



Workshop Report

Skin sensitisation – Moving forward with non-animal testing strategies for regulatory purposes in the EU[☆]

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ABSTRACT

In a previous EPAA-Cefic LRI workshop in 2011, issues surrounding the use and interpretation of results from the local lymph node assay were addressed. At the beginning of 2013 a second joint workshop focused greater attention on the opportunities to make use of non-animal test data, not least since a number of *in vitro* assays have progressed to an advanced position in terms of their formal validation. It is already recognised that information produced from non-animal assays can be used in regulatory decision-making, notably in terms of classifying a substance as a skin sensitiser. The evolution into a full replacement for hazard identification, where the decision is not to classify, requires the generation of confidence in the *in vitro* alternative, e.g. via formal validation, the existence of peer reviewed publications and the knowledge that the assay(s) are founded on key elements of the Adverse Outcome Pathway for skin sensitisation. It is foreseen that the validated *in vitro* assays and relevant QSAR models can be organised into formal testing strategies to be applied for regulatory purposes by the industry. To facilitate progress, the European Partnership for Alternative Approaches to animal testing (EPAA) provided the platform for cross-industry and regulatory dialogue, enabling an essential and open debate on the acceptability of an *in vitro* based integrated strategy. Based on these considerations, a follow up activity was agreed upon to explore an example of an Integrated Testing Strategy for skin sensitisation hazard identification purposes in the context of REACH submissions.

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1. Introduction

Whereas toxicology in the last century relied almost exclusively on the use of surrogate animal models, there is now a transformation occurring in which these *in vivo* models are being steadily replaced by *in vitro* alternatives. The endpoint of skin sensitisation is

arguably at the epicentre of this change in the year 2013, the year when such testing, at least for demonstrating the safe use of cosmetic ingredients specifically in relation to the Cosmetics Regulation, has been banned in the European Union (EU, 2003; Adler et al., 2011; Basketter et al., 2012a). This action is anticipated however to have wider ramifications for many other industry sectors, not least since the measure of success for *in vitro* methods is their appearance in the test guidelines of the Organisation for Economic Cooperation and Development (OECD). OECD test guidelines have a very wide applicability, extending far beyond the evaluation of

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cosmetic ingredients. Indeed, this brings into focus the differences between the requirements of the legislation that relates specifically to cosmetics and the wider regulations, which apply to industrial chemicals, notably the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (Commission of the European Communities, 2006).

Experience has taught us that current *in vivo* methods for the predictive identification and characterisation of skin sensitisers have their limitations and indeed this was the focus of an earlier EPAA-Cefic LRI workshop (Basketter et al., 2012b). Consequently, as skin sensitisation toxicology appears poised to migrate to *in vitro* alternatives, it was timely to engage in discussions between toxicologists from various industry sectors, several EU member states, the OECD, EURL ECVAM and the European Chemicals Agency (ECHA). The primary questions addressed were:

1. What are regulatory requirements for the acceptance of non-animal test methods under REACH? (e.g. as weight of evidence; as a stand alone method; as part of a testing strategy)
2. What would regulators see as necessary requirements for non-animal integrated testing strategies (ITS) for skin sensitisation that would help them to take regulatory decisions in a confident way?
3. Until non-animal approaches become available at a regulatory level, what is needed in the interim for facilitating regulatory decision-making on the basis of existing non-animal ITS?
 - a. Can such strategies be accepted already? (e.g. if published in peer reviewed journals; what are the number of chemicals needed?)
 - b. What can be used prior to OECD guideline publication? (e.g. what is required?; what is needed after publication?; are legal adjustments to current regulation/annexes needed?)
4. What can industry and others do to facilitate acceptance procedures?

The first half of the workshop was designed as a training session. It was devoted to platform presentations from industry and regulators detailing current practice in the prediction of skin sensitisation potential, new concepts and the validation of non-animal testing methods, and the strategies for hazard/risk assessment without animal testing. This information generated some discussion, but its primary purpose was to set the scene for the second part of the workshop, involving two break-out groups and a round-table discussion. In the material that follows, what is presented are the main points and recommendations that embodied the primary outcome(s) of the workshop discussions on the four questions above. It should be noted that for certain of these questions, notably the first and second, there was an inevitable overlap in the considerations, which was consequently reflected in the conclusions. A further point to keep in mind is that the principal focus of the workshop and the discussions was hazard identification and regulatory classification; the vital need for information on the potency of identified skin sensitizers to enable risk assessment was also considered, but as a subsidiary theme.

