric pairs of type I and type II keratins which make up the cytoskeletal keratin intermediate filament network in epithelia. In human epidermis, K5 and K14 are found in the basal layers, whereas suprabasal layers express another heterodimeric keratin pair, K1 and K10, which are extensively crosslinked and far less likely to have available Cys residues for haptenation (Moll et al. 2008). Cys 54 of K5 was shown as one of the main bromobimane targets. These two remarkable studies have shown that



haptentation in living cells and tissues is relatively rare event, given that protein concentration far exceeds that of the hapten. Another study located a specific fluorescent tetramethylrhodamine isothiocyanate (TRITC) modification on unusually reactive N-terminal proline of migration inhibitory factor (MIF) in lymph nodes and blood of mice topically treated by TRITC, suggesting MIF may have an important immunomodulatory role in sensitization (Karlsson et al. 2018; Ndreu et al. 2022). This additionally demonstrates utility of the fluorescent adduct approach for studying haptentation of sensitizers which react with amine based nucleophiles (Patlewicz et al. 2002), but remains applicable only to electrophiles which make fluorescent adducts.

Studying correlation between reactivity and sensitization potency, Natsch and colleagues investigated the anti-inflammatory effect Michael acceptors are exhibiting *via* inhibition of nuclear factor-kappa B (NF $\kappa$ B) signaling, which appears to dampen their pro-inflammatory sensitizing effect and appear less potent. Cyclopentenone prostaglandins (Natsch et al. 2011), 4-hydroxy-2-nonenal (4HNE) (reactive carbonyl species (RCS), which are end product of lipid peroxidation (Ji et al. 2001)) and parthenolide (Kwok et al. 2001) modify I $\kappa$ B kinase (IKK), whereas sesquiterpene lactones directly target p65 of NF $\kappa$ B (Lyss et al. 1998; García-Piñeres et al. 2001). This is in contrast to S<sub>N</sub>2 and S<sub>N</sub>Ar reactive sensitizers, which are not inhibiting this signaling cascade (Natsch et al. 2011). Clearly, studying specific haptentation targets may help us interpret the reactivity of sensitizers more accurately in the context of sensitization potency.

Elbayed et al. (2013) showed the utility of the high-resolution magic angle spinning nuclear magnetic resonance (HR MAS NMR) to compare haptentation of human serum albumin (HSA) and reconstituted human epidermis (RHE) by a model sensitizer methyl dodecanesulphonate. The critical comparison of haptentation “in solution” using a model protein Vs haptentation in a 3D model of human epidermis showed a considerably faster haptentation in tissue (detectable by 24 h compared to days with HSA). Chemoselectivity for the protein nucleophiles differed significantly between models, with Lys being targeted on HSA, and His, Met, and Cys found modified in RHE. Two further studies using this approach (with  $\alpha$ -methylene- $\gamma$ -butyrolactone (Debeuckelaere et al. 2015) as well as 2-methylisothiazolin-3-one (MI) and 5-chloro-2-methylisothiazolin-3-one (MCI) (Debeuckelaere et al. 2016)) confirmed the initial kinetic and chemoselectivity findings of Elbayed and colleagues and demonstrated the importance of reactivity toward multiple nucleophilic residues, namely His. While the HR MAS NMR studies detect the nucleophilic residues and do not identify haptentated proteins as such, they clearly demonstrate that nucleophile targeting and the dynamics of the haptentation are not fully represented in the current *in chemico* reactivity assays. However, previous efforts to include His in reactivity assays, have demonstrated poor sensitivity of His peptide depletion as well as poor predictivity compared to Cys and Lys peptides (Gerberick et al. 2004).

The sparse protein haptentation was detected using sensitive stable isotope labeling, initially with single proteins and relevant protein mixtures (HaCaT cell and human skin lysates) (Parkinson et al. 2014, 2018, 2020a). This method was further

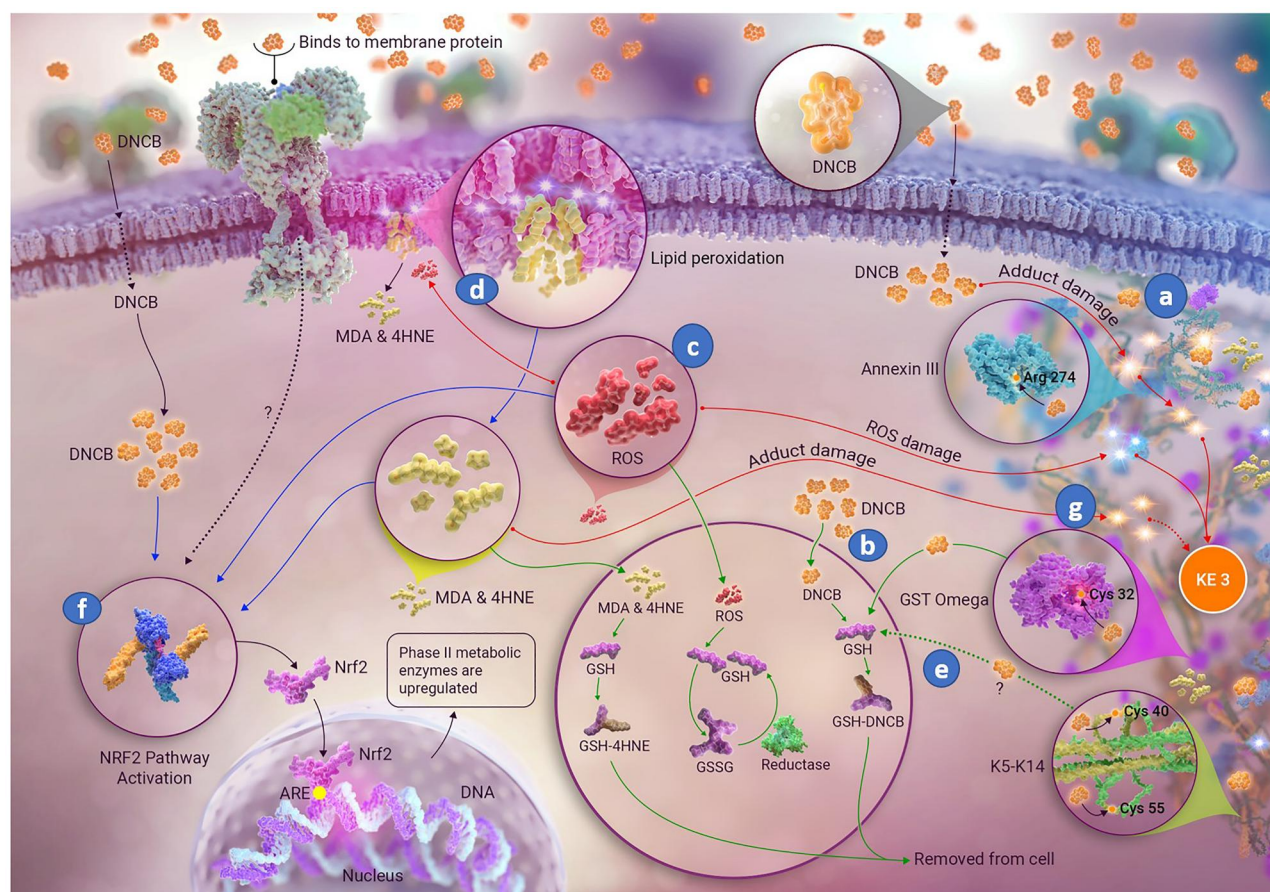
utilized with living HaCaT cells treated with DNCB, adding the quantitative analysis of proteome (Parkinson et al. 2020b). Minimal changes in overall protein differential expression were observed in HaCaT cells while DNCB haptentated approximately 0.25% of all available nucleophiles when applied at a subtoxic concentration (10  $\mu$ M) for 4 h. This vanishingly low level of haptentation by an extremely potent sensitizer is in contrast with observations from reactivity assays, where DNCB depletes 100% of target peptides. The sites of modification on individual proteins were detected, however, haptentation could not be directly quantified. While reactive with most nucleophiles in reactivity assays, DNCB showed a preference here for Cys residues, despite the considerably higher concentration of amine-based nucleophiles. Although a proportion of highly abundant proteins were haptentated, including K5 and K14 (seen haptentated by bromobimanes previously), haptentation was also detected on low abundant proteins. Interestingly, maximum haptentation occurred at 2 h and was negligible at the end of the time course (48 h), indicating that DNCB haptentation may have been reversed. This is in contrast to the current consensus opinion that the covalent modifications are irreversible (OECD 2014). Similar dynamics were mirrored by a study using DNCB at the identical subtoxic concentration and same cell line which quantitatively investigated time course of the main phase II metabolism of DNCB, binding to glutathione (GSH) (Jacquilleot et al. 2015). In the glutathione-s-transferase (GST) catalyzed process, 2 h timepoint showed maximal depletion of GSH, with levels recovering from 4 h onward. A more complex study of GSH cycle upon repeated DNCB treatment was using RHE model and has shown a complete recovery of the levels of GSH after three consecutive daily doses of DNCB (Spriggs et al. 2016).

Despite only a few distinct chemicals having been investigated in the complex studies described above, new hypotheses are emerging from these insights. It is evident that haptentation develops immediately post-exposure to the electrophilic chemical which concomitantly triggers a variety of associated events in epidermal cells. Some of this knowledge has already been utilized for development of predictive assays (e.g. KeratinoSens<sup>TM</sup> (Natsch and Emter 2016; OECD 2022a)). Other associated events, such as damage to proteins by elevated reactive oxygen species (ROS), lipid peroxidation (LP) and secondary damage to proteins from end products of LP (RCS) have not had as much attention.

Three emerging areas of interest which are either a direct consequence of chemical reactivity of sensitizers or are events associated with the initial external electrophilic stress are discussed below with suggestions how exploring these events mechanistically and quantitatively may be useful in predicting sensitizer potency (Figure 3).

### 3.1.1. Skin metabolism

Metabolism research in skin sensitization has been focused on phase I metabolic processes which may be responsible for activation of pro-haptens. Conceptually, the formation of reactive metabolites by phase I metabolism in human skin is



**Figure 3.** Molecular initiating event (MIE) in skin sensitization by 1,4-dinitro-2-chlorobenzene (DNCB). Besides antigen formation ((a) covalent binding of DNCB to specific cell proteins), additional concomitant and associated events ((b) detoxification, (c) oxidative balance disturbance, (d) lipid peroxidation, (e) covalent modification reversal, (f) activation of the Nrf2 pathway) and (g) covalent protein damage from reactive carbonyl species (RCS, end products of lipid peroxidation) are shown. Image: NEXU Science communication.

well established, although *in vitro* demonstration is still relatively limited (Niklasson et al. 2014; Reynolds et al. 2021).

However, current understanding of phase I skin metabolism and abiotic activation (e.g. air oxidation) have been used in the adaptations to existing reactivity assays, aiming to incorporate “activation” of nonelectrophilic chemicals to their electrophilic metabolites which then bind to the model nucleophiles (e.g. Peroxidase Peptide Reactivity Assay (PPRA) (Gerberick et al. 2009; Troutman et al. 2011; Ryan et al. 2020). The utility of current *in chemico* and *in vitro* assays for detection of pre- and pro-electrophiles has been a subject of reviews (Patlewicz et al. 2016; Urbisch et al. 2016). To date, little emphasis has been placed on the activity of phase II metabolizing enzymes in epidermis which are responsible for effective “removal” of a variety of electrophilic molecules by either binding them to substrates generating non-reactive products or converting the electrophiles to related unreactive molecules.

Oesch and colleagues updated their comprehensive review of skin metabolizing enzymes in 2014 (Oesch et al. 2014), reviewing and comparing numerous studies which investigated either mRNA levels, protein levels or activity of a vast array of metabolizing enzymes (including non-CYP metabolizing enzymes, such as aldehyde oxidases/dehydrogenase (AO/ALDH), NAD(P)H quinone reductase (NQR), hydrolases (epoxide hydrolase (EH), esterases/amidases), and

conjugating enzymes, such as glutathione-s-transferases (GSTs), UDP-glucuronosyl transferases (UGTs), sulfotransferases (SULTs) and N-acetyl transferases (NATs)). The broader role of detoxifying enzymes in drug hypersensitivity was subject of a review (Sánchez-Gómez et al. 2016), highlighting the evidence supporting the involvement of GSTs and aldo-keto reductases in development of a variety of allergic responses as well as interaction of these key detoxifying enzymes with oxidative stress molecules, both endogenous and external electrophiles and inflammatory mediators. While it can be challenging to confidently detect and measure activity of some metabolizing enzymes in human skin, 3D skin/epidermal models and skin relevant cell lines, it is accepted that majority of relevant metabolizing enzymes are present and detoxification is observable *in vitro* and *ex vivo* (Manevski et al. 2015; Kazem et al. 2019). However, although stable in models, levels of protein expression of certain metabolizing enzymes and consequently their metabolic activity may vary in individuals as well as at a population level (Spriggs et al. 2018; Buratti et al. 2021).

The phase II metabolism of electrophilic molecules is closely linked to the reactivity mechanism. For example,  $S_N2$  chemicals and MAs are conjugated to GSH, removed out of the cell and processed to the relevant mercapturic acid conjugate for excretion, whereas SB formers and acylators are metabolized by AO/ALDH (Table 1). When a sensitizer has



**Table 1.** Likely phase II metabolic fate of different mechanistic groups of sensitizers.

Reaction mechanism	Associated detoxification mechanism	Enzyme(s) involved
Michael addition (MA)	GSH conjugation	GSTs
Acylation (Ac) and Schiff base formation (SB)	Conversion of aldehyde to carboxylic acid	AO/ALDH
$S_N2/S_NAr$	GSH conjugation	GSTs
Other examples (e.g. PPD)	N-acetylation	N-acetyl transferase(s)

Note: The two most prominent enzyme systems are glutathione-S-transferases (GSTs) and aldehyde oxidases/dehydrogenases (AO/ALDHs) both serving to mitigate the effects of externally or endogenously induced oxidative and electrophilic stress.

more than one electrophilic center, there may be more than one mechanism involved in completely removing the sensitizing potential of that molecule. For example, cinnamaldehyde reacts *via* MA to thiols and *via* SB formation to amine nucleophiles. Thus, cinnamaldehyde  $\alpha,\beta$ -unsaturated moiety undergoes conjugation to GSH catalyzed by GSTs and its carbonyl moiety is dealt with by an AO/ALDH converting it to cinnamic acid.

The phase II metabolic reactions are inevitably faster than spontaneous protein haptenation, due to enzyme catalysis and high concentrations of cofactors (e.g. cellular GSH concentration is approx. 5 mM in most cells (Pizzorno 2014)). Most electrophiles are subject to these cellular defense mechanisms, effectively preventing the damage they may exert by covalently modifying proteins. This can explain the vanishingly low and, until recently, almost undetectable level of haptenation by non-cytotoxic concentration of even a very potent sensitizer, such as DNCB (Parkinson et al. 2020b).

Considering the reactivity of sensitizer in the context of detoxification may help explain extreme potency of certain sensitizers. For example, DNCB is an extreme sensitizer and depletes 100% of Cys peptide in standard reactivity assays (Gerberick et al. 2004). However, DNCB is found to covalently modify the active center of GST- $\omega$  (Figure 3, b), the enzyme which catalyzes the phase II metabolism of DNCB, possibly disabling the enzyme (Bailey et al. 2021). Another extreme sensitizer, 5-Chloro-2-methylisothiazol-3-one (MCI), is activated by thiols, including GSH and two further highly reactive intermediates are generated in this process (Alvarez-Sánchez et al. 2004a; 2004b). Thus, instead of effective detoxification, the GSH acts as a catalyst, increasing the reactivity of MCI. These two extremely potent sensitizers appear to impede their removal from the cell. Further instances of sulphhydryl based (e.g. GSH) activation of sensitizers have recently been shown for isothiocyanates (Karlsson et al. 2016) and 2-bromo-2-(bromomethyl)glutaronitrile (MDBGN) (Ndreu et al. 2020). It is also known that some GSH conjugates can be converted into reactive, toxic intermediates (Anders 2008; Cooper and Hanigan 2018).

Main phase II metabolic mechanisms apply to both externally applied and endogenously generated electrophilic molecules (e.g. RCS). These are mainly aldehydes, dialdehydes or  $\alpha,\beta$ -unsaturated aldehydes, and it is likely that their increased level would impact the capacity of GST and AO/ALDH detoxification mechanisms. It may be important to determine the level of RCS generated by exposure to sensitizers to be able to fully assess the detoxification capacity and how this influences the potency for skin sensitizers competing for the same detoxification mechanisms.

Quantifying the phase II metabolic capacity of the human keratinocytes for the main mechanistic domains of reactive chemicals should enable understanding of how this process competes with protein haptenation and at which concentration the electrophilic chemical could overwhelm the cellular defense mechanisms. Reactivity rates of reaction of sensitizers with simple nucleophiles are already available *in chemico* (e.g. aldehydes reactivity with butylamine (Natsch et al. 2018) and electrophiles with glutathione (Schultz et al. 2005)), although inclusion of enzymatic activity (in skin extracts or using isolated enzymes) might give a more relevant estimation of detoxification *in vivo*. This can be applied to the chemicals with a single mechanistic domain as well as those with more than one reaction mechanism.

### 3.1.2. Oxidative stress and end products of lipid peroxidation (LP)

Oxidative stress is part of keratinocyte inflammation, currently considered under the KE2 in the AOP. This is a direct consequence of electrophilicity of sensitizers, and a molecular basis for secondary inflammatory effects also described in KE2. Electrophile presence in keratinocytes disturbs the redox balance, either by direct binding to cellular antioxidants (such as GSH), oxidative dimerization of thiols (e.g. GSH to GS-SG) or *via* radical mechanisms (directly or following activation, e.g. hydroperoxides). Disruption of redox balance by engaging the main cellular antioxidant inevitably leads to increase in ROS (Figure 3, c), which, in turn, disturbs cellular homeostasis *via*: (1) irreversibly damaging cellular proteins (attacking the nucleophilic sites and the protein backbone); (2) attacking the polyunsaturated fatty acids (PUFAs) (including disrupting the cell membrane) and initiating LP (Figure 3, d) which results in formation of endogenous electrophiles (or RCS) as well as (3) additionally activating the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (Figure 3, f), ultimately increasing the transcription of the phase II metabolizing enzymes in the cell.

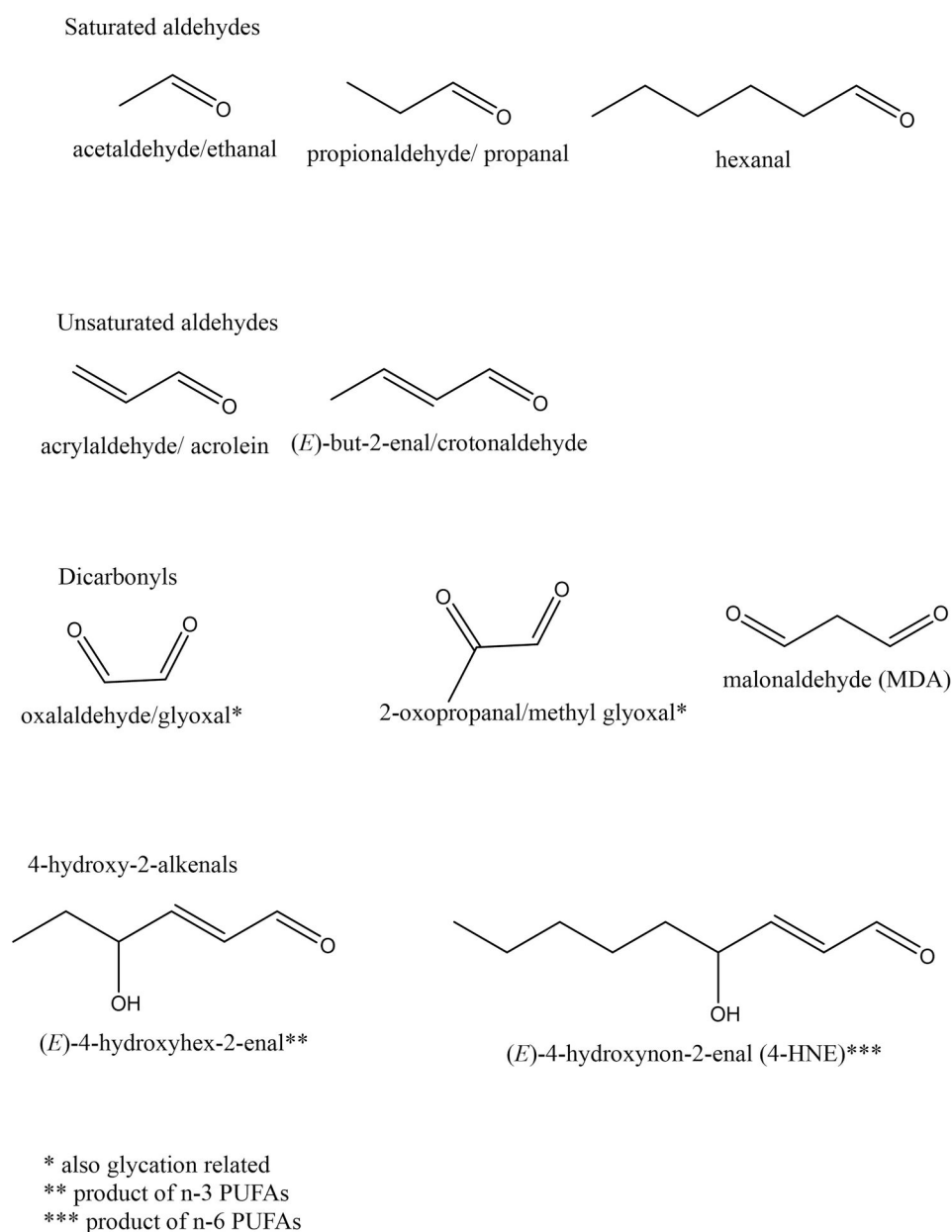
Increased ROS results in the accumulation of the unfolded proteins in the endoplasmic reticulum (ER) and activation of the unfolded protein response (UPR) (Read and Schröder 2021). This ROS dependent phenomenon has been demonstrated in a THP-1 cell line treated with a potent skin sensitizer 1-fluoro-2,4-dinitrobenzene (DNFB) (Luís et al. 2014). Increase in ROS is, therefore, likely to speed up the processing of haptenated proteins.

Redox balance disruption deepens the damaging effect by enabling elevated ROS to interact with cellular and organelle membranes, where a phospholipid bilayer is made up of

hydrophilic head group and hydrophobic acyl chains of saturated, monounsaturated, and polyunsaturated fatty acids. Epidermis and keratinocyte cell cultures contain a large proportion of saturated fatty acids (FAs), and of the polyunsaturated fatty acids (PUFAs), only linoleic acid (LA, 18:2  $\omega$ -6) and arachidonic acid (AA, 20:4  $\omega$ -6) are detected at higher levels and vary considerably between freshly isolated epidermis and cultured keratinocytes (Ponec et al. 1988; Schürer et al. 1993). LP involves ROS attack on the carbon-carbon double bonds of the PUFAs (Figure 3, d), often in the phospholipid bilayer of membranes (Pamplona 2011; Łuczaj et al. 2017). ROS initiates LP, however, the progression of this reaction is not further impacted by ROS levels. It is, instead, driven by the proximity of the peroxidizable fatty acid chains (Zimniak 2011). Self-propagating fatty acid peroxy radicals of  $\omega$ -6, but

also  $\omega$ -3 PUFAs, ultimately produce, *via* series of cleavages, a collection of over 30 biologically active structurally related electrophilic molecules known as RCS (end products of LP) (Figure 4) (Kawai et al. 2007; Negre-Salvayre et al. 2008). Unlike ROS, RCS have longer half-life and are capable of covalent modification of other biomolecules, in particular proteins, causing “endogenous electrophilic stress” (Marnett et al. 2003).

RCS have multiple roles, ranging from cell signaling and preventing irreversible oxidative protein damage at low concentrations to damage inducing at high concentrations (Domingues et al. 2013; Castro et al. 2017; Mol et al. 2017; Zhang and Forman 2017). RCS covalently modify proteins *via* MA reactions and SB formation, much like some externally applied sensitizers, which they resemble. Proteins covalently



**Figure 4.** Structures of some reactive carbonyl species (RCS), end products of lipid peroxidation (LP). The most abundant and most often studied RCS are malondialdehyde (MDA) and 4-hydroxynonenal (4HNE). A large body of literature details extensive investigations of generation, known metabolic routes and effect on certain signaling events, cellular Pool of protein and pathologies associated with production of MDA and 4HNE (e.g. Alzheimer's disease, Parkinson disease, liver disease, diabetes, cardiovascular disease and cancer) (reviewed by but not limited to) (Ayala et al. (2014)).

modified by RCS (carbonylated proteins), are often processed by the proteasome 20S, but can be ubiquitinated and processed by the proteasome 26S or, indeed, by lysosomes where, due to low pH and active acid hydrolases, SB adducts can be reversed (Zhang and Forman 2017).

From a structure and reactivity perspective, RCS resemblance of well-known skin sensitizers raises the obvious question of the immunogenicity of their protein adducts. Indeed, there is accumulating evidence that malondialdehyde (MDA) modifications of certain proteins act as oxidation-specific epitopes capable of triggering both innate and adaptive immune response (Papac-Milicevic et al. 2016). Only two RCS molecules have been tested in sensitization assays and classified as human sensitizers (glyoxal (Gerberick et al. 2005; Basketter et al. 2014) and hexanal in DPRA (Kawakami et al. 2020)). It is easy to assume that, if tested, RCS could be determined as topical sensitizing chemicals, however, realistically, the sensitizing concentrations should far exceed those that are generated endogenously. Under oxidative stress conditions, keratinocytes are likely producing a variety of RCS, which are collectively damaging, but individually should not reach the concentration required to provoke a specific immune response. Additionally, certain LP end products are metabolized and routed into other cellular processes (e.g. MDA and acetaldehyde are converted to acetate; methyl glyoxal is converted into pyruvate and both enter the citric acid cycle (Negre-Salvayre et al. 2008; Ayala et al. 2014)), potentially lowering the chances of eliciting a skin reaction in an individual topically sensitized to an LP end product.

The type and level of individual RCS generated under oxidative stress conditions is entirely dependent on the PUFA composition of the cells (Zimniak 2011). The susceptibility of PUFAs to peroxidation increases proportionately with their level of unsaturation and can be determined by calculating the “peroxidation index” for a known PUFA composition (Hulbert et al. 2014). This could be one of the important sources of variability in individual sensitization thresholds.

All cells, including keratinocytes, have adapted and developed defense mechanisms to rapidly sequester RCS or metabolize them into other useful molecules and repair the damage they cause (Marnett et al. 2003; Sousa et al. 2017). However, it is important to consider the level and type of RCS generated during the induction of sensitization, given that their reactivity is resulting in cellular interactions chemically mirroring those of sensitizers (Figure 3, g). Dependent on the type and level of RCS generated, they may act as competition for detoxification mechanisms, impairing the cellular ability to remove the sensitizer effectively; contribute to further activation of the UPR and “speed up” the processing of haptenated proteins and/or as additional signal to increase the cellular ability to remove the electrophiles, for example by activating the Nrf2 pathway.

### 3.1.3. Stability of covalent adducts

We explored the literature for evidence of (in)stability and reversibility of covalent modifications both in skin sensitization field and beyond, namely in studies examining reactions of LP end products, RCS.

Time course studies of DNCB haptenation (Parkinson et al. 2020b) and GSH cycle in DNCB treated HaCaT cells (Spriggs et al. 2016) show that protein haptenation peaks at 2 h, coinciding with maximum depletion of GSH. The level of haptenation is negligible at 48 h, indicating that either the previously haptenated proteins have been removed somehow from the samples or that the modifications have been reversed. The latter scenario seems more plausible for several reasons: (1) the haptenation experiments were conducted in a closed system; (2) the whole sample was lysed at a given time point and, thus, it contains the entire proteome, including proteins that might have been “expelled” from the cells; (3) there was no obvious way to systematically remove the haptenated proteins; and (4) it is energetically more favorable for the cell to repair the damage to haptenated proteins by removing the covalently bound hapten than to undergo *de novo* protein synthesis. While none of the above reasons constitutes a proof that covalent modifications by DNCB are reversed, it points us strongly in the direction of exploring the possibility of this occurring (Figure 3, e).

Natsch and Emter exemplified occurrences of “sequential” reactivity of electrophiles with different amino acid residues (Natsch and Emter 2017). Using MCI, methyl isothiazolinone (MI), oxazolone, isocyanate, isothiocyanate, and an anhydride as case studies, an initial reaction with thiol (protein Cys residue or GSH) is described, followed by a final, more stable adduct detected on an amine (Lys or His residues on proteins). The article does not explicitly discuss haptenation reversibility in the context of hapten removal and ultimate detoxification, however, it does point out lack of full understanding of the complex reactivity of sensitizers.

Outside of skin sensitization field, experimental reversal of SB adducts is found in literature reporting measurements of “free” and “total” levels of RCS where acidification is often used experimentally to remove the SB conjugated MDA and subsequently capture it by thiobarbituric acid (TBA) (e.g. Spickett et al. 2010). Haptenated proteins are processed in an acidic environment of lysosomes, where acid hydrolases are active at low pH (Cooper 2000), likely reversing SB adducts. Furthermore, to prevent the known spontaneous reversibility of SB adducts in biological samples, sample preparation (e.g. for proteomic analyses) often includes “stabilization” step (e.g. Han et al. 2007; Eggink et al. 2008). This involves a reaction with sodium borohydride ((Billman and Diesing 1957)), which reduces the  $-C=N-$  bond in SB to a more stable  $-CH_2-NH-$  making the adduct less likely to be hydrolyzed and, therefore, more likely to be detected.

Reversibility of several types of GSH adducts has been discussed as a spontaneous event (Baillie and Kassahun 1994). A recent study investigating 4-hydroxynonenal (4HNE) site specific binding in human colon carcinoma cell line treated with an analogue of 4HNE, showed that MA adducts rapidly disappear in intact cells (Yang et al. 2015). The mechanism of this potential reversal remains unknown, however, sporadic evidence shows that some MA adducts can be reversed by excess thiol (often GSH) (Mol et al. 2017). Some reports detail design of reversible MA electrophiles (Lee and Grossmann 2012; Krishnan et al. 2014). Another important component of cellular defense against ROS, thioredoxin (Trx) and its



associated enzyme thioredoxin reductase (TrxR) comprise a system capable of reducing the disulfide bonds of oxidized Trx, but also other chemical entities, such as lipid hydroperoxides (Nordberg and Arnér 2001). There is, however, no direct evidence that this system can reduce S-C bonds formed during MA reaction.

Certain strong or extremely potent sensitizers (Gerberick et al. 2005) have more than one reaction mechanism or react *via* complex mechanisms. It is plausible that complex adducts and crosslinks are less likely to be reversed (or are energetically “costly” to reverse) and that this could explain their high sensitization potency. For example, glutaraldehyde is a strong sensitizer capable of formation of strong crosslinks between (and within) proteins (Bowes and Cater 1968), making it a very useful reagent in many experimental procedures (Migneault et al. 2004). Another potent sensitizer, formaldehyde, makes initial methylol adduct with amine nucleophiles which is dehydrated to a SB and known to be unstable. However, formaldehyde adducts continue to react beyond this initial step with other amino acids within close proximity forming methylene bridges and imidazolinone adducts (Metz et al. 2004, 2006). Further complex crosslinks following interaction with formaldehyde were recently described (Michiels et al. 2020). An extreme sensitizer, MCI, mentioned earlier, reacts *via* complex mechanism to protein nucleophiles (Alvarez-Sánchez et al. 2004a, 2004b).

Mechanistically, it is unclear how covalent adducts could be reversed in skin for majority of reactions in sensitization and whether this phenomenon has a quantitative impact on kinetics of antigen formation. It is evident that some adducts are more readily reversible than others and plausible that reversing covalent adducts is, in fact, an “extended arm” of cellular detoxification mechanism.

### 3.1.4. Quantitative combination of early reactivity-related events

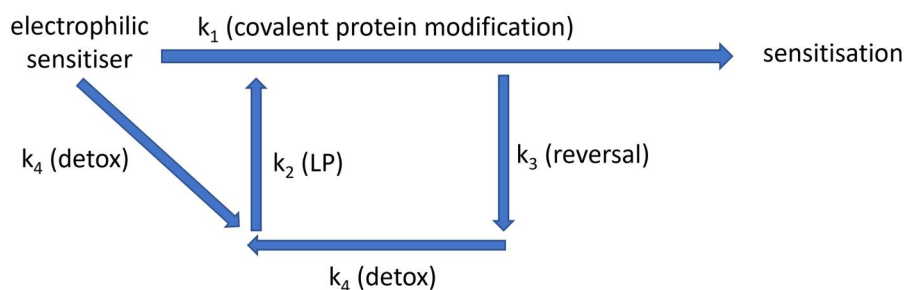
The impact of skin exposure to reactive chemicals reaches beyond antigen generation, triggering the additional events described above. Concomitant nature of these events may explain how sensitization occurs only when cellular defense systems are overwhelmed (Figure 5). Developing assays to quantify certain events (e.g. detoxification, redox balance disturbance and generation of endogenous electrophiles, reversibility of covalent modifications) in addition to existing

reactivity assays may help determine the true relationship between reactivity and sensitization potency of a chemical.

### 3.2. Epidermal inflammation

Keratinocytes and antigen presenting cells (APCs) are triggered *via* innate immune mechanisms during induction of skin sensitization (OECD 2014; Martin 2017), resulting in epidermal inflammation. Often referred to as “danger signal” (Ainscough et al. 2013), molecules released in response to sensitizer-induced cellular injury act as the link between the innate and the adaptive arms of the immune system. Current validated assays addressing KE2 have focused on the activation of the key inflammatory pathway in keratinocytes as a readout of the epidermal inflammation (KeratinSens<sup>TM</sup> (Emter et al. 2010; Natsch and Emter 2016) and LuSens (Ramirez et al. 2014), included in the OECD TG 442D (OECD 2022a)). The assays rest on the principle that electrophiles modify Cys residues on Kelch-like ECH-associated protein (Keap 1) which leads to release of the Nrf2. Released Nrf2, no longer targeted for ubiquitination and proteosomal degradation, migrates to the nucleus and binds to the electrophile/antioxidant response element (EpRE/ARE), thus activating the transcription of cytoprotective genes, mainly encoding for metabolic phase II enzymes (Natsch and Emter 2016). Nrf2 pathway activation represents a cumulative effect of electrophilic sensitizer, ROS, GSH, and endogenous electrophiles (RCS), all of which react with Cys residues of Keap 1 (Figure 3, f). Subject of much investigation, it has been proposed most recently that the potency of a chemical may be inversely proportional to the amount of Nrf2 protein available (Vallion and Kerdine-Römer 2022).

Besides Nrf2 pathway activation, epidermal inflammation in skin sensitization is a culmination of multiple molecular signals from both keratinocytes and APCs (Kimber et al. 2002, 2011). The entire set of molecular components of epidermal inflammation is not completely understood, but it is evident that a substantial overlap in the inflammatory signaling pathways exists in these two cell types. Recent advances in understanding inflammation in both keratinocytes and APCs as well as potential ways of utilizing this information are discussed below.



**Figure 5.** Simplified diagram showing how electrophilic chemicals may interact with cell defense systems, which act in concert to prevent a threshold for sensitization being reached. Electrophilic molecule is likely to encounter cell detoxification mechanisms ( $k_4$ ) faster than be able to covalently modify proteins ( $k_1$ ). It is likely that some type of covalent modifications can be reversed ( $k_3$ ) and subsequently removed by cellular detoxification mechanisms ( $k_4$ ). However, detoxification ( $k_4$ ) may lead to disturbance of redox balance and increase lipid peroxidation (LP,  $k_2$ ), which, in turn, also covalently modifies cellular proteins, potentially speeding up antigen processing and activating unfolded protein response (UPR).

### 3.2.1. Cytokines in keratinocytes and APCs

Cytokine secretion is a hallmark of epidermal inflammation in response to skin sensitizer exposure and is regulated at both transcriptional and post translational levels. Increased serum levels of IL 18 and enhanced expression of IL 1 $\beta$ , IL 1Ra, IL 36a, IL 36b, IL 36c, and IL 33 in the involved skin of ACD patients have been observed (Mattii et al. 2013). Both human keratinocytes and APCs significantly increased the production of IL 1 $\beta$ , IL 18 as well as Interleukin Converting Enzyme (ICE) (Zepter et al. 1997; Matos et al. 2005). ICE or Caspase 1, which cleaves both IL 1 $\beta$  and IL 18 into their biologically active forms, is regulated by inflammasome in both keratinocytes and APCs (Naik et al. 1999; Mee et al. 2000). Pro-inflammatory cytokines also induced in both cell types include tumor necrosis factor alpha (TNF  $\alpha$ ) and IL 8 (Barker et al. 1991; Rambukkana et al. 1996; Nukada et al. 2008). Interestingly, the IL 8 assay, which is the only OECD validated cytokine assay (Watanabe et al. 2007; Sand et al. 2018) was developed as a marker for APC activation (KE3) rather than keratinocyte inflammation (KE2), possibly due to the cell type used in the assay (OECD 2023c).

### 3.2.2. Damage associated molecular patterns (DAMPs)

Both intracellular and extracellular danger signals contribute to the epidermal inflammation *via* cytokine regulation (Schaefer 2014). Upstream to the cytokine expression and processing, extracellular DAMPs such as Low Molecular Weight Hyaluronic Acid (LMWHA) fragments signal through the Toll-like Receptors (TLRs) to trigger inflammatory responses (Scheibner et al. 2006; Jiang et al. 2011). Electrophiles increase the degradation of High Molecular Weight HA (HMWHA) to LMWHA fragments by either modulating the expression of Hyaluronidase isoforms or Hyaluronan Synthase levels in human keratinocytes (Nikitovic et al. 2015). Electrophiles also upregulate TLR 4 and NF $\kappa$ B leading to further modulation of inflammatory cytokines, such as IL 18 and IL 8 (Galbiati et al. 2014; van der Veen et al. 2016; Kavasi et al. 2019). High Mobility Group Protein B1 (HMGB1), a non-histone chromatin binding protein, is an agonist of TLR 4 that is released from keratinocytes on exposure to electrophilic chemicals (Galbiati et al. 2014). LMWHA fragments have also been shown to activate the TLR 4 signaling pathway in DCs, including facilitating phosphorylation of p38/p42/44 MAP kinases and nuclear translocation of NF $\kappa$ B (Termeer et al. 2002). However, covalent modification of either IKK or p65 of NF $\kappa$ B by MA sensitizers mentioned earlier has been shown to inhibit this signaling pathway and have an anti-inflammatory effect (Natsch et al. 2011). Signaling through MAPK is another key component that regulates inflammation through various touch points in both keratinocytes and DCs (Trompezinski et al. 2008).

Adenosine triphosphate (ATP) contributes to inflammation, but also to the maturation of APCs. Chemically injured keratinocytes release ATP and in the presence of extracellular ATP, APCs show a transient increase in endocytosis, up-regulation of CD86, CD54, and major histocompatibility complex (MHC) class II, secretion of IL 12 as well as exhibit an improved

stimulatory capacity for allogeneic T cells (Schnurr et al. 2000; Mizumoto et al. 2003).

Both keratinocytes and APCs elevate ROS upon exposure to skin sensitizers (Bruchhausen et al. 2003; Mehrotra et al. 2005; Martin et al. 2011). This can lead to the activation of inflammasome and release of active IL 1 $\beta$  and IL 18 in keratinocytes (Galbiati et al. 2014). ROS-induced oxidative breakdown of the extracellular matrix results in generation LMWHA, which activate the TLR and NF $\kappa$ B signaling cascade (Martin et al. 2011). As discussed earlier, ROS additionally activates the Nrf2 pathway, which mounts an antioxidant response. Nrf2 pathway cross talks with the NF $\kappa$ B and inflammasome signaling which contributes to the dynamics of pro and anti-inflammatory effects (Ade et al. 2009; Helou et al. 2019). ROS are generated during mitochondrial oxidative metabolism, which implies a key mitochondrial function in the sensitizer-induced inflammatory response and a further burden to the cellular detoxification systems.

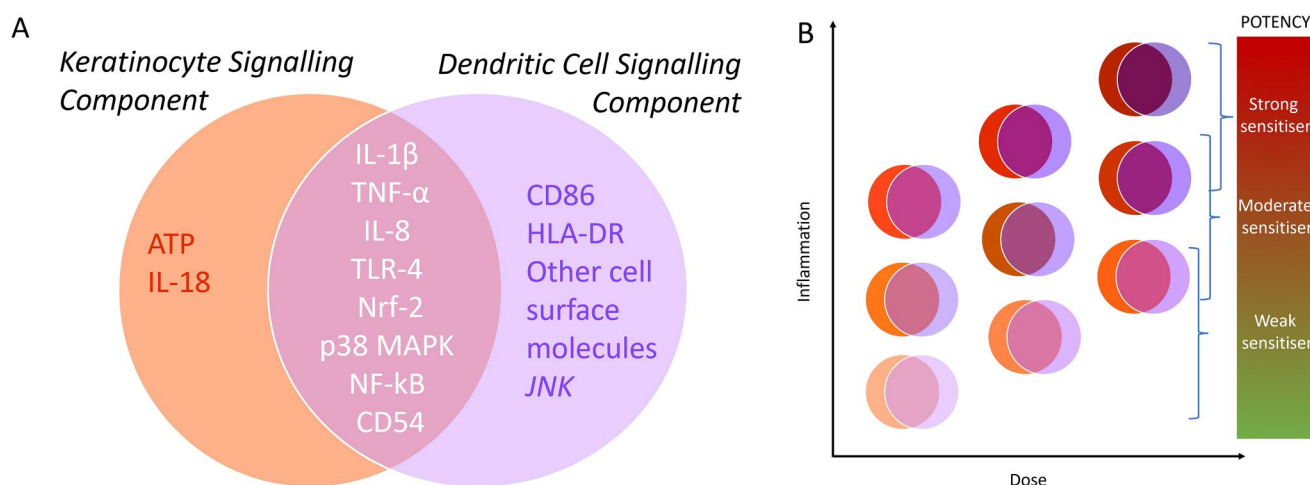
Additional protective systems, upregulated in response to protein damage by electrophiles, have not received much attention (e.g. heat shock inducible factor (HSF1) and hypoxia inducible transcription factor (HIF1 $\alpha$ ), controlling heat shock and hypoxia responses, respectively (Cyran and Zhitkovich 2022)). A recent study has suggested a specific mechanistic role for the heat shock protein 90 (Hsp90) in sensitization to DNCB, identifying several DNCB modified Hsp90 peptide fragments (Kim et al. 2022).

### 3.3.3. Biomarker signatures

Genes downstream of the Nrf2 pathway and other cellular stress markers have been evaluated as signatures for sensitizer-induced transcriptional changes in novel *in vitro* assays, such as SenCeeTox®, SENS-IS, EpiSensA, and VITASENS (Hooyberghs et al. 2008; Lambrechts et al. 2010; McKim et al. 2012; Cottrez et al. 2015; Saito et al. 2017), as well as *ex vivo* skin (van der Veen et al. 2015). In addition to Nrf2 targets, the SENS-IS assay also considers select biomarkers with redox/detox functions within the set of ARE genes (24 biomarkers) and an additional set of 42 genes with varied functionalities (apoptosis, cell death, heat shock, cell stress, inflammation, MHC, proteasome, and tissue repair pathways). Specific criteria applied to a combination of gene expression from both sets is used to classify sensitizers of varying potency. In MUTZ3 CD34+ DC progenitors treated with sensitizers, Nrf2 and ARE genes were found to be part of the predictive gene signature (Johansson et al. 2011). This has been explored further through the development of GARD<sup>TM</sup>skin assay platform and indicates the role of genes related to metabolism and cell cycle in addition to oxidative stress response (Albrekt et al. 2014; Zeller et al. 2017; Johansson et al. 2019; Gradin et al. 2021).

### 3.3.4. Combining multiple inflammatory signals from keratinocytes and APCs

It is difficult to envisage how any measurement of single components of the inflammation process in keratinocytes and/or APCs could provide a useful readout for improving



**Figure 6.** (A) A Snapshot of unique and overlapping signaling components of keratinocytes (KCs) and dendritic cells (DCs) (B) Schematic showing possible dose-dependent interplay and additive effects of inflammatory signals from KCs and DCs feeding into different levels of potency of sensitizing compounds.

our ability to predict potency of sensitizers. Given the numerous signaling pathways and markers characteristic of the sensitizer-induced inflammatory response and the substantial overlap of these cascades between keratinocytes and APCs, there is ample opportunity to explore how combined inflammatory parameters may influence sensitizer potency. Additionally, understanding how reactivity may modulate inflammation response (directly (e.g. MA sensitizers inhibiting NF $\kappa$ B signaling) or by proxy (increase in ROS and initiation of LP, forming RCS)) may be vital in improving our ability to predict sensitizing potency. A potential approach to quantitatively combine key inflammatory signals from keratinocytes and APCs is suggested in Figure 6(A,B).

### 3.4. Keratinocyte-immune cells communication

In addition to the unique and overlapping components of signaling that takes place within keratinocytes and DCs, there is evidence of “cell-to-cell” communication that generally takes place in the epidermis as well as specifically during allergic sensitization. This intercellular communication takes place *via* a variety of mechanisms.

#### 3.4.1. Exosomes

Exosomes have been shown to aid intercellular communication by transporting key information in the form of mRNA, miRNA, DNA and proteins (Bang and Thum 2012; Jella et al. 2018) as well as mitochondria (Hough et al. 2018). Exosomes from murine keratinocyte cell line MPEK were readily taken up by bone marrow-derived DC *in vitro* resulting in a matured phenotype, accompanied by increased CD40 expression as well as by the production of large amounts of IL 6, IL 10, and IL 12 (Kotzerke et al. 2013). HaCaT cells can induce T cell proliferation *via* indirect contact through exosomes that contain major histocompatibility complex (MHC) I and II (Cai et al. 2017). In addition to cells using a systemic transit of exosome-like nanovesicles to deliver a chosen inhibitory miRNA to target effector T cells in a contact hypersensitivity model, functional cell targeting by free extracellular RNA has

also been shown to take place by transfecting companion cell exosomes that then transfer RNA cargo to the acceptor cells (Bryniarski et al. 2013; 2015). Such mechanisms may play a role in modulating cellular/tissue homeostasis and the host immune system in allergic diseases (Hough and Deshane 2019).

#### 3.4.2. Nanotubes

Su and Igyártó demonstrated that epidermal Langerhans cells (LCs) contained many keratinocyte specific molecules (keratins, adhesion molecules, mRNA and protein) and were more prone to migration. These keratinocyte-specific signatures were transferred from keratinocytes to LCs through an exosome independent mechanism that likely involved tunneling nanotubules and was not unidirectional (Su and Igyártó 2019). Nanotubular structures have been shown to facilitate the selective transfer of membrane vesicles and organelles, thereby serving as a key mechanism of intercellular transfer of organelles (Rustom et al. 2004). Additionally, nanotubules enable myeloid cells to communicate with both targeted neighboring or distant cell, as well as other cell types, therefore, creating a complex variety of cellular exchanges, including pathogen spread (Dupont et al. 2018).

#### 3.4.3. Blebs

Mentioned previously, Bauer et al. demonstrated a keratinocyte mechanism where neoepitopes (covalently modified proteins generated upon bromobimanes exposure) are released *via* keratinocyte blebbing. This form of cellular communication is likely the main source of haptenated protein for the APCs which engulf and process the material contained within the blebs (Bauer et al. 2011).

Although potentially interesting for understanding the mechanistically complete picture of induction of sensitization, it is unclear how quantifying the various means of “cell-to-cell” communication may be informative in estimating sensitization potency.



### 3.5. Antigen presenting cells activation, migration, and maturation

The activation, migration and overall maturation of DCs are the hallmarks of the KE3 of the skin sensitization AOP. The bridging event between the innate and adaptive immunity results from a cascade of several upstream cellular processes. Apart from inflammatory response, LCs undergo numerous additional changes. These include internalization of MHC Class II molecules into endosomal compartments (for loading of the haptenated peptides on to the MHC II molecules (Girolomoni et al. 1990; Becker et al. 1992)), followed by increased tyrosine phosphorylation (Kühn et al. 1998), increase in the expression of surface markers such as CD86 and CD54, stabilization of the HLA-DR molecules on the surface, and downregulation of cell adhesion molecules (Aiba et al. 1997) resulting in their migration to the local lymph nodes. While the MHC restricted antigen presentation is accepted as the main antigen presentation pathway for skin sensitization, there is emerging evidence that CD1 presentation, involving skin lipids, may also have a role to play (Betts et al. 2017).

Validated assays using human DC-like cell lines or monocytic cell lines that can be differentiated into DC are hCLAT (Ashikaga et al. 2006), USENS<sup>TM</sup> (Piroird et al. 2015), IL 8 Luc assay (Takahashi et al. 2011) and GARD<sup>TM</sup>skin (Johansson et al. 2011, 2013), all included in the OECD TG 442E (OECD 2023c). While read outs from all these assays have been aligned to KE3, some of these assays are also representative of the events leading up to KE3. New knowledge related to DC maturation and a mechanistic basis to integrate inputs from the various signaling cascades, cellular processes as well as different dendritic and other immune cell populations are discussed below.

#### 3.5.1. Multiple signaling pathways

There is substantial evidence pointing to oxidative stress and MAP Kinase signaling as key intracellular triggers leading to activation of DCs (Mizuashi et al. 2005; Nakahara et al. 2006; Trompezinski et al. 2008). Further genomic evidence, as seen in the GARD<sup>TM</sup>skin hazard prediction signature, shows that numerous genes with known functions underpin the biology of cellular and molecular events that occur within DCs on sensitizer exposure (e.g. small molecule biochemistry, cell death, lipid metabolism, hematological system development, cell cycle, molecular transport, cellular growth and proliferation, carbohydrate metabolism, canonical pathways (e.g. Nrf2 mediated oxidative response), xenobiotic metabolism signaling, protein ubiquitination pathway, lipopolysaccharide (LPS)/IL 1 mediated inhibition of retinoid X receptor (RXR) function, aryl hydrocarbon receptor signaling and protein kinase A signaling pathways) (Johansson et al. 2011). Genomic signatures indicate that metabolic processes, cell cycling and oxidative stress responses are engaged differently depending on the reactivity mechanisms or potency of the sensitizer, as well as showing regulation of specific short, endogenous noncoding RNA molecules, miRNAs (Albrekt et al. 2014; Zeller et al. 2017). Number and type of regulated miRNAs appears

to rely on individual structural and/or reactivity properties of chemical sensitizers (Lindberg et al. 2019). Furthermore, new insights have emerged from these investigations about proteins involved in cholesterol biosynthesis, homeostasis and autophagy (Lindberg et al. 2020; de Ávila et al. 2022, 2023b). Although not all mentioned pathways are biomarkers for DC activation, as some precede the KE3, consideration of these changes together with the commonly quantified DC activation markers in an integrated manner can add more understanding to the mechanistic basis of varying levels of potency amongst skin sensitizers.

#### 3.5.2. Antigen presenting cell (APC) subtypes

The human skin is resident to several subtypes of APCs of which the epidermal LCs preferentially prime and expand the CD8<sup>+</sup> or cytotoxic T lymphocytes (CTLs) (Kissenpfennig et al. 2005; Klechevsky et al. 2008; Furio et al. 2010). Dermal DCs are also capable of presenting antigens to T cells, albeit not as efficiently as LCs in inducing the CTLs into potent effectors (Kaplan et al. 2008; Klechevsky et al. 2008). There is also evidence of recruitment of blood-borne Langerin<sup>+</sup> DC precursor cells to the dermis, dependent on chemokine ligands such as C-C chemokine receptor 2 (CCR2) and Selectins expressed in vascular endothelial cells (Ginhoux et al. 2007; Merad et al. 2008). Thus, not only epidermal LCs, but DCs of dermal and vascular origin, are also capable of antigen presentation and priming of T cells. It is, however, unclear which factors determine the recruitment and involvement of additional DCs.

#### 3.5.3. Changes in cell adhesion and migration

A key aspect of LCs activation is their migration to the draining lymph nodes, for which alteration of inter-cellular adhesion within the epidermis is important (Griffiths and Nickloff 1989; Barker et al. 1991; Groves et al. 1995). E-cadherin mediates the adhesion between keratinocytes and LCs (Blauvelt et al. 1995; Mayumi et al. 2013) and yet, there is conflicting evidence whether it is indispensable in LC activation and migration to the draining lymph nodes (Jakob and Udey 1998; Brand et al. 2020). Whether other cell adhesion molecules such as integrins, selectins or immunoglobulins, some of which are expressed in the epidermis (D'Arcy and Kiel 2021), play a role in regulating migration remains unclear and may be worthy of investigation.

APC activation, maturation and migration rightly remain a vibrant area of research, however, it is difficult to envisage utility of, thus far overlooked, indicators of these events in the context of sensitizer potency.

### 3.6. Cellular immune response

As described in the skin sensitization AOP, the development of ACD is a biphasic event consisting of an induction phase characterized by priming of antigen specific T cells and an elicitation phase characterized by clinical symptoms such as erythema, itching and burning (Figure 1). T cell proliferation, clonal expansion of primed antigen specific T cells, is described in the KE4 of the skin sensitization AOP. This KE is

the hallmark of the delayed type hypersensitivity, a point at which the individual is sensitized to a chemical, although involvement of the B cells in skin sensitization has also been investigated.

### 3.6.1. T cell proliferation

Currently, the only validated assay measuring T cell proliferation resulting from the induction of skin sensitization is the local lymph node assay (LLNA, (OECD 2010a)). However, animal data can no longer be generated for the purpose of cosmetic regulation in the European Union (EU 2009). While there may, indeed, be opportunities to explore development of informative assays with this output (Kimber et al. 2012; van Vliet et al. 2018), currently there are no NAMs addressing the activation and proliferation of a T cell response which are sufficiently progressed for implementation in OECD TG or for use in a NGRA approach (van Vliet et al. 2018). It remains difficult to envisage robust, sensitive, and reproducible assays addressing this KE *in vitro* in the near future.

### 3.6.2. B cell involvement

Historically, there have been attempts to investigate the level of involvement of B cells in pathophysiology of ACD. This is still an area generating considerable interest. Both LCs and dermal DCs have the ability to polarize naïve CD4<sup>+</sup> T cells either to secrete Th2 cytokines or differentiate into T cells that can induce naïve B cells to secrete large amounts of immunoglobulins (Igs) (Klechevsky et al. 2008). If B cells are presented with specific IgE to a hapten-protein complex together with the specific antigen, they have been shown to internalize the IgE-antigen complex *via* a CD23 mediated mechanism and cross-present the antigen to DCs *in vitro* (Engeroff et al. 2018). The involvement of B cells could potentially modulate the efficiency with which DCs present the antigens to T cells leading to immunological priming and generation of memory T cells. A relatively recent review indicates that, despite technical difficulties, more investigation, particularly in humans, is required into B cell activation and proliferation as well as hapten specific antibody production (Singleton et al. 2016). This represents a unique link to respiratory sensitization, where B cell response is dominant (Sullivan et al. 2017). The sparse knowledge of the nature of hapten protein conjugate is perhaps what hampers efforts in understanding the B cell and hapten specific antibody response in skin sensitization. The relative strength of the B cell response may be related to the type of antigen generated during KE 1. For example, certain skin sensitizers are capable of crosslinking proteins or otherwise generating “larger” antigens (e.g. *p*-phenylenediamine (PPD), glutaraldehyde, formaldehyde). Such complex antigens are likely to be less transient, not readily reversed, more persistent and thus, perhaps more likely to be recognized by an anti-hapten antibody. This warrants further investigation as antibodies could influence the thresholds in induction of skin sensitization.

### 3.7. Adverse outcome

ACD, characterized by clinical symptoms ranging from mild (such as erythema, itching and burning) to severe (blistering, severe edema and cracking of the skin), is a common, widespread ailment which can become chronic and disabling, causing a public health issue (Uter et al. 2020). In recent studies it has been estimated that over 20% of the general population have acquired contact allergy to at least one environmental allergen (Alinaghi et al. 2019; Scheinman et al. 2021). ACD is diagnosed by specialist dermatological investigations involving patch testing to identify the allergen to which an individual is allergic and enable allergen avoidance. In addition, the clinical patch test information can be used to detect emerging issues and identify predisposing factors for development of ACD (Schwensen et al. 2015). A number of opportunities may exist to make better use of the clinical evidence and experience within the safety assessment of contact allergens (Gilmour et al. 2019). Recently, clinical experience and established trends in contact allergy have been used within the SARA model DA to enable a prediction of the risk of induction of skin sensitization to a given exposure arising from a consumer product (Reynolds et al. 2022). Further utility of the clinical data and evidence could be found in understanding the sources of interindividual and/or population variability in susceptibility to sensitization. Understanding differences in individual phase II metabolizing enzymes may shed a light on differences in susceptibility to sensitization by chemicals ultimately removed by those enzymes (Sánchez-Gómez et al. 2016). For example, NAT1 genotypes containing a rapid acetylator NAT1\*10 allele were potentially associated with reduced susceptibility to PPD sensitization (Blömeke et al. 2009). Similarly, differences in epidermal PUFA make-up may help understand individual susceptibility to sensitization. These questions can potentially be answered by studying key molecular components on a population level.

## 4. Discussion and concluding remarks

When trying to understand the sensitizing potential of chemicals and make risk assessment decisions, particularly for those molecules for which the current tools provide limited or conflicting information, there is considerable value in further mechanistic characterization of the molecular events in skin sensitization. Quantitative information about certain molecular events or models utilizing multiple quantitative outputs, discussed above, could be instrumental in this endeavor.

The most promising area for thorough exploration remains chemical reactivity of sensitizers. Natsch and Emter (2017) made a strong case for better utilization of knowledge of chemical reactivity of sensitizers and made very useful suggestions on what future assays determining reactivity may look like. This study served as a catalyst for the current review of the AOP and strengthened the resolve to keep the mechanistic knowledge up to date and useful for non-animal risk assessment of skin sensitization potential and potency. The authors noted that the emphasis in the development of new assays for skin sensitization was almost exclusively

placed on the biological assays and that this bias is not mechanistically fully justified.

Cellular reaction following exposure to an electrophile is a dynamic process during which an electrophilic chemical interacts with several components of the cell defense systems, which work in concert to prevent protein damage and, in this case, induction of sensitization. It is, therefore, likely that induction of sensitization occurs only once these defense systems are overwhelmed. While the defense mechanisms of cells are unlikely to change, they will differ for different chemicals, depending on their electrophilicity and reaction mechanism to protein and other cellular nucleophiles. Following on from this, quantitatively studying ways of how cell defense mechanisms are orchestrated together to prevent sensitization is the most promising path to explore and expand our understanding. Quantitative understanding of relevant components of cell defense may ultimately allow us to consider sensitizer potency across all mechanistic domains.

While the MIE in sensitization is now better understood, not all reactivity relevant events which have the potential to influence sensitization threshold are currently always considered in risk assessment. Questions remain about the quantitative impact of oxidative stress and LP on induction of sensitization. It is further unclear if and how the RCS impact the kinetics of antigen generation and whether PUFA make-up of individual's keratinocytes may be influencing their susceptibility to sensitization. Furthermore, as skin lipids are proportionally the highest constituents of human skin (Knox and O'Boyle 2021) and evidence is emerging of their involvement in an alternative antigen presentation pathway (Betts et al. 2017), it is prudent to investigate further how skin lipids may contribute to induction of sensitization. Another phenomenon which has not received much attention is the stability or reversibility of covalent protein adducts and what impact this may have on antigen formation. Both LP (and, therefore, levels of RCS) and cellular capacity to reverse covalent modification are likely to be stable in models and could potentially be determined, at least for the best known mechanisms. However, they may differ qualitatively and quantitatively between individuals and populations.

The repertoire of reactivity-driven events secondary to antigen formation is often regarded as part of inflammatory response and is, traditionally, considered in KE2. These events occur as a direct consequence of electrophile reactivity, either prior to or concomitantly with MIE in sensitization. It is, therefore, vital to understand their quantitative relevance to antigen formation. The established concept of direct relationship between chemical reactivity and sensitizing potency could be deepened by expanding the quantitation of reactivity from a solely "antigen generating" event to a more holistic "interaction with epidermal cells." This encompasses detoxification, redox balance disturbance, initiation of LP, and reversibility of covalent adducts. It is possible that several of these elements could influence the potency of a sensitizer: (a) electrophilic reactivity of the chemical (including the complexity and chemoselectivity), (b) reversibility of covalent adducts formed with skin proteins, (c) type and capacity of detoxification, (d) level of redox imbalance and immediate

oxidative stress induced and (e) amount of LP (and, therefore, RCS). A very potent sensitizer should be highly reactive with multiple nucleophiles and/or have complex reactivity mechanism (making adducts in several steps and/or crosslinking proteins), generate less readily reversible adducts, be subject to an exhaustible mode of detoxification and generate rapid and extensive redox balance disturbance, resulting in immediate effect on proteins and lipids, in turn generating large amount of RCS. Conversely, sensitizer at the weak end of potency spectrum should be less reactive with fewer nucleophile types, have simpler reactivity mechanism without ability to cross link, make readily reversible adducts, be subject to effective and less exhaustible (or multiple) modes of detoxification and generate less redox balance disturbance and lipid peroxidation and, therefore, less RCS.

It is plausible that simple, *in chemico* assays could be developed (or adapted) for detoxification mechanisms currently best understood. The sensitivity and specificity of analytical techniques is continually improving and it is likely that phenomena such as LP and reversibility mechanisms/rates could be quantitatively studied in this context using omics approaches (such as HR MAS NMR metabolomics (e.g. Moussallieh et al. 2020) or adapting existing proteomic methods (e.g. Lu et al. 2022). Utilization of the quantitative data of KEs and additional elements of AOPs may ultimately be achieved through quantitative AOP models (qAOP) (Zgheib et al. 2019; Spinu et al. 2020), which may help us avoid the linear interpolation of the quantitative outputs of the KEs in the AOP and adopt a more mechanistically relevant interpolation. Emphasis should be firmly on quantifying highly informative parts of the AOP in such models, rather than attempting to fully quantify the entire set of KEs. While the current approaches for skin sensitization risk assessment of chemicals serve us well, the ability to derive more mechanistic information on chemicals that have been identified as sensitizers may ultimately allow more informed decisions on a chemical-specific basis.

Finally, besides skin sensitization, electrophilic chemicals are implicated in other pathologies. Numerous examples exist of toxicity of electrophiles (or their metabolites), ranging from respiratory sensitization (Cochrane et al. 2015; Ferreira et al. 2018), systemic toxicity of certain antibiotics, anticonvulsants and antiretrovirals (e.g. Evans et al. 2004; Zhou et al. 2005; Hernandez-Jaimes et al. 2022), specific organ toxicities (liver and kidney) caused by non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. acetaminophen (Bessemers and Vermeulen 2001)) to mixed modes of toxicity. Some of the phenomena discussed above, including ways to utilize the assays with quantitative output should ultimately be applicable to variety of exposure scenarios to electrophilic chemicals or electrophilic metabolites.

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## Declaration of interest

The submitted manuscript was inspired by the publication by Natsch and Emter in 2017 (Natsch and Emter 2017). MA conceived the original idea, which was further developed in many discussions with RR, NG, SS and RA with a view to review the most up to date mechanistic insights into induction of sensitization in humans. Our motivation is to emphasize the most promising areas of research suitable for development of novel assays with capacity to determine sensitizing potency and aid risk assessment decisions. The authors are employees of Safety and Environmental Assurance Centre, Unilever and no external funds were used in the preparation of this manuscript. The authors do not imply competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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