



Workshop report

Can currently available non-animal methods detect pre and pro-haptens relevant for skin sensitization?



Grace Patlewicz^{a,*}, Silvia Casati^b, David A. Basketter^c, David Asturiol^b,
David W. Roberts^d, Jean-Pierre Lepoittevin^e, Andrew P. Worth^b, Karin Aschberger^b

^a US Environmental Protection Agency, National Center for Computational Toxicology, Research Triangle Park, NC, USA

^b European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Chemical Safety and Alternative Methods Unit, Ispra, VA, Italy

^c DABMEB Consultancy Ltd, Sharnbrook, Bedfordshire, UK

^d Liverpool John Moores University, School of Pharmacy and Biomolecular Sciences, Liverpool, UK

^e Institute of Chemistry, CNRS UMR 7177 and University of Strasbourg, Strasbourg, France

ARTICLE INFO

Article history:

Received 10 August 2016

Accepted 18 August 2016

Available online 26 August 2016

Keywords:

Adverse Outcome Pathway

Skin sensitization

Pre-hapten

Pro-hapten

DPRA

h-CLAT

KeratinoSens™

Reaction mechanistic domain

ABSTRACT

Predictive testing to characterize substances for their skin sensitization potential has historically been based on animal tests such as the Local Lymph Node Assay (LLNA). In recent years, regulations in the cosmetics and chemicals sectors have provided strong impetus to develop non-animal alternatives. Three test methods have undergone OECD validation: the direct peptide reactivity assay (DPRA), the KeratinoSens™ and the human Cell Line Activation Test (h-CLAT). Whilst these methods perform relatively well in predicting LLNA results, a concern raised is their ability to predict chemicals that need activation to be sensitizing (pre- or pro-haptens). This current study reviewed an EURL ECVAM dataset of 127 substances for which information was available in the LLNA and three non-animal test methods. Twenty eight of the sensitizers needed to be activated, with the majority being pre-haptens. These were correctly identified by 1 or more of the test methods. Six substances were categorized exclusively as pro-haptens, but were correctly identified by at least one of the cell-based assays. The analysis here showed that skin metabolism was not likely to be a major consideration for assessing sensitization potential and that sensitizers requiring activation could be identified correctly using one or more of the current non-animal methods.

Published by Elsevier Inc.

1. Introduction

Skin sensitization remains an important endpoint for risk assessment purposes, however predictive testing still relies on *in vivo* assays. The most notable of these is the local lymph node assay (LLNA) which provides a quantitative measure of skin sensitization potential and potency. Since the advent of EU regulations such as REACH (EC, 2006) and the Cosmetics Regulation (EC, 2009), there has been a call for non-animal methods to be developed to assess skin sensitization potential. Significant progress has been made to develop test methods and to evaluate their validity for use (Adler et al., 2011; Goebel et al., 2012; Zuang et al., 2015). In parallel,

there has also been an effort to construct a framework to facilitate the development, interpretation and application of mechanistic information within Adverse Outcome Pathways (AOPs) (Edwards et al., 2016; Kleinstreuer et al., 2016). The AOP for skin sensitization, published by the OECD in 2012 (OECD, 2012), describes the series of events that are needed to induce skin sensitization. Three test methods in particular have undergone a significant validation process and are now OECD test guidelines. Two of these methods, the direct peptide reactivity assay (DPRA) and the KeratinoSens™, have been adopted by the OECD in 2015 as TG 442C (OECD, 2015a) and TG442D¹ (OECD, 2015b), respectively. A third *in vitro* method, the human Cell Line Activation Test (h-CLAT), was adopted by the OECD in 2016 as TG 442E. The DPRA characterizes the first key event (KE) or the molecular initiating event – the covalent binding

* Corresponding author. EPA, Office of Research and Development, National Center for Computational Toxicology (NCCT), 109 T W Alexander Dr, RTP, NC 2711, USA.

E-mail address: patlewicz.grace@epa.gov (G. Patlewicz).

¹ Note the guideline representing the KeratinoSens™ is known as the ARE-Nrf2 Luciferase Test Method.

with skin proteins. Briefly, the DPRA readout is the percentage of peptide depletion, after 24 h incubation with the test chemical, calculated from the unreacted peptide concentration in the reaction mixture relative to the average concentration of peptide in the control. The percentage depletion is used as a measure of inherent reactivity (Gerberick et al., 2004, 2007). Subsequent events describe the activation of keratinocytes (KE 2) and dendritic cells (KE 3) which can be detected by KeratinoSens™ and h-CLAT, respectively. Keratinocyte exposure to sensitizing chemicals results in the induction of the antioxidant/electrophile response element (ARE/EpRE)-dependent pathways (Natsch and Emter, 2008). Reactive chemicals bind to KEap1 (Kelch-like ECH-associated protein 1) provoking the dissociation of the nuclear erythroid 2-related factor (Nrf2) from the protein. Released Nrf2 translocates from the cytoplasm to the nucleus where it promotes the activation of ARE/EpRE dependent pathways (Natsch and Emter, 2008). An *in vitro* luciferase reporter gene assay based on activation via the ARE/EpRE response element as covered by KeratinoSens™ has been shown to be responsive to known sensitizers in HaCaT keratinocytes (Emter et al., 2010). The activation, maturation of Langerhans cells and their migration to the draining lymph nodes are detected in part by measuring the expression of markers within the h-CLAT. The h-CLAT quantifies *in vitro* changes in the expression of the CD86 and CD54 membrane phenotypic markers in a human monocytic leukemia cell line (THP-1 cells) (Ashikaga et al., 2006). THP-1 cells have been shown to produce DC-like responses following exposure to skin sensitizing chemicals (e.g. upregulation of surface markers (e.g. CD86 and CD54) and cytokine production (e.g. TNF- α). The CD86 (a co-stimulatory molecule) and the CD54 (an adhesion molecule) are upregulated in activated Dendritic Cells (DC) and play a critical role in DC presentation of antigens to T cells (T-cell priming).