1.1. About LRI

Launched 15 years ago, the Long-Range Research Initiative (LRI) is one of the major voluntary initiatives of the European chemical industry to support its competitiveness and innovation potential. LRI aims to identify and fill gaps in our understanding of the hazards posed by chemicals and to improve the methods available for assessing the associated risks. LRI sponsors high quality research, published in peer reviewed journals, and seeks to provide scientific advice on which industry and regulatory bodies will draw

to respond effectively to the public concerns. Details can be found on the organisation's website: www.cefic-lri.org.

1.2. About EPAA

The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a Public-Private Partnership between the European Commission and Industry members from seven industry sectors. Launched in 2005, it brings together 36 companies, 7 European trade federations and 5 Directorates-General of the European Commission.

During its second mandate for 2011–2015, the EPAA continues to promote the application of Russell and Burch's 3Rs principles (replacement/reduction/refinement of animal testing) in the framework of regulatory compliance procedures (Russell and Burch, 1959). The EPAA contributes to the uptake of 3Rs approaches by assessing their implementation and scope in different industry sectors, mainly at European level. The EPAA fosters a holistic approach, from research & development and the use of 3Rs methods in regulation, to communication and dissemination. Further details can be found on the EPAA website: www.epaa.eu.com.

2. Regulatory requirements for the acceptance of non-animal test methods under REACH

In this context, "acceptance" refers to the use of non-animal test methods for the complete replacement of the existing *in vivo* assays. This includes the adoption of both positive and negative results as a sufficient set of information to determine whether a substance should or should not be classified as a skin sensitiser. The overwhelming view at the workshop was that in the current situation it was highly unlikely that a single *in vitro* alternative would wholly replace the current animal tests. Notwithstanding what might be demonstrated in a formal validation activity concerning predictive accuracy, it was concluded that the limitations of the *in vitro* methods currently under validation with respect to metabolic capability (Chipinda et al., 2011; Hennen et al., 2011) and concerning the problem (shared in common with many *in vitro* methods) of the compatibility of hydrophobic substances with the test matrix (typically an aqueous cell culture medium) could prove a substantial barrier to standalone acceptance for all chemical classes. However, it was considered plausible that the *in vitro* methods covering the key mechanistic steps of skin sensitisation, as now set out in an Adverse Outcome Pathway (AOP) description, when organised into a testing and assessment strategy, may be used to replace the current *in vivo* test.

In part, the above conclusion was driven by the perception that the experiences with *in vitro* alternatives in skin irritation/corrosion, and possibly for phototoxicity, suggested that reliance on the predictive accuracy of a single alternative method had led to a number of problems (Basketter and Kimber, 2007; Schrage et al., 2010, 2011; Jírová et al., 2010; Eskes et al., 2012). Therefore, a greater time was devoted to consider the next question, which was on the topic of ITS.

Lastly in this section, a comment that arose during this discussion, as well as in response to questions concerning an ITS, was the quality and source of the data on which any validation is based. In this respect, it was perceived very positively that the current ECVAM and related validation activity used substances whose classification was based on consistent human and animal data (Casati et al., 2009). Indeed, there was general consensus that, as the overall purpose of classification is to protect human health, then *in vivo* and *in vitro* predictive tools should reflect what is known concerning their effects in humans and therefore should not be primarily

benchmarked against *in vivo* data alone. Exemplifying this had been the decisions already made to regard nickel, a well known human skin sensitiser, as a false negative in the local lymph node assay (LLNA), whereas sodium lauryl sulphate, which is not a human sensitiser, is rightly regarded as a false positive in the LLNA. With careful review, it will be necessary to make similar decisions for other materials in order that an ITS and the on-animal methods on which it is built are not distorted to produce incorrect predictions for humans. An example of how the human information can be deployed has been proposed (Basketter et al., 2013). Of course, in this respect, human data is generally much more likely to be available for existing chemicals than for newer substances.

3. Requirements for a non-animal Integrated Testing Strategy (ITS)

As already noted, both industry and regulatory participants were of the opinion that a combination of information from *in vitro* methods was likely to be necessary for confident replacement of *in vivo* assays for the identification of the presence or absence of skin sensitisation hazard. Furthermore, it was noted that even the output from an ITS would best be placed within a weight of evidence argument concerning classification. It should be noted that it was not the intention of this workshop to consider in what ways the *in vitro* alternatives currently under validation might be combined into an ITS, although there was an awareness of proposals that had been made in that area (Basketter and Kimber, 2009; Natsch et al., 2009, 2013; Bauch et al., 2011, 2012; Nukada et al., 2013).

At its most basic level, it was agreed that any ITS should comprise methods which had a mechanistic rationale, e.g. their place in the Adverse Outcome Pathway (AOP) for skin sensitisation could be identified (OECD, 2012). In terms of the methods closest to validation at EURL ECVAM, three steps essential to the induction of skin sensitisation are represented: the direct peptide reactivity assay (DPRA) for Key Event 1, covalent binding to protein; the Keratinosens assay for Key Event 2, keratinocyte inflammatory responses; and the human cell line activation test (h-CLAT) for Key Event 3, the activation of dendritic cells. It was noted that raw data generated by these methods is expected to be robust, such that interpretation of the final outcome is consistent. Assuming that each of these methods is given a positive recommendation from the bodies involved in validation procedures and are accepted by the OECD, an ITS built on that foundation would in principle be acceptable. Under certain circumstances, results from other methods, i.e. those not (yet) fully validated, could also be integrated into such a strategy, but data should have already been published, allowing evaluation of the methods and the results. However, an essential objective of the ITS is that the output would, as a minimum, permit both positive and negative classification and labelling decisions to be taken.

Although the shape of an ITS based on the *in vitro* methods mentioned above was not discussed in any detail, there was a view that it should have a limited degree of flexibility. For example, it should accommodate the ability to select a first pass test or tests, supported by a scientific rationale, rather than stipulating a fixed order of test conduct. Furthermore, even with an accepted ITS based on validated *in vitro* assays, it was anticipated that the output would be placed within a weight of evidence argument and thus it would be the conclusion of that argument, rather than the ITS outcome in isolation, which determined whether or not a substance should be classified. In principle, this is no different to the current situation with *in vivo* assays. In addition to the WoE framework for using the data, there may be alternative concepts/approaches in the longer term, such as validation of ITS and direct

application of the “results” of the ITS in regulatory schemes. Furthermore, the ITS, or perhaps the WoE framework into which it feeds, should also include, as at present, the possibility to incorporate *in silico* and other non-standard data.

4. Acceptance of non-animal test methods pre-validation

At the time of the workshop (and of writing this report of the proceedings), neither the individual *in vitro* assays of an ITS, nor of course the ITS, had gained regulatory acceptance. Nevertheless, data from these assays increasingly will become available, added to which there are clear provisions in European legislation which encourage the use of such information for regulatory submission (Commission of the European Communities, 2006). Accordingly, the workshop considered what regulators would see as necessary requirements for acceptance of *in vitro* data, particularly given the pressures faced by all partners in terms of the evaluation of substances prior to the next REACH deadline in 2018 applicable to many thousands of phase-in substances produced at 1–100 tonnes per annum.

Among all the discussions that took place, this seemed to be the area that prompted the most calls for continued communication between all the stakeholders. Novel *in vitro* approaches and the data they generate can be of great use, particularly if they have a solid mechanistic foundation (i.e. they fit into the AOP), and have good supporting data with reference substances. Information on the methods should have been published in peer-reviewed journals. Where the method is used by multiple industries, this provides further reassurance concerning its merits. However, critical to data acceptance, particularly to support a negative outcome, is demonstration that the substance under investigation falls within the “applicability domain” of the assay, i.e. that the substance is known to be capable of being tested meaningfully. In essence, regulators saw it as the role of an industry submitter to provide a framework of information around data from non-validated methods which would provide a level of confidence which might be characterised in this way: ideally any toxicologist examining the information package would arrive at the same conclusion concerning skin sensitisation classification. Ultimately therefore, data is most likely to be accepted if it is derived from *in vitro* methods which are well advanced in the process of formal validation, which have the benefit of multiple peer-reviewed publications and for which there already exists some consensus in industry that they deliver meaningful results.

In this area, it is possible to draw a parallel with the acceptance of the LLNA. After the formalisation of the assay in 1992 (Kimber and Basketter, 1992), but prior to validation several years later, a body of work appeared in the peer-reviewed literature, which helped ensure that by the time of the OECD Guideline (OECD, 2002), there was already a considerable familiarity with the assay, including its strengths and limitations. A similar position might reasonably be expected for the *in vitro* assays and certainly is regarded as desirable, particularly by the regulatory authorities.

5. How to facilitate acceptance

Many of the aspects already discussed can be seen as essential to acceptance, but perhaps the most important to be made here is that validation is not acceptance; validation is generally a necessary prerequisite for acceptance, but by no means guarantees acceptance. The key to acceptance, even after formal validation, is that the regulatory community has confidence in both the positive and negative results from *in vitro* assays (and the safety assessments made therefrom). This means that there has to be published evidence of the performance of the assay(s) and the

ITS with a wide range of substances that fill the spectrum of chemistry known to be associated with skin sensitisation as well as those that are not. That published evidence should be in the form of peer-reviewed material, i.e. either in suitable journals or via an independent validation authority. In addition, experience will accumulate when regulators examine the first attempts to apply the *in vitro* assays and ITS; as the experience is positive this examination will also build the confidence of the assays/ITS. In effect, acceptance is facilitated best by the building of confidence in the assay/ITS. One facet of this, which to date has taken second place to the validation of individual component assays, is the ITS itself. In this respect, one simple example is offered in Fig. 1: central to this ITS is the regulatory decision that has to be taken concerning a substance. Currently, *in vivo* methods can deliver essential information on which this decision is based, noting nevertheless that in the final analysis, the decision depends on the weight of all the evidence. In the near future, the feed-in data will be derived from an overall conclusion on the outcome of *in vitro* assays, still combined with any other evidence. So clearly, gaining acceptance from both regulators and industry on the suitability of such an *in vitro* ITS and how it fits in to the decision making process forms an essential ingredient that facilitates acceptance. In this respect, and as indicated in Fig. 1, it is worth noting that whereas *in vivo* methods can deliver information that permits potency sub-categorisation of skin sensitisers, it is at present unclear whether the *in vitro* methods most advanced in terms of validation can offer this benefit.

Confidence can arise from a number of sources in addition to that mentioned above, notably, presentation of the strengths and especially the limitations of the methods, especially the latter, in open forums, scientific meetings, as well as in publications. This activity then not only begins to build confidence, but also enables regulators to consider how the output information can be matched

to the legislation and the regulatory decisions that have to be made. In this respect, the recent institution of a process whereby ECVAM seeks inputs from stakeholders, including member states, concerning the practical utility of *in vitro* methods proffered for validation represents a very positive step forward (EURL ECVAM, 2013a,b). Acceptance of alternatives where member states' experts have been closely involved in the decision-making process should be enhanced. Of course, this helps within Europe, but does not address the global theatre of operations and regulations which confront many industries.

6. Concluding remarks

The most prominent message, one that came from all participants in this workshop, was that the most important requirement for facilitating adoption of an ITS based on validated *in vitro* alternatives was the building of confidence in those who would need to use the outputs, whether they came from industry, the regulatory community or elsewhere. In the interim, there was a good consensus that *in vitro* data submission, prior to validation/ITS development and adoption of OECD Guidelines, would still be considered as a key part of a weight of evidence submission. In such a case, comprehensive details of the methods and a rationale, as well as of the substance specific data would need to be included in the dossier. Given the 2018 registration deadline or REACH (with the high number of substances and tight testing schedules involved) for lower tonnage substances, early submission of dossiers including non-animal and/or ITS strategies is encouraged, the earlier the better. Such submissions help to build experience, highlight challenges and encourage exchange between industry and regulators, as well as informing those responsible for validation and the development of a formal ITS of issues that need to be addressed. To this

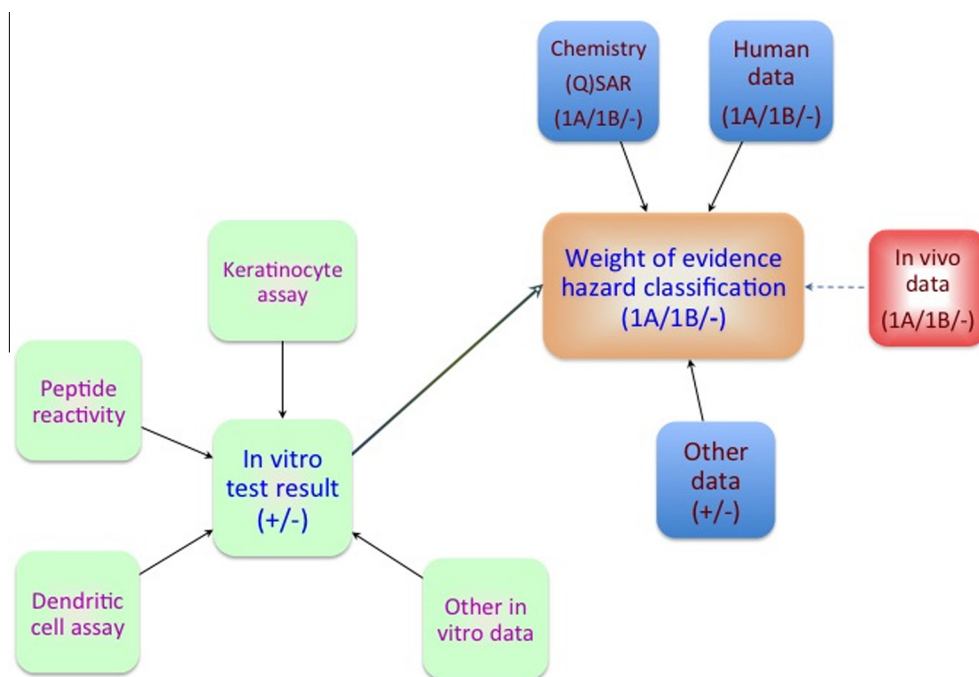


Fig. 1. An example of an ITS for regulatory classification of skin sensitisation. The core of this example is the regulatory weight of evidence decision concerning whether a substance is a sensitiser (1), and if so, whether it is potency sub-category 1A or 1B. The simple ITS example for *in vitro* methods (green background) will replace the *in vivo* input (red background). Note that whereas the *in vivo* methods, and even human data, may permit the determination of sub-categories, it is possible that the *in vitro* methods, at least for the present, may not be sufficiently developed to enable this, leaving classification as a binary decision of either positive or negative. Where this is the case, classification simply as a category 1 skin sensitiser is indicated.

end, it was agreed that current strategies (e.g. Bauch et al., 2012) be explored to gain insights into their strengths and limitations as well as to see what in practice, the real requirements of the regulatory community would be. This action was seen as desirable also in terms of enhancing familiarity with such approaches (and is currently in progress).

Importantly, there was broad consensus that *in vitro* models and an associated ITS fit for regulatory purposes, such as REACH, would not yet be able to replace fully the characterisation of sensitising potency (delivered largely via the LLNA) that is required for risk assessment. In this respect it seems probable that more detailed work to understand at a mechanistic level what the key drivers of skin sensitisation potency are, to develop methods which reflect this knowledge and then calibrate those outputs against a catalogue of substances characterised on the basis of the relative sensitising potency in humans (Basketter et al., 2013).

On the key question of what can industry and others do to facilitate acceptance, the responses of the groups were more varied, but nevertheless contained core themes of openly sharing information, including by peer reviewed publication, of continuing dialogue and of the preparation of guidance documentation, e.g. on use of human data and applicability domains. In this respect, industry volunteered to organise an informal, small scale follow-up discussion to investigate pragmatic solutions and make interim recommendations. This has already been set up for the end of April in Brussels with the key stakeholders.

Finally, one of the most significant aspects of this workshop is it paved the way for, and perhaps even set the model for, future collaborations between CEFIC-LRI, EPAA, ECHA and the national authorities in charge of implementing alternative approaches to animal testing. The positive atmosphere of the event and the valuable progress made, was surprising to some, but in reality a necessity for the prompt adoption of skin sensitisation *in vitro* alternatives that can deliver the same degree of human health protection currently afforded by the *in vivo* methods. Thus, it is felt that this Skin Sensitisation training workshop was a breakthrough in international collaboration on 3Rs which will promote their adoption and use in regulatory toxicology.

Conflicts of interest

DAB was paid by the EPAA for the preparation of this workshop report. The remaining authors are paid employees of their respective organisations.

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