IDEA Pre- and Pro-Haptens Workshop
October 16 and 17, 2019

Courtyard by Marriott Brussels Hotel
Avenue des Olympiades 6,
1140 Brussels, Belgium

Rapporteur's Report

REPORT ON THE IDEA WORKSHOP ON PREHAPTENS - October 16th 2019

IDEA has held several previous workshops on pre- and prohaptens. The moderator pointed out that the aim of the present one was to ensure a holistic view on the issue, aiming to hopefully better understand the different facets of the issue and to arrive at some concrete conclusions and actions.

Identification and characterisation of fragrance materials that are pre- and/or prohaptens might be sufficiently addressed in the original QRA1. However, the hazard assessment methodology has since changed very substantially, it is therefore appropriate to review the topic again. Fragrance materials (FMA's) potentially fall into one of five categories:

- Not allergenic (NS). This appears to be the case for the great majority of FMA's.
- Directly allergenic (i.e. a hapten).
- An allergen if subjected to certain chemically induced changes (i.e. a prehapten).
- An allergen if subjected to certain biological processes (i.e. a prohapten). Biological change may occur either directly due to the activity of the xenobiotic metabolising enzymes or indirectly, for example as a result of irritation related oxidative stress in the skin resulting in FM oxidation.
- Both a pre- and a pro- hapten.

In principle, a prehapten might still be suitable for use in products used by consumers provided their chemical conversion to a hapten can be prevented or managed in a way that there is an acceptable risk for the consumer. However, to ensure this, prehaptens need to be identified and the potential modes of conversion recognised. It appears that the main issue remains with substances that appear to be slowly oxidised.

The workshop focussed on hydroperoxides as chemical oxidation products of Linalool and Limonene. Linalool can be converted to both its 6- and 7- hydroperoxides while limonene gives both 1- and 2- hydroperoxides. These have different chemical reactivities. Limonene -1 and -2 hydroperoxides show different potencies in the patch test, but so far no comparison of the patch test reactions to the two Linalool hydroperoxides have been published. There are big differences between dermatology clinics in their conclusions on the potency assessment of these hydroperoxides and

IDEA Management Team
Avenue des Arts, 6
1210 Brussels, Belgium
Tel: +32-2 214 20 61
Fax: +32-2 214 20 69

www.ideaproject.info

in terms of the clinical picture resulting from patch testing with an oxidized mixture, not the pure hydroperoxides. An overview of these studies indicates that:

- most patients do not react at all to either hydroperoxide.
- specifically in the case of limonene,-females appear to react more often positive than males (which is likely due to higher exposure).
- -most patients affected respond to a particular hydroperoxide only (hardly any cross reactions) .
- -there are patients who react to both the oxidized mixtures of Linalool and Limonene.
- there is limited information on reproducibility of reactions

A number of reasons for these variations were put forward. It was noted that for some individuals massage was a very significant form of exposure which is one of those not taken into account in the current QRA exposure scenario. Potentially some accumulation of the fragrance material could occur in the skin as a consequence. There is growing consensus that Limonene and Linalool containing products under the control of the fragrance industry may not be the main cause of the induction of contact allergy today. Other Limonene/Linalool exposure scenarios are more likely to be the major causes and need to be identified.

Hydroperoxides of allylic chemicals have been shown to arise due to air oxidation although the process is generally relatively slow. In skin, catalytic factors such as availability of iron and levels of dissolved oxygen were mentioned as important promoters. There is also new data indicating that oxidised forms of other fragrance materials, like Linalyl acetate, Nerol, Citral, Geraniol have been found to be more potent dermal allergens than the parent FM. It should be noted that many natural oils contain one or more of these FMs.

Hydroperoxides may react directly with proteins or could become first converted to hydroxyl radicals. Although induction is evidently dose related, the relationship between chemical reactivity and potency for induction of dermal sensitisation is not a simple one. A number of factors need to be investigated in order to explain the potential relationship, including: the oxidative defence in the relevant area of skin (e.g. enzyme activities e.g. catalase, natural antioxidants levels e.g. glutathione), skin penetration rates and retention (N.B.: it was noted that SLS increases the risk of induction/elicitation) and the nature and location of the target proteins.

Since oxidative attack for both Linalool and Limonene could result in more than one product the analytical methodology for the identification of hydroperoxide products is important. A number of different methods have been used. In a study sponsored by IDEA and reported by Natsch (Natsch et al, Food and Chemical Toxicology 127 (2019) 156–162) on a number of consumer product samples of various ages, low levels were found in some samples. The results of the analytical investigation indicate that limonene and linalool hydroperoxides in fragranced consumer products could explain to a certain extent elicitation in sensitised individuals but do not explain the source of induction. It appeared also that hydroperoxide levels did not increase with the age of the sample. It could be that in neat oils the potential for hydroperoxide formation is considerably greater, however this was not investigated. These analytical results raise a number of questions. One of which is whether hydroperoxide formation can occur on the skin. It was suggested that if oxidative stress occurs in the area of application then this could trigger prehapten conversion to a hydroperoxide.

The above findings appear to cast doubt on the value of animal tests for identifying FM's that are slowly oxidising pre-haptens and/or will not necessarily give a clear indication of the relative potency of the true hapten which emerges from the prehapten.

Looking at the clinical data established with patch testing of oxidized linalool and limonene, there appears to be a high number of false positive reactions Nonetheless without any doubt there is a number of cases of patients with a true allergy to the oxidised mixtures and likely the contained hydroperoxides. The question remains what the cause is and

therefore the importance of identifying and characterising both induction and irritation was emphasised with SLS being a valuable irritant standard. It was also suggested that prehaptens present in fragranced consumer products may be more important for elicitation than for inducing of dermal sensitisation.

A number of recommendations for next steps e.g. with regard to clinical work were made that are summarized in the key conclusions of the workshop.

IDEA
International Dialogue for the Evaluation of Allergens

REPORT ON THE WORKSHOP ON PROHAPTENS – October 17th, 2019.

The induction of an allergic response may be due to the dermally applied substance itself or the initiating hapten may be formed chemically (prehaptens) or biologically (prohaptens). Prehaptens may be formed prior to application or on the surface of the skin, whereas prohaptens are formed in the skin. The significance of this, if any, for the availability of a new hapten is not known.
The workshop on November 16 (see separate report) provided an update on prehaptens, this one focused on pro-haptens.
This workshop looked at the evidence for biological formation of (toxic) metabolites, based on studies of xenobiotics of many kinds, not just fragrance materials. The principal themes of this workshop were to review key aspects of active metabolite formation as part of xenobiotic metabolism (XM), to recognise the modifications in the activities of the enzymes involved in cell cultures used for *in vitro* testing purposes, the use of *in chemico* methods to predict the potential for hapten formation and the evidence that any prohapten is also likely a prehapten. However, the workshop did not address how the QRA1 methodology was previously used to identify either pre-or prohaptens.
Xenobiotic metabolism typically occurs in two phases:

- phase 1 -oxidation, reduction and or hydrolysis resulting in the insertion or uncovering of a functional group.
- phase 2 (conjugation with various endogenous substances to form glucuronates, acetates, sulphates, glutathione, acetylcysteine and glycine conjugates, etc.), It should be born in mind however that some chemicals can go directly to phase 2 and become activated at that stage, without having to undergo phase 1 because they already have functional groups that are able to undergo conjugation.

The opening presentation (Helmut Greim) reviewed what is known about active metabolite formation from chemicals in general and the importance of the identification of this to anticipate and understand mechanisms of toxicity. It was emphasised that although oxidation is the most common initial fate of exposure to a xenobiotic, active metabolites can be formed by other phase 1 pathways. Several examples were given of such active metabolite formation leading to activation and subsequent deactivation. It was also noted that the oxidation products generated chemically (i.e. products of prehaptens) may differ from those produced by the oxidative enzymes. Moreover, conjugation in some cases also results in active metabolite formation. Thus, a sequence of steps may be involved in the generation of some active metabolites. This is important because the predictive tools tend to be focused only on a single stage oxidative metabolism. A further issue is that in addition to direct oxidative metabolism, oxidation of a xenobiotic can also arise indirectly because of oxidative stress on a cell due to irritation. This oxidation process may be mediated by hydroxyl radicals or other reactive oxygen species. This is a much less studied mode of active metabolite formation (it hasn't been discussed in previous workshops) but could be very significant for chemicals that are dermal irritants.

Jean-Pierre Lepoittevin reported about known fragrance prohapten and their metabolic pathways leading to activation and other factors affecting it. He reminded the participants of the history of introducing the terminology of pre- and prohaptens. Metabolism in general results in detoxification. Chemicals that are not reactive per se in phase 1 of the conversation need to have a functional group inserted or revealed so that phase 2, conjugation and detoxification can occur. In the generation of such functional groups, electrophiles may be formed, which can be reactive with tissue macromolecules. Toxicity may occur if the conjugation process is overwhelmed. It was noted that when it comes to metabolism pathways and activities, we often extrapolate to the skin from what is known in the liver. However, he proposed that the detoxication/toxication balance as well as the reactivity of skin sensitisers can be observed and quantified in situ and he presented the respective approach in reconstituted human epidermis. Although the signal is less good, he concluded that the overall picture is the same.

The enzymes responsible for xenobiotic metabolism and their activities in various *in vitro* preparations were considered (Franz Oesch). It was emphasised that these enzymes may increase or decrease the activity of a xenobiotic. Unfortunately, there is insufficient data on the activity of these enzymes in human or mouse ear skin to be used for

comparison purposes with *in vitro* preparations. However, from studies of cell cultures from other tissues it is apparent that they tend to lose xenobiotic metabolising capability on culturing. For the purposes of the workshop the activity of xenobiotic enzymes in the cell culture preparations used to identify and characterise inducers of dermal allergy were of particular interest. Evidence was presented that showed that cells typically used in KE2 tests had reasonably stable activity of esterases, aldehyde dehydrogenase and N-acetyl transferase. However, of these activities aldehyde dehydrogenases were not detected in the cell types used for KE3 tests. In cell lines used for KE2 and lines used for K3 tests activities of the most important family of oxidative enzymes (the P450's) along with flavin monooxygenases were below detection, while alcohol dehydrogenase activity was below the level of quantitation (with the possible exception of THP-1, used in the h-CLAT assay, where - in 1 out of 3 passages tested - quantifiable levels of alcohol dehydrogenase were observed).

In several other areas of *in vitro* toxicity tests addition of a metabolism system (e.g. liver S9 or liver cells) has been made to enable active metabolite formation to occur. In one study enzymes in the culture cells were induced with butyrate. Whether the same approach is needed for the identification of potential prohapten fragrance materials was discussed briefly but no conclusion reached.

David Basketter reviewed the current knowledge on fragrance materials that have been identified as pre and prohaptens. He discussed the role of air oxidation in the formation of haptens and the implications of this. He considered that there was minimal evidence supporting a strict separation between pre- and pro - haptens, emphasising that any prohapten would also be likely be a prehapten and suggested to rather talk about indirectly acting haptens. He reiterated the conclusion that he expressed at some previous workshops that the majority of indirect acting haptens were still positive in the mouse LLNA and / or human studies. He therefore assumed that there was sufficient metabolic activation without understanding all mechanistic details. With regard to AAT, ECVAM in its recent report reassured that the available methods would not miss hazard identification of relevant sensitizers. He concluded that AATs are doing a sufficiently good job for hazard identification, but the most relevant issues remain potency assessment and the identification of slowly oxidised prehaptens. In this context it should be noted that slowly oxidised prehaptens are non-sensitizers (unless they also have features that enable them to act as direct haptens or fast-activated pre/pro-haptens, in which case the animal tests and the alternatives, applied to the pure chemical, should pick them up). So he concluded that the problem, insofar as it is a problem at all, is firstly to identify whether a non-sensitising chemical is capable of being slowly oxidised to a directly allergenic chemical, and if so how capable. This appears mainly a chemistry and less a biological problem that might be approached by computational chemistry and experimental chemistry. The problem is secondly to assess how much slow oxidation needs to take place in order for the mixture of original chemical and oxidation products to become a sensitization risk.

The use of *in chemico* methods to identify chemicals that could undergo active metabolite generation was reviewed (Steve Enoch). He discussed the uses of several established data bases. It was noted that the data bases generally are focussed on oxidative fate. The data bases cover a range of chemical structure with rather few reference chemicals that are fragrance material related. Nonetheless it was clear that *in chemico* methods can provide important information for the hazard assessment. He also questioned keeping the terminology of pre- and prohaptens. The aim of the predictive tools is to answer the question whether electrophiles are likely to be formed and how reactive they might be, rather than trying to understand the whole pathway in detail.

In the follow up general discussion consideration was given to the how QRA1 methodology. identifies pre and prohaptens and how such identification can be achieved using non-animal-based hazard assessment. Integration of *in vitro* and *in chemico* findings was not addressed. It was evident that this aspect of QRA2 requires further work.

The key conclusions of the workshop as agreed by all participants are available in a separate document.
Jim Bridges

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LIST OF PARTICIPANTS:

Academia participants:

Don Belsito	Columbia University, United States
Brunhilde Blömeke	Trier University, Germany
Johanna Bråred Christensson	University of Gothenburg, Sweden
Steven Enoch	Liverpool John Moores University, UK
David Gawkrodger	University of Sheffield, UK
Elena Giménez-Arnau	Strasbourg University, France
Ann-Therese Karlberg	University of Gothenburg, Sweden
Jean-Pierre Lepoittevin	Strasbourg University, France
Franz Oesch	University of Mainz, Germany
Ahmed Ramzi	University of Stockholm, Sweden
David Roberts	Liverpool John Moores University, UK
Axel Schnuch	Göttingen University, Germany

Industry participants:

Anne Marie Api	RIFM
David Basketter	DABMEB Consultancy
Fanny Boisleve	Chanel
Carsten Göbel	Coty
Peter Griem	Symrise
Xing Han	IFF
David Hauck	Dr.Hauck R&D GmbH
Amaia Irizar	IFRA
Petra Kern	P&G
Andreas Natsch	Givaudan
Neil Owen	Givaudan
Claudia Turek	Wala / Dr. Hauschka Cosmetics

Observers:

Qasim Chaudhry	SCCS Chair
Georgios Manikas	European Commission – DG Grow

Members of the IDEA Supervisory Group:

Helmut Greim	Member of the IDEA Supervisory Group
Jim Bridges	Chair of the IDEA Supervisory Group

Members of the IDEA Management Team:

Hans Bender	IDEA Management Team, Moderator of IDEA Workshops
Cécile Gonzalez	IDEA Management Team
Peter Griem	IDEA Management Team
Matthias Vey	IDEA Management Team