Performance metrics of each of the individual test methods relative to the LLNA have been published that have provided sufficient scientific confidence that skin sensitizers are correctly identified (see EC EURL-ECVAM, 2013; 2014; 2015). However, it is stated in the test guidelines that a combination of these non-animal test methods will be needed to subcategorize skin sensitizers into the UN GHS (subcategories 1A and 1B), or indeed to predict sensitizing potency for safety assessment decisions. As such, there are ongoing efforts to develop integrated approaches to assess both skin sensitization potential and potency. An EPAA/Cefic LR/Cosmetics Europe cross sector workshop report describes many of the efforts to date (Basketter et al., 2015).

For REACH, there has been a concerted move to incorporate these 3 non-animal test methods into an integrated testing strategy or weight of evidence approach to address the skin sensitization requirements for substances greater than 1 tonne per annum that need to be registered in light of the 2018 registration deadline (ECHA, 2016). A particular concern that was raised as part of the discussions to revise the ECHA guidance (ECHA, 2016) was the ability of the non-animal test methods to predict substances that needed to be activated to induce sensitization, i.e. indirectly acting sensitizers. The DPRA is a cell free assay and therefore does not have any metabolic capacity. The two *in vitro* methods on the other hand, use cells which do possess some metabolic capacity, though do not fully represent the *in vivo* metabolic situation (Urbisch et al., 2016). N-acetyl transferase (NAT-1) and esterase activities have been detected in both cell lines by Urbisch et al. (2016). They also detected aldehyde dehydrogenase (ALDH) activities in KeratinoSens™ cells; but, cytochrome P450 (CYP) and flavin-dependent monooxygenase (FMO) activities were determined to be below the limit of detection. It should be noted that cutaneous enzymes are in general active at significantly lower constitutive levels than their hepatic counterparts. A background on the relative activities in

comparison with hepatic activities can be found in Smith and Hotchkiss (2001). More recent references include Wiegand et al. (2014).

Indirectly acting sensitizers can be further subcategorized as pro- or pre-haptens (Lepoittevin, 2006). Pre-haptens undergo activation externally before coming into contact with the skin or on the skin (e.g. through air oxidation processes – autooxidation). Pro-haptens undergo activation within the skin, mainly through metabolic processes. Many pre-haptens can also act as pro-haptens and this potential should be considered as and when a pre-hapten has been identified. The same haptens can sometimes be formed from both activation pathways even though the mechanisms may be different. Activation of pre-haptens often occurs via a radical pathway with the formation of highly sensitizing hydroperoxides as the primary oxidation products (Karlberg et al., 2008, 2013). Some of these hydroperoxides are unstable; often they cannot be detected and are only identified as a result of secondary oxidation products being formed (Karlberg et al., 2008, 2013). The secondary oxidation products can also be sensitizers and have for some substances, (e.g. geraniol (Hagvall et al., 2007, 2008) and alpha-terpinene (Bergstrom et al., 2006; Rudbäck et al., 2012) been shown to be identical with those formed via the metabolic pathway. It is important to remember that not all products formed through oxidation processes result in sensitization.

A current uncertainty is whether the non-animal methods available to date can adequately identify indirect skin sensitizers, or whether a detailed investigation of metabolic products is necessary. In order to address this question, we put into context the incidence of skin sensitizers that require activation and secondly assessed the performance characteristics of the 3 test methods to determine their fitness-for-purpose to correctly identify indirect skin sensitizers.

The two objectives of this study were therefore to: 1) quantify the incidence of indirectly acting sensitizers using a dataset of high quality skin sensitization information; and, 2) assess the ability of the non-animal test systems to identify correctly the sensitization response for the indirect sensitizers. The role and utility of *in silico* systems for the prediction of transformation products is also discussed in brief.

These questions were discussed as part of an European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) expert meeting held in November 2015 at JRC, Ispra, Italy. A short technical report was published in March 2016 summarizing the main findings (Casati et al., 2016). This manuscript describes the evaluation performed in greater detail.

2. Materials and methods

Two datasets were considered to address objective 1. The first of these (the comparative dataset) was published by Gerberick et al. (2005) and Kern et al. (2010). Kern et al. (2010) published a dataset of 108 LLNA results to augment an earlier set of 211 substances published by Gerberick et al. (2005). Collectively these comprised 319 LLNA results. Each chemical evaluated had been previously assigned into one of several reaction mechanistic domains using the principles from Roberts and Aptula (2008). The incidence of indirectly acting sensitizers as taken from Kern et al. (2010) and Gerberick et al. (2005) was expected to provide a comparative benchmark for the dataset in this current study.

The dataset for this study was compiled by EURL ECVAM and consisted of 127 chemicals with DPRA, KeratinoSens™ and h-CLAT data. The dataset was considered high quality in that it had been carefully curated and evaluated as part of the respective test method validation exercises (see EC EURL-ECVAM, 2013; 2014; 2015). The study dataset was complemented with LLNA outcomes

and human response data. The LLNA outcomes were taken from submissions to EURL ECVAM as well as scientific publications whereas the human response data was taken from [Basketter et al. \(2014\)](#). The EURL ECVAM dataset is also the subject of a complementary study (see [Asturiol et al., 2016](#)).

All experimental outcomes were denoted as positive (1) or negative (0) for skin sensitization. The principles as outlined in [Roberts and Aptula \(2008\)](#) were used to categorize each of the chemicals in turn: firstly, to determine whether they were directly acting or required activation – metabolic (pro) and/or abiotic (pre); and secondly, to assign their reaction mechanistic domain(s). These domains are Michael acceptors (MA), S_N2 (nucleophilic substitution bimolecular), Acylating agents, Schiff base formers (SB), S_NAr and special cases. Whilst *in silico* tools such as the profilers within the OECD Toolbox (<http://www.qsartoolbox.org/>), the expert systems Tissue Metabolism Simulator for skin sensitization (TIMES-SS) ([Dimitrov et al., 2005](#); [Patlewicz et al., 2007, 2014](#)) or Derek Nexus (<http://www.lhasalimited.org/products/derek-nexus.htm>), the Toxtree rulebases (https://eurl-ecvam.jrc.ec.europa.eu/laboratories-research/predictive_toxicology/qsar_tools/toxtree), could have been used to systematically identify electrophilic centres either directly or as precursors, in this case the assignments were made by expert judgement by 4 of the participants at the EURL ECVAM meeting. The experts (namely J-P Lepoittevin, DW Roberts, A-T Karlberg and G Patlewicz) considered each substance on the basis of its chemical structure to determine whether it was reactive and if so whether it was directly acting or indirectly acting. This was carried out by identifying whether there were any electrophilic centres within the structure or whether plausible transformations could occur that would result in the formation of a reactive centre. This determination was based on their experience and prior study. A large number of the substances in the dataset had been studied previously and their chemical mechanisms were investigated experimentally or discussed in prior publications by some of the authors. The substances that have been considered previously are noted in the dataset with their associated reference(s). The complete dataset is provided in [Asturiol et al. \(2016\)](#), here we provide a summary set with the reaction mechanistic domains as supplementary information.

3. Results and discussion

In the [Kern et al. \(2010\)](#) and [Gerberick et al. \(2005\)](#) datasets collectively, approximately 25% of the 319 substances were reported to be pre- or pro-haptens. Of the 127 substances considered here as evaluated by the LLNA, a similar percentage was found. This is not altogether surprising since the datasets did significantly overlap, 109 of the 127 substances fell within the two published sets from [Kern et al. \(2010\)](#) and [Gerberick et al. \(2005\)](#).

Twenty eight substances were designated as pre- and/or pro-haptens (28/127 = 22%). Of the 28 substances, 6 were assigned by expert judgement as pro-haptens alone i.e. requiring metabolic activation, 12 were exclusively pre-haptens, and the remaining 10 were assigned as capable of acting by more than one pathway ([Table 1](#)).

3.1. Pro haptens

The substances assigned as pro-haptens alone were 3-aminophenol, 4-allylanisole, ethylene diamine, resorcinol, dihydroeugenol (2-methoxy-4-propyl-phenol) and 3-dimethylamino propylamine. The experimental data for these substances are recorded in [Table 2](#). All 6 were correctly identified as sensitizers on the basis of the h-CLAT. The KeratinoSens™ correctly identified 3 of the set and the DPRA only 1.

3-Aminophenol is proposed to undergo hydroxylation followed by oxidation to form a quinone-imine capable of reacting via Michael addition as shown in [Scheme 1](#).

4-Allylanisole is expected to undergo demethylation and form a quinone-methide that could act as a Michael acceptor. Ethylenediamine and 3-dimethylaminopropylamine are proposed to act as pro-Schiff base electrophiles by conversion of the CH–N entity into a (di)aldehyde. The conversion to a (di)aldehyde was a working hypothesis based on a proposal by Benezra, quoted by [Foussereau et al. \(1983\)](#).

Resorcinol and dihydroeugenol are both proposed to act as pro-Michael acceptors, i.e. hydroxylation in the para position would result in the formation of a hydroquinone precursor which could then act by Michael addition.

Albeit based on evaluation of a limited set, a feasible assessment strategy might be to: 1) identify the reaction domain using the principles outlined in [Roberts and Aptula \(2008\)](#) or using an *in silico* tool (assuming it possessed the ability to simulate transformation products) and if a robust assignment of pro-hapten could be made, 2) perform confirmatory testing of the parent substance in the h-CLAT. If a conclusive assignment of pro-hapten was not feasible but activation was nonetheless predicted, step 1 would be to test in the DPRA. A negative outcome would be followed by step 2.

3.2. Pre-haptens

Twelve substances fell into the category of pre-haptens. All of the pre-haptens were correctly identified by at least one of the 3 assays (experimental results are recorded in [Table 3](#)).

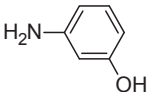
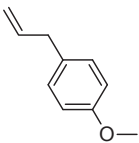
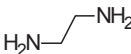
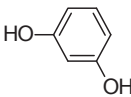
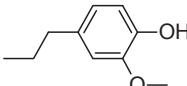
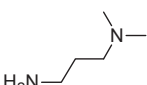
The DPRA failed to identify linalool correctly. The KeratinoSens™ missed d-limonene and linalool and the h-CLAT missed abietic acid and isoeugenol. Eight of the twelve substances were assigned as being activated to react as Michael acceptors (pre-MA). Most of these could form a quinone or quinone-imine derivative (1,4-phenylenediamine, 2,5-diaminotoluene, 2-aminophenol, hydroquinone, isoeugenol, lauryl gallate and propyl gallate). 1-Naphthol was proposed to act via Michael addition via its keto-tautomer (discussed in [Roberts et al., 2007a](#)). Three contained alerts for autooxidation to hydroperoxide formation (abietic acid, d-limonene, and linalool). 1,2-Dibromo-2,4-dicyanobutane (MDGN) was assigned as a pre-Michael acceptor (pre-MA) or possibly a pre- S_N2 – loss of HBr could result in either a vinylic bromide that could undergo Michael addition or an allylic bromide that could react via S_N2 . The mechanistic pathways were discussed in [Patlewicz et al. \(2008\)](#).

If a robust assignment of a pre-hapten could be made by reference to the reaction domains, testing in the DPRA was likely to be sufficient to conclude on sensitizing potential – only linalool was missed but would have been picked up by confirmatory testing in the h-CLAT. It is also worth noting that abietic acid, linalool and limonene are challenging for any assay to identify since the pure chemicals may be weak or non-sensitizing themselves ([Karlberg et al., 2008, 2013](#)). The outcomes of the assays depend on how much opportunity the test substance has had to become oxidized before it is tested.

Table 1
Distribution of substances within the dataset.

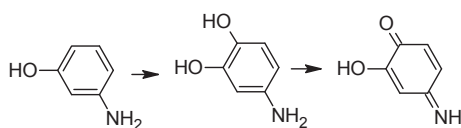
Type	#
Pre	12
Pro	6
Pre/Pro	9
Pre or Direct acting	1
Direct acting (DA)	59 directly + LLNA positive
False positives in the LLNA	4

Table 2
Pro-haptens in the dataset.

Name	Structure	In vivo		Cell free in chemico	Cell based <i>in vitro</i>		Reaction mechanistic domain
		LLNA	Human data	DPRA	KeratinoSens™	h-CLAT	
3-Aminophenol		1	NA	0	0	1	pro-MA
4-Allylanisole		1	NA	1	0	1	pro-MA
Ethylenediamine (free base)		1	1	0	1	1	pro-SB
Resorcinol		1	1	0	0	1	pro-MA
Dihydroeugenol (2-methoxy-4-propyl-phenol)		1	NA	0	1	1	pro-MA
3-Dimethylamino propylamine		1	1	0	1	1	pro-SB ^a

Notes: NA – not available, 1 = positive, 0 = negative, SB = Schiff base former, MA = Michael acceptor.

^a 3-Dimethylamino propylamine was originally assigned as a pre-SB in the original EC EURL-ECVAM report (Casati et al., 2016). It has been reconsidered as a pro-SB electrophile.



Scheme 1. Proposed reaction pathway for 3-aminophenol.

3.3. Pro/pre-haptens

Nine substances were assigned to the category of pro/pre-haptens. More than one possibility existed for each substance, but no concrete evidence was available to rationalize which might be the more dominant pathway leading to skin sensitization induction (Table 4).

All nine substances were correctly identified as sensitizers by the h-CLAT. The KeratinoSens™ and the DPRA mis-categorized 4 of the sensitizers. 2-Methoxy-4-methylphenol was only picked up in the h-CLAT, it was hypothesized to be a pro- or pre-Michael acceptor. 2-Nitro-1,4-phenylenediamine and 4-ethyl-N-2-methanesulphomamido-ethyl-2-methyl-1,4-phenylenediamine (CD3) and Bandrowski's Base (N,N-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine) were identified correctly as sensitizers by all 3 assays, and were categorized as pre- or pro-Michael acceptors. Cinnamyl alcohol was also correctly identified as sensitizing by all 3 tests. It could become activated to react via a Michael addition, alternatively evidence for a pro-S_N2 mechanism by OH sulphation might be feasible as proposed by Moss et al. (2016). Formation of hydroperoxides is also possible. Eugenol has been discussed to a great extent elsewhere (Roberts et al., 2007a). It was assigned as a pro- or pre-Michael acceptor; the DPRA and h-CLAT correctly

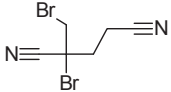
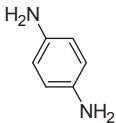
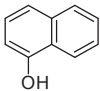
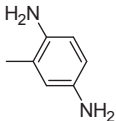
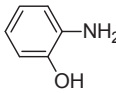
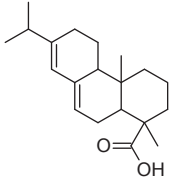
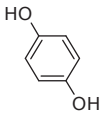
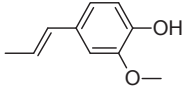
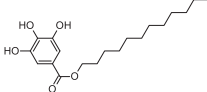
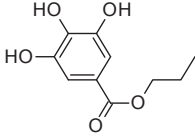
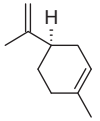
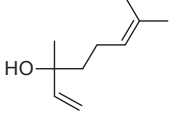
assigned its sensitizing ability whereas the KeratinoSens™ predicted it to be negative. Aniline was only picked up as sensitizing by the h-CLAT. Several pathways appear possible, including ring oxidation to a pre- or pro-quinone imine and subsequent reaction as a Michael acceptor. Alternatively, the NH₂ group could undergo oxidation to form a nitroso compound which would behave as a pseudo Schiff base former. A free radical route is another option. Geraniol was identified correctly by the KeratinoSens™ and the h-CLAT – it was assigned as a pro-S_N2 via sulphation as well as pre- and pro-Michael acceptor. Alternatively, formation of hydroperoxides and epoxides as discussed in Hagvall et al. (2008) would allow for a pre activation and free radical mechanism. Chlorpromazine hydrochloride contains a diamine moiety which could be released to form a dialdehyde and hence Schiff base formation could occur.

In the absence of being able to determine whether a pro and pre-hapten drive the sensitization response, a positive outcome in the DPRA would tend to indicate a pre-hapten whereas a negative outcome could be followed up with the h-CLAT.

3-Propylidenephthalide was assigned as a potential acyl transfer former but also could potentially act by a free radical route, i.e. a direct acting hapten or a pre-hapten. It was positive in both the DRPA and the h-CLAT but incorrectly predicted by the KeratinoSens™.

To put into context the performance of the assays for indirectly acting sensitizers, it is worth considering their performance for the entire dataset. A more detailed analysis of the relative performance of the individual assays and their combinations is the subject of a companion manuscript (Roberts and Patlewicz, in prep), here we only draw attention to the false negative and false positive rates of the individual assays for this dataset. The false negative rates for h-CLAT, KeratinoSens and DRPA were 17%, 24% and 22% respectively.

Table 3
Pre-haptens in the dataset.

Name	Structure	In vivo		Cell free in chemico	Cell based <i>in vitro</i>		Reaction mechanistic domain
		LLNA	Human	DPRA	KeratiNoSens™	h-CLAT	
1,2-Dibromo-2,4-dicyanobutane (MDGN)		1	1	1	1	1	pre-MA/S _N 2
1,4-Phenylenediamine		1	1	1	1	1	pre-MA
1-Naphthol		1	NA	1	1	1	pre-MA
2,5-Diaminotoluene sulphate (PTD)		1	1	1	1	1	pre-MA
2-Aminophenol		1	1	1	1	1	pre-MA
Abietic acid		1	1	1	1	0	pre
Hydroquinone		1	1	1	1	1	pre-MA
Isoeugenol		1	1	1	1	0	pre-MA
Lauryl gallate		1	1	1	1	1	pre-MA
Propyl gallate		1	1	1	1	1	pre-MA
d-Limonene		1	0	1	0	1	pre
Linalool		1	1	0	0	1	pre

Notes: NA = not available, 1 = positive, 0 = negative, MA = Michael acceptor, pre = free radical mechanism.

The h-CLAT and the KeratinoSens had a false positive rate of 35%, compared to 24% for the DPRA. This does suggests that the three test methods are unlikely to miss sensitizers at the expense of overclassifying for sensitization.

All substances that were assigned as non-reactive or insufficiently reactive to sensitize were also looked at to examine whether any insights could be inferred about the types of substances that tended to be mispredicted by the assays and whether these were similar between the assays. Thirty two substances were assigned as non-reactive (25 substances) or insufficiently reactive enough (7 substances) to sensitize; all were negative in the LLNA but many gave rise to positive outcomes in one or more of the non-animal tests. For the non-reactive substances, the h-CLAT gave rise to 10 false positive outcomes whereas the KeratinoSens™ gave rise to 9 false positives and the DPRA, 4 false positives. The substances for which the DPRA was incorrect coincided for 3 of the 4 substances with the h-CLAT namely 2-acetyl-cyclohexanone, 3-

phenoxypropiononitrile and salicylic acid. The KeratinoSens™ incorrect predictions only overlapped with the h-CLAT in three instances; 2-acetyl-cyclohexanone, benzaldehyde and propyl paraben. Only 2-acetyl-cyclohexanone was incorrect across the 3 assays. No specific insights could be extracted to rationalize the sorts of substances that were misclassified in the 3 assays for this dataset.

Four substances were assigned as false positives in the LLNA. The four false positives in the LLNA were xylene, sodium lauryl sulphate (SLS), nonanoic acid and oxalic acid. For xylene and SLS, none of the non-animal assays were positive. Nonanoic acid and oxalic acid gave rise to positive responses in the h-CLAT. Oxalic acid elicited a positive response in the KeratinoSens™.

One of the common criticisms around non-animal test methods in general is their apparent lack of metabolic capacity (NAS, 2015). In this case, lack of metabolic capacity could mean that true sensitizers are not correctly identified due to the inability to adequately account for possible transformation products. However,

Table 4
Pre/Pro-haptens in the dataset.

Name	Structure	In vivo		Cell free in chemico		Cell based <i>in vitro</i>		Reaction mechanistic domain
		LLNA	Human	DPRA		KeratinoSens™	h-CLAT	
2-Methoxy-4-methylphenol		1	NA	0		0	1	pro/pre-MA
2-Nitro-1,4-phenylenediamine		1	1	1		1	1	pro/pre-MA
4-(N-Ethyl-N-2-methanesulphonamido-ethyl)-2-methyl-1,4-phenylenediamine (CD3)		1	NA	1		1	1	pre/pro-MA
Aniline		1	1	0		0	1	pre/pro-MA or pseudo SB
Chlorpromazine hydrochloride		1	1	0		0	1	pro/pre-SB
Cinnamyl Alcohol		1	1	1		1	1	pre/pro-(MA or S _N 2)/pre
Geraniol		1	1	0		1	1	pro/pre-(MA or S _N 2) or pre
Bandrowski's Base (N,N-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine)		1	NA	1		1	1	pro/pre-MA
Eugenol		1	1	1		0	1	pro/pre-MA

Notes: NA = not available, 1 = positive, 0 = negative, MA = Michael acceptor, SB = Schiff base former, pre = free radical mechanism.

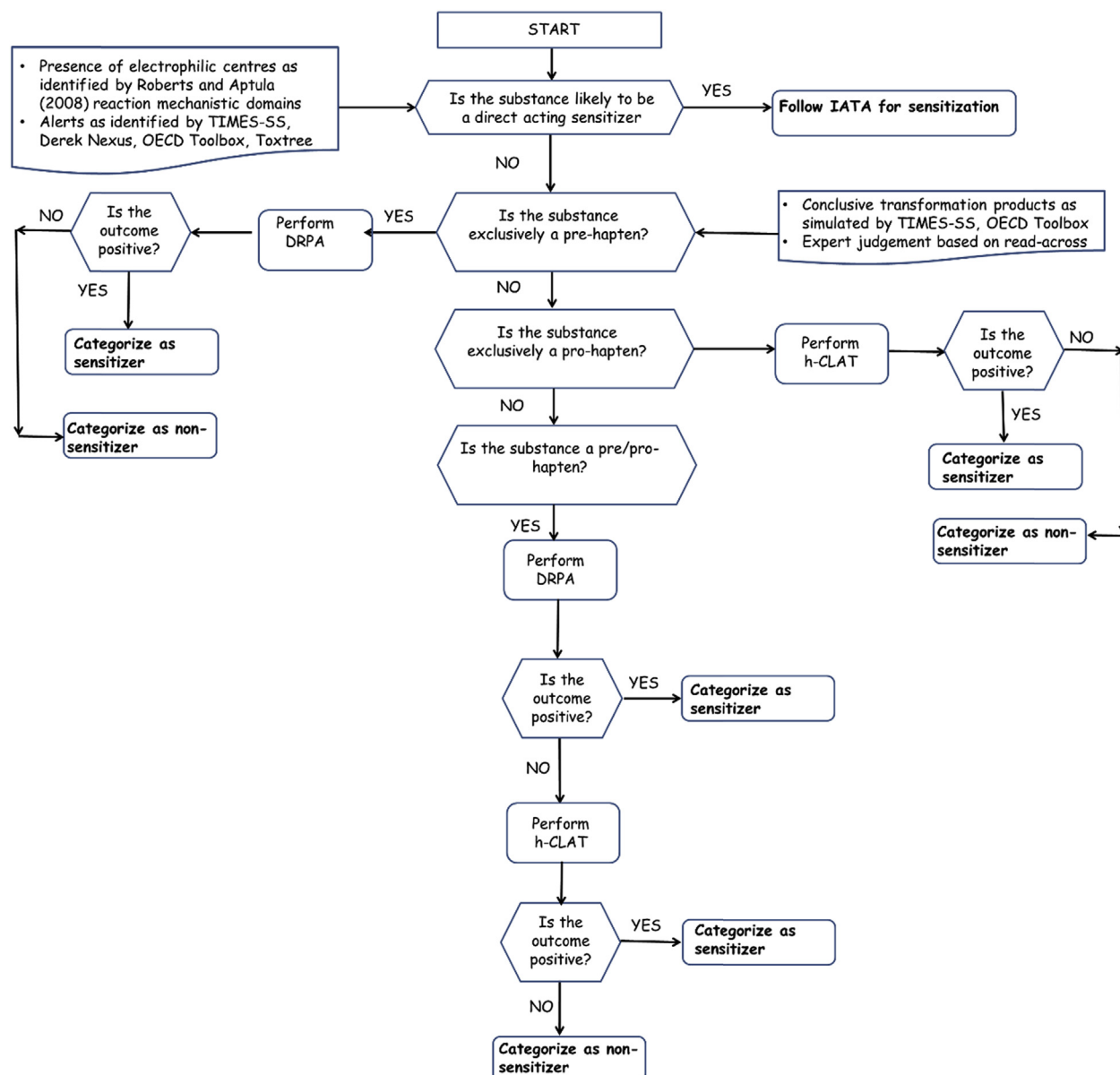


Fig. 1. Workflow summarizing possible testing and assessment strategies to address indirectly acting sensitizers.

there is wealth of knowledge regarding the reaction chemistry to rationalize sensitizers (see Aptula et al., 2005; Roberts et al., 2007a, b as examples) and much of this knowledge has been implemented as structural rules in *in silico* tools such as TIMES-SS, Derek Nexus, OECD Toolbox to facilitate routine use. These tools can be very helpful in identifying the reaction mechanistic domain and/or potential transformation pathways to determine whether a substance is likely to be directly acting or will require activation (either abiotic or biotic). If a determination of the reaction domain can be made for a given substance, then the DPRA could be used first to characterize its inherent reactivity. If transformation through metabolism needs to occur, as were identified in 6 cases in this dataset, the h-CLAT could be potentially used to identify these cases as part of a tiered approach with the DPRA.

4. Conclusions

Assessing skin sensitization potential remains a critical consideration for risk assessment. Under REACH, three non-animal test

methods can now be used to fulfil the information requirements for skin sensitization at tonnages greater than 1 tonne per annum as laid out in column 1 of Annex VII (see ECHA draft guidance http://echa.europa.eu/documents/10162/13643/ir_csa_r7a_r7-3_caracal_draft_en.pdf). A particular concern that was raised as part of the discussions to revise the ECHA guidance (ECHA, 2016) was the ability of the non-animal test methods to predict substances that need to be activated to induce sensitization, i.e. indirectly acting sensitizers. This issue was discussed at an EURL ECVAM expert meeting specifically convened to consider whether the metabolic capacity within the current test systems were fit-for-purpose in terms of predicting sensitizers correctly. A large dataset compiled by EURL ECVAM was evaluated for which outcomes from the LLNA and the 3 established non-animal test methods were available. Each of the substances was assigned by 4 of the authors into one of 5 reaction domains using the reaction chemistry principles by Roberts and Aptula (2008). Twenty two percent of substances evaluated were found to require some level of activation. This was consistent with the outcome from the collective compilation of

Kern et al. (2010) and Gerberick et al. (2005). The indirectly acting sensitizers were assigned by reaction domain. Reaction pathways were postulated to determine whether metabolism or chemical transformation would result in the formation of an electrophile capable of binding with skin proteins. It was found that of the 28 substances identified; 12 were clearly identified as pre-haptens. The majority of these either resulted in the formation of reactive Michael acceptors upon activation or could be oxidized to form reactive hydroperoxides or epoxides. Exploring the non-animal test assay outcomes suggested that these pre-haptens could best be identified using the DPRA.

For 9 substances, no determination could be made to differentiate whether a pre- or pro-pathway would drive the sensitization induction response; the substances were found to be correctly identified using the DPRA and h-CLAT. Pro-haptens were best identified using the h-CLAT. These findings are summarized by a workflow (Fig. 1).

Based on the dataset herein studied, we concluded that most indirectly acting sensitizers could be correctly detected using non-animal test methods thus obviating the need for *in vivo* testing. More non-animal test data are needed to substantiate this finding more generally.

Conflicts of interests

The authors declare no competing financial interest. The authors alone are responsible for the content and writing of this article.

Disclaimer

The views expressed in this article are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency.

Acknowledgements

The perspectives in this manuscript were a product of the expert group meeting held at the JRC in November 2015. We would like to thank Ann-Therese Karlberg and Sabcho Dimitrov for their contributions during the meeting and in drafting the EURL Technical Report. This work was presented as a poster at the 17th International Conference on QSAR in Environmental and Health Sciences (QSAR 2016), held 13–17 June 2016 at Miami Beach, Florida, USA.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.yrtph.2016.08.007>.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.yrtph.2016.08.007>.

References

Adler, S., Basketter, D., Creton, S., Pelkonen, O., van Benthem, J., Zuang, V., Andersen, K.E., Angers-Loustau, A., Aptula, A., Bal-Price, A., Benfenati, E., Bernauer, U., Bessems, J., Bois, F.Y., Boobis, A., Brandon, E., Bremer, S., Broschard, T., Casati, S., Coecke, S., Corvi, R., Cronin, M., Daston, G., Dekant, W., Felner, S., Grignard, E., Gundert-Remy, U., Heinonen, T., Kimber, I., Kleinjans, J., Komulainen, H., Kreiling, R., Kreysa, J., Leite, S.B., Loizou, G., Maxwell, G., Mazzatorta, P., Munni, S., Pfuhler, S., Phrakonkham, P., Piersma, A., Poth, A., Prieto, P., Repetto, G., Rogiers, V., Schoeters, G., Schwarz, M., Serafimova, R., Tähti, H., Testai, E., van Delft, J., van Loveren, H., Vinken, M., Worth, A., Zaldivar, J.M., 2011. Alternative (non-animal) methods for cosmetics testing:

current status and future prospects-2010. *Arch. Toxicol.* 85 (5), 367–485. <http://dx.doi.org/10.1007/s00204-011-0693-2>.

Aptula, A.O., Patlewicz, G., Roberts, D.W., 2005. Skin sensitization: reaction mechanistic applicability domains for structure-activity relationships. *Chem. Res. Toxicol.* 18, 1420–1426.

Ashikaga, T., Yoshida, Y., Hirota, M., Yoneyama, K., Itagaki, H., Sakaguchi, H., Miyazawa, M., Ito, Y., Suzuki, H., Toyoda, H., 2006. Development of an *in vitro* skin sensitization test using human cell lines: the human Cell Line Activation Test (h-CLAT) I. Optimization of the h-CLAT protocol. *Toxicol. In Vitro* 20, 767–773.

Asturiol, D., Casati, S., Worth, A., 2016. Consensus of classification trees for skin sensitisation hazard prediction. *Toxicol. In Vitro*. <http://dx.doi.org/10.1016/j.tiv.2016.07.014> pii: S0887-2333(16)30143-6.

Basketter, D.A., Alépée, N., Ashikaga, T., Barroso, J., Gilmour, N., Goebel, C., Hibatallah, J., Hoffmann, S., Kern, P., Martinozzi-Teissier, S., Maxwell, G., Reisinger, K., Sakaguchi, H., Schepky, A., Tailhardat, M., Templier, M., 2014. Categorization of chemicals according to their relative human skin sensitizing potency. *Dermatitis* 25, 11–21.

Basketter, D., Ashikaga, T., Casati, S., Hubsch, B., Jaworska, J., de Knecht, J., Landsiedel, R., Manou, I., Mehling, A., Petersohn, D., Rorije, E., Rossi, L.H., Steiling, W., Teissier, S., Worth, A., 2015. Alternatives for skin sensitisation: hazard identification and potency categorisation: report from an EPAA/CEFC LRI/Cosmetics Europe cross sector workshop, ECHA Helsinki, April 23rd and 24th 2015. *Regul. Toxicol. Pharmacol.* 73 (2), 660–666. <http://dx.doi.org/10.1016/j.yrtph.2015.10.005>.

Bergstrom, M.A., Luthman, K., Nilsson, J.L.G., Karlberg, A.T., 2006. Conjugated dienes as prohaptens in contact allergy: *in vivo* and *in vitro* studies of structure – activity relationships, sensitizing capacity, and metabolic activation. *Chem. Res. Toxicol.* 19, 760–769.

Casati, S., Aschberger, K., Asturiol, D., Basketter, D., Dimitrov, S., Dumont, C., Karlberg, A.T., Lepoittevin, J.P., Patlewicz, G., Roberts, D.W., Worth, A., 2016. Ability of non-animal methods for skin sensitisation to detect pre- and pro-haptens: Report and recommendations of an EURL ECVAM expert meeting; EUR 27752 EN. <http://dx.doi.org/10.2788/01803>.

Dimitrov, S., Low, L., Patlewicz, G., Kern, P., Dimitrova, G., Comber, M., Phillips, R., Niemela, J., Bailey, P., Mekenyan, O., 2005. Skin sensitization: modeling based on skin metabolism simulation and formation of protein conjugates. *Int. J. Toxicol.* 24, 189–204.

EC, 2006. Regulation (EC) No 1907/2006 of the European parliament and of the council of 18 december 2006 concerning the registration, evaluation, authorisation and restriction of chemicals (REACH), establishing a European chemicals agency, amending directive 1999/45/EC and repealing council regulation (EEC) No 793/93 and commission regulation (EC) No 1488/94 as well as council directive 76/769/EEC and commission directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. *Off. J. Eur. Union* L396/1 of 30.12.2006. Commission of the European Communities.

EC, 2009. Regulation (EC) No 1223/2009 of the European parliament and the council of 30 november 2009 on cosmetic products. *Off. J. Eur. Union* L342, 59–209.

EC EURL-ECVAM, 2014. Recommendation on the KeratinoSens™ Assay for Skin Sensitisation Testing, p. 42. Available at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvamrecommendations/recommendation-keratinosens-skin-sensitisation.

EC EURL-ECVAM, 2013. Recommendation on the Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing. Available at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvamrecommendations/eurl-ecvam-recommendation-on-the-direct-peptide-reactivity-assaydpdra.

EC EURL-ECVAM, 2015. Recommendation on the human Cell Line Activation Test (h-CLAT) for Skin Sensitising Testing. Available at: https://eurl-ecvam.jrc.ec.europa.eu/news/news_docs/eurl-ecvam-recommendation-on-the-human-cell-line-activation-test-h-clat-for-skin-sensitisation-testing.

ECHA, 2016. Guidance on Information Requirements and Chemical Safety Assessment. Chapter R.7a: Endpoint Specific Guidance. Version 5.0. Under revision. http://echa.europa.eu/documents/10162/13632/information_requirements_r7aen.pdf.

Edwards, S.W., Tan, Y.M., Villeneuve, D.L., Meek, M.E., McQueen, C.A., 2016. Adverse outcome pathways-organizing toxicological information to improve decision making. *J. Pharmacol. Exp. Ther.* 356 (1), 170–181. <http://dx.doi.org/10.1124/jpet.115.228239>.

Emter, R., Ellis, G., Natsch, A., 2010. Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers *in vitro*. *Toxicol. Appl. Pharmacol.* 245, 281–290.

Foussereau, J., Samsoen, M., Hecht, M.T., 1983. Occupational dermatitis to Ampholyt G in hospital personnel. *Contact Dermat.* 9, 233–234.

Gerberick, G.F., Vassallo, J.D., Bailey, R.E., Chaney, J.G., Morrall, S.W., Lepoittevin, J.P., 2004. Development of a peptide reactivity assay for screening contact allergens. *Toxicol. Sci.* 81, 332–343.

Gerberick, G.F., Ryan, C.A., Kern, P.S., Schlatter, H., Dearman, R.J., Kimber, I., Patlewicz, G.Y., Basketter, D.A., 2005. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis* 16 (4), 157–202.

Gerberick, G.F., Vassallo, J.D., Foertsch, L.M., Price, B.B., Chaney, J.G., Lepoittevin, J.P., 2007. Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. *Toxicol. Sci.* 97, 417–427.

- Goebel, C., Aeby, P., Ade, N., Alépée, N., Aptula, A., Araki, D., Dufour, E., Gilmour, N., Hibatallah, J., Keller, D., Kern, P., Kirst, A., Marrec-Fairley, M., Maxwell, G., Rowland, J., Safford, B., Schellau, F., Schepky, A., Seaman, C., Teichert, T., Tessier, N., Teissier, S., Weltzien, H.U., Winkler, P., Scheel, J., 2012. Guiding principles for the implementation of non-animal safety assessment approaches for cosmetics: skin sensitisation. *Regul. Toxicol. Pharmacol.* 263 (1), 40–52. <http://dx.doi.org/10.1016/j.yrtph.2012.02.007>.
- Hagvall, L., Backtorp, C., Svensson, S., Nyman, G., Borje, A., Karlberg, A.T., 2007. Fragrance compound geraniol forms contact allergens on air exposure. Identification and quantification of oxidation products and effect on skin sensitization. *Chem. Res. Toxicol.* 20, 807–814.
- Hagvall, L., Baron, J.M., Börje, A., Weidolf, L., Merk, H., Karlberg, A.T., 2008. Cytochrome P450-mediated activation of the fragrance compound geraniol forms potent contact allergens. *Toxicol. Appl. Pharmacol.* 233, 308–313.
- Karlberg, A.T., Bergström, M.A., Börje, A., Luthman, K., Nilsson, J.L., 2008. Allergic contact dermatitis—formation, structural requirements, and reactivity of skin sensitizers. *Chem. Res. Toxicol.* 21, 53–69.
- Karlberg, A.T., Börje, A., Duus Johansen, J., Liden, C., Rastogi, S., Roberts, D., Uter, W., White, I.R., 2013. Activation of non-sensitizing or low-sensitizing fragrance substances into potent sensitizers – prehapten and prohaptens. *Contact Dermat.* 69, 323–334.
- Kern, P.S., Gerberick, G.F., Ryan, C.A., Kimber, I., Aptula, A., Basketter, D.A., 2010. Historical local lymph node data for the evaluation of skin sensitization alternatives: a second compilation. *Dermatitis* 21, 8–32.
- Kleinstreuer, N.C., Sullivan, K., Allen, D., Edwards, S., Mendrick, D.L., Embry, M., Matheson, J., Rowlands, J.C., Munn, S., Maull, E., Casey, W., 2016. Adverse outcome pathways: from research to regulation scientific workshop report. *Regul. Toxicol. Pharmacol.* 76, 39–50. <http://dx.doi.org/10.1016/j.yrtph.2016.01.007>.
- Lepoittevin, J.-P., 2006. Metabolism versus chemical transformation or proverbs prehapten? *Contact Dