IDEA
International Dialogue for the Evaluation of Allergens

Rapporteur's Progress Report on the IDEA Expert Workshop on

Pre- and prohaptens

June 16th, 17th, 2015

Château du Lac
Avenue du Lac, 87
1332 Genval, Belgium

1. Introduction and workshop objectives

Pre- and prohaptens were the subject of a workshop in May 2013. Clinical evidence presented at this first workshop showed that prehaptens, including those used as fragrance raw materials, are a significant cause of contact allergy as indicated by positive patch test reactions. A number of the conclusions of this workshop were included in the QRA2 submission to the Commission Services (DG SANCO) in July 2014 for comment by the JRC. The JRC in their comments reiterated the importance of the integration of the assessment of the potential of fragrances to act as pre- and/or prohaptens in the QRA. A summary of the issues relating to pre- and prohaptens in QRA2 is attached as Appendix 1.

The primary aims of this workshop were:

i) To focus on scientific advances in the understanding of the relationship between abiotic and biotic hapten production and the initiation of induction (NB: study of the relationship between induction and elicitation will be a subject for another workshop). In particular to focus on the generation, measurement and effects of oxidation products of monoterpenes (e.g. limonene and linalool).
ii) To identify the progress in addressing the recommendations of the first workshop and the suggestions of the JRC.
iii) To consider in more depth specific issues for which the time was not sufficient at the first workshop.
iv) To identify priorities for further work on pre- and pro-haptens.

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2. Identifying prehaptens based on physicochemical properties

A number of fragrance ingredients have the potential to be oxidized to more reactive forms that may serve as haptens. Based on their physicochemical properties (e.g. chemical stability, lipophilicity and volatility) alone in many cases it is possible to predict the likelihood that abiotic reactive products will be formed, although predicting the extent of such transformations is more challenging. Tools available for this purpose include:

i) Use of structural alerts and SAR
ii) Quantitative mechanistic modelling and QSAR

Reactive products of relevance include: hydroperoxides, aldehydes and ketones, epoxides, amines and quinone like substances. An increasing number of examples of each type are being identified. In addition to a change in the fragrance material itself in principle, the fragrance material (or more likely a reactive product of it) could generate a hapten by causing a chemical activation of a body constituent (e.g. tryptophan) or of a formulation component.

The ability to form a hapten may be affected by the co-formulated components. This needs to be considered when investigating the potential of a fragrance material to act as a prehapten. Challenges include the identification of suitable standardized testing conditions, establishment of suitable methodology for hapten identification and its quantification.

3. Chemical analysis of haptens generated from pre- and prohaptens

Haptens must be chemically reactive in order to bind to the target protein(s). Consequently the identification and quantitation of haptens is often a challenging task.

After the first workshop a task force was set up to establish a reliable methodology for the measurement of hydroperoxides, as hydroperoxides were considered to be one of the most common haptens. .An intensive inter-laboratory comparison was made at two concentration levels and in matrices of increasing complexity. Six laboratories were involved each using their own methodology. Overall there were 10 different methods (including variants of the same method). The findings from this inter-laboratory comparison was that while identification of the hydroperoxide was achieved by all participants there were major differences in quantitation. This was attributed to the instability of hydroperoxide in some matrices, unexpected reactivity and potentially interfering substances. The lessons learned have important implications not only for the analysis of hydroperoxides, but also for the analysis of other haptens. A follow up is in progress to identify the interfering substances in the hydroperoxide analysis followed by a repeat inter-laboratory study that takes on board the lessons learned.

4. Skin absorption.

A number of preparations were considered suitable to mimic the uptake of compounds by human skin in vivo. These include:

i) Human skin ex vivo
ii) Pig (ear) skin

iii) Organotypic constructs (3D) of normal skin epidermis (RHE) or of full thickness human skin (RHS). A number of commercial preparations are available including EPISKIN, SkinEthic and EpiDerm.

Rodent skin is generally not suitable because it is more permeable than human skin. The explanation for this is not entirely clear but does not appear to be related to the properties of the compound tested (e.g. water solubility, log P or molecular mass).

For the purposes of assessing skin penetration the viability of the skin preparation is much less important than it is for determining skin metabolism. However surface integrity and freedom from contamination is important. Skin disease may result in modification of dermal uptake of compounds however the importance of factors such as age, sex and disease on skin uptake requires further study. There are a number of studies that have investigated the impact of co formulated compounds on uptake. Some studies indicate that nanoparticles may enhance penetration. This requires further investigation in view of the increasing use of nanoparticles in cosmetics products.

5. Skin metabolism and transport

Many organisms have a range of enzymes whose role appears to be the metabolism of lipophilic non nutrients to more water soluble metabolites that can be cleared in the excreta. These enzymes are often referred to as the 'drug metabolizing enzymes'. Commonly these enzymes are categorized as phase 1 enzymes (oxidation, reduction and hydrolysis) and phase 2 enzymes (conjugation with endogenous molecules such as glucuronic acid, sulphate, acetate, glutathione, glycine). These reactions occur in a number of tissues in man and other mammals although they predominate in the liver. Many studies have demonstrated that in the liver, reactive metabolites can be formed as intermediates and that as a consequence toxic reactions such as mutagenesis may occur. Thus it is important to ascertain for human skin:

i) Which enzymes (and which isoforms of these enzymes) are present
ii) Their activity (including saturation thresholds)
iii) The relative proportions of each enzyme in different cell types
iv) How the activities of these enzymes are affected by the presence of other substrates (other compounds to which the skin may be simultaneously exposed)
v) Inter-individual differences in the above and their relevance to hapten generation and detoxication.

Drug metabolizing enzymes shown to be present in human skin include:

- Cytochrome P450's
- Flavin dependent mono-oxygenases
- NADPH dependent reductases.
- Various dehydrogenases (eg alcohol dehydrogenase)
- Acetyl transferases
- Epoxide hydrolases
- Other esterases
- Glucuronyl transferases

- Sulpha transferases
- Glutathione transferases.

These categories of enzymes are very similar to those found in other body tissues such as the liver, gut and lung. However for most of these enzymes, various isoforms exist in the body with different substrate specificities and activities. Thus it is important to identify which isoforms are present in the skin and in which cell types. A number of these enzymes are susceptible to induction and/or inhibition by lipophilic compounds. Thus the potential of co-formulated substances to induce or inhibit specific drug metabolizing enzymes needs to be considered. In the case of cytochrome P450 evidence was presented that different isoforms of P450 have different cellular locations. This is presumably also the case for a number of the other enzymes. This may be an important factor governing the potential of a compound to form reactive metabolites at a location that facilitates the initiation of induction of sensitization.

A number of transporter proteins have also been located in skin cells, their contribution to hapten interaction with target proteins is under active study.

Particular areas where further clarification is needed are:

- What evidence is needed to confirm that a compound is biotically converted to a hapten and that this is critical to its ability to induce sensitization. At present this conclusion generally relies on exclusion criteria.
- Which co-formulated compounds (and in what concentrations) are likely to cause sufficient changes in the metabolic fate of a fragrance to affect the induction of sensitization potential.

A review of the criteria and methodology used to identify and characterize prohaptens in skin and to elucidate the key toxicokinetic factors in man that influence induction of sensitization is required. The use of high resolution magic angle spinning nuclear magnetic resonance offers great potential for the real time tracking of reactive intermediates in skin preparations. This methodology has already demonstrated its potential. In the case of cinnamyl alcohol the widely held assumption that it acts as a hapten through the formation of cinnamaldehyde is now under challenge. At the WS it was suggested to broaden the scope of skin models (going beyond keratinocytes) to better understand what might happen in real human skin.

6. Sensitisation and cross reactivity

In QRA2 the issue of aggregate exposure was comprehensively addressed but the topic of cumulative exposure (cross reactivity) was only very briefly touched on. A good understanding of the potential for cross reactivity between fragrance ingredients is therefore crucial.

Cross reactivity was defined as ‘the receptor of a memory cell for antigen 1 cannot distinguish between antigen 1 and an antigen 2 created by another hapten and will thus react also to antigen 2. The following situations therefore need to be considered:

i) Haptens A and B have very similar structures
ii) Hapten A is metabolized to a compound similar to compound B
iii) Hapten B is metabolized to a compound similar to compound A

iv) Haptens A and B are metabolized to the same hapten.

Currently our understanding of the potential for cross reactivity relies on animal models (especially the guinea pig test). Clinical studies can only provide indications as there is no control on exposure.

Although a limited range of fragrances have been assessed for cross reactivity it is reasonable to conclude that no general cross reactivity is likely even among fragrance ingredients with a common functional group. For example in 29 individuals sensitized to colophony only 1 in 29 reacted to more than one hydroperoxide. Nonetheless where there is a very close structural resemblance in the two haptens some cross reactivity is likely. Thus among 352 patients sensitized to either oxidized linalool or oxidized limonene on 255 reacted to both. Key issues for the future include:

- the importance of utilizing the real hapten formed from the pre or pro- hapten,
- the need to find a reliable non –animal test(s),
- the inclusion of a wider range of structures in the assessment of cross reactivity.

7. Target proteins, reservoirs for retention of haptens and inactivation of effects due to non-specific binding

No new information on these topics was presented at the workshop. They will be addressed subsequently.

8. Modelling of the induction of skin sensitisation

The development of in silico methods to predict the potential of fragrances to act as pre- and pro- haptens is important. On such model is the OASIS TIMES model which is an expert system describing structure –metabolism and structure – toxicity relationships. It involves a step wise approach using physicochemical properties, structural domains including functional groups associated with skin sensitization and drug metabolism estimates. It was considered to be in a form that can be used to compare the model findings with data from laboratory and clinical testing.

9. Overall conclusions

A much greater emphasis has been given to prehaptens than to prohaptens. This is reasonable because, assuming that mouse skin has broadly comparable distribution of drug metabolizing enzymes to those in human skin, the current LLNA test would be expected to pick up most prohaptens. It is recognized however that metabolic capability will be an important consideration in the suitability of potential replacement tests. More work is required to identify the drug metabolizing capability of human skin, how this can be affected by factors such as disease, co-exposure to other compounds etc. and how stable the key enzymes are in various in vitro preparations.

There is insufficient data to provide a realistic estimate of the overall importance of prehaptens as inducers of sensitization. It appears that oxidation is the most common mechanism by which prehaptens are converted to haptens. The resultant haptens are chemically reactive. Consequently identifying and quantifying the relevant hapten is a challenging task. More work is needed to develop robust methods suitable for this purpose. Other chemical analytical requirements are:

- Determination of changes occurring in fragrance stability tests

- Improved understanding of the chemistry taking place on the surface of the skin following fragrance application

The application of modelling techniques such as OASIS TIMES may assist in predicting likely haptens.

The ultimate question is which of the potentially chemically reactive species that are generated are of primary importance in reacting with the target protein for induction. The use of magnetic resonance has indicated that assumptions on the most important active form based on gross metabolism or chemical conversion studies may lead to misleading conclusions e.g. that the hapten of cinnamyl alcohol is cinnamaldehyde. Other methodologies need to be brought to bear on this important issue.

It is important too in order to advance our understanding of pre and pro haptens to draw much more extensively on studies of skin sensitization involving non-fragrance ingredients.

Jim Bridges Rapporteur for the workshop July 2015

APPENDIX 1: QRA AS OF JULY 2014 ON PRE- PRO- HAPTENS.

The QRA 2 text on pre and prohaptens in QRA 2 that was submitted to Commission services in July 2014 was based largely on the conclusions of the first pre and prohapten workshop which was held in Brussels from the 28th to the 29th of May 2013. The key aspects of the relevant section of the submission are set out below.

- **Working definitions**

Pre- and prohaptens are by definition hapten precursors.

A prehapten is a chemical that needs to be chemically (abiotically) activated before it can elicit induction.

A prohapten is a chemical that must penetrate the surface of the skin and gain access to the so-called 'drug metabolizing enzymes' to be activated and form a hapten.

- **Importance in terms of induction**.

The number and range of structures of fragrance ingredients in general use that can act as pre- or prohaptens is unknown. Both laboratory research and clinical studies have generally focused on a very limited number of commonly used fragrances, in particular limonene, linalool, isoeugenol, eugenol, geraniol, cinnamyl alcohol and cinnamal.

- **Properties required of a hapten**.(see figure 1)

To be a hapten a chemical needs to:

1) gain access in sufficient concentration to the target protein(s) in the skin that are responsible for the initiation of sensitization;
2) be sufficiently reactive with the critical target sites of the protein(s);
3) have limited reactivity with other cellular targets that would result in cytotoxicity or other substantial cell damage.

- **Activation of prehaptens**

Activation to form a hapten can in principle arise during processing or storage of a raw material/formulation, or on the surface of the skin as a result of application, or within the skin. The most common pathways for hapten formation from precursors are generally considered to be oxidation and hydrolysis). Activation of a prehapten can arise due to oxidation of a double bond to form an epoxide, a catechol or hydroquinone to form a quinone, an allylic or benzylic group to form a hydroperoxides. In addition, reactions such as oxidative dehalogenation and photo-oxidation can occur. The following factors have been identified as having a potentially important influence on hapten formation from pre-haptens:

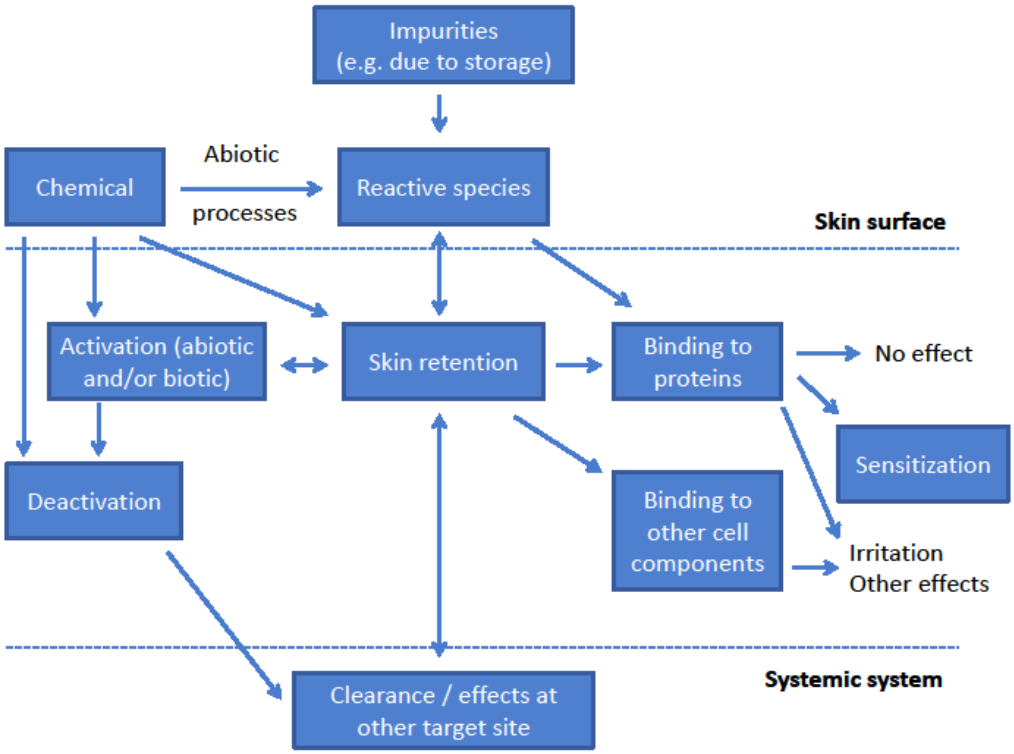

Figure 1: Distribution, transformation and effects of pre- and pro-haptens.

- Oxygen availability: This may be reduced if there are other components in the medium/formulation that compete for the oxygen or act as antioxidants.
- Temperature: As in all chemical reactions, the temperature plays an important role in hapten formation according to the Arrhenius equation.
- Lipophilicity: To enable the resultant hapten to reach the target protein(s) for induction.
- Alternative abiotic and biotic pathways for the chemical that do not result in hapten formation.

- **Activation of prohaptens**

In principle this may arise due the activity of enzymes located in the skin (often termed the drug metabolizing enzymes) or due to the microflora present on the skin surface. The focus of attention has been on the drug metabolizing enzymes. These enzymes are present in a number of body tissues including the liver, gut, lung, kidney and skin. They are generally classified into two groups:

- phase 1 enzymes (those that carry out oxidation, hydrolysis or reduction transformations). Many of the phase 1 products are the same as those formed from prehaptens albeit the concentrations many be very different.
- phase 2 enzymes (conjugation of phase 1 products with endogenous constituents such as glucoronate, sulphate, glutathione, acetate, glycine). In the process of generating phase 1 metabolites reactive intermediates are often formed (some of which in the skin will serve as haptens). Phase 2 reactions are

generally considered to produce detoxification however some conjugates are in fact reactive. Many of the drug metabolizing enzymes exist in a range of isoforms that vary in their tissue location and activity levels. There is a large body of work showing that these drug metabolizing enzymes can initiate toxicity due to their ability to form reactive metabolites. However they also play a vital role in detoxification too.

The following factors have been identified as having a potentially important influence on hapten formation from pre-haptens:

- Ability of the pro-hapten to reach an appropriate enzyme; lipophilicity has been identified as an important property for pro-hapten access to skin epidermal cells.
- Concentration of the hapten achieved.
- Proximity of the hapten generation to the target protein.
- Rate of detoxification of the hapten by biotic and/abiotic mechanisms.
- Availability of any relevant cofactors e.g. NADPH.

- **Identification and characterization of the pre- and pro-haptens**

Physicochemical data.

This requires information on physicochemical properties, in particular stability under relevant conditions. The development of a validated structure activity relationship (SAR) model for predicting pre- and pro- haptens is needed.

In vitro studies using skin.

Studies on biotic and abiotic transformation by the skin, animal or human skin biopsy samples or homogenates or cell fractions of these samples may be used to provide additional information on conversion pathways and relative rates of formation of reactive products for individual fragrances of interest.

In vivo animal data

The test method for hapten identification and potency assessment used so far has been the local lymph node assay (LLNA) in mice. The way the test is conducted does not seek to identify metabolites of the test fragrance. However data on the metabolic capability of mice indicate that many of the drug- metabolizing enzymes present in human skin are also present in the mouse. Consequently this test is likely to result in a positive reaction for many pro- and haptens.

In vivo human data

In addition, the propensity of a substance to act as a prohapten is already taken into account in the confirmatory HRIPT where the same epidermal metabolic processes exist in the volunteers undertaking this test and as in consumers.

APPENDIX 2 WORKSHOP PROGRAMME

IDEA Expert Workshop

Pre- and Pro-haptens

June 16-17th, 2015

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Program

Monday, June 15th – Arrival and Registration

16:00 -18:30 Welcome and registration

18:30 - 21:30 Standing buffet (Château du Lac, Grand Salon du Lac)

Tuesday, June 16th (09:00 to 17:00) – Day 1 (Geneviève plenary meeting room)

09:00 - 09:15 Workshop opening – *Hans Bender and Matthias Vey*

<u>First session – Update on developments since the last pre- and pro-hapten WS</u>

09:15 - 09:45 Clinical developments: How do clinical data help in the identification and characterization of pre- and pro-haptens? Status of knowledge on 'cross reactivity' when patch testing with oxidized materials. What are the main knowledge gaps?

Speaker: Ann-Therese Karlberg (University of Gothenburg)

09:45 – 10:15 Analytical chemistry developments: Development of an analytical methodology specific to hydroperoxides – report from the Hydroperoxide TF

Speaker: Alain Chaintreau (Firmenich)

10:15 – 10:45	Moderated discussion
10:45 – 11:00	Coffee break
11:00 - 11:30	Abiotic transformation developments: pre-hapten activation and consumer products (The methodological challenges. What type of structures / structural groups among fragrance ingredients are particularly vulnerable to conversion to haptens? What are the main knowledge gaps?) *Speaker: Dave Roberts (Liverpool John Moores University)*
11:30 – 12:00	Skin absorption developments. Relevance of current test systems to man. *Speaker: Monika Schäfer Korting (Free University of Berlin)*
12:00 - 12:30	Moderated discussion
12:30 - 13:15	Lunch
13:15 - 13:45	Biotic transformation developments: pro-hapten activation (The methodological challenges – what is known about prohapten activation of fragrance materials in the *in-vivo* and *in-vitro* test methods we use for induction) *Speaker: David Basketter (Consultant)*
13:45 - 14:00	Moderated discussion

<u>Second session – The way forward: important knowledge gaps and prioritization in filling them</u>

14:00 - 14:30	Predicting pre- and pro- electrophilic activation of chemicals in skin sensitization assessment *Speaker: Chanita Kuseva (University of Bourgas)*

14:30 - 15:00	The skin as a metabolizing organ of pro-haptens (Understanding of the fate of reactive chemicals in the skin) *Speaker: Hans Merk (University of Düsseldorf)*
15:00 – 15:30	Moderated discussion
15:30 - 15:45	Coffee break
15:45 - 16:15	Pro-hapten activation and subsequent interaction with proteins: Mechanistic understanding and quantitative follow up *Speaker: Jean-Pierre Lepoittevin (University of Strasbourg)*
16:15 - 16:30	Moderated discussion

Conclusions of Day I

16:30 – 17:00	Preliminary progress report *Speaker: Rapporteur of the workshop (Jim Bridges)*

End of Day I

19:00 – 22:00	Diner (Château du Lac, grand Salon du Lac)

Wednesday, June 17th (09:00 to 15:00) – Day 2 (Geneviève plenary meeting room)

09:00 - 09:15 Wrap-up of Day 1 and confirmation of Working Groups for day 2

Third session – Risk management opportunities

09:15 – 09:45 Minimization of pre-hapten conversion (focus on oxidation) to haptens by improved formulation, storage and packaging

Speaker: Andreas Natsch (Givaudan)

09:45 – 10:00 Coffee break

Fourth session – Working groups discussion

10:00 – 12:00 The participants will be subdivided into up to three working groups

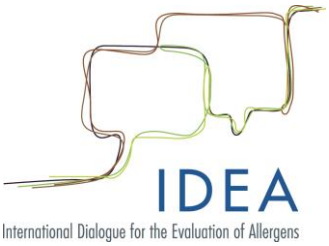
12:00 – 12:45 Lunch break

12.45 – 14.15 Presentation of the conclusions / recommendations of the working groups

14:15 – 14:45 Conclusions of the workshop and next steps

End of Day II

15:00 End of Day 2 and workshop closing



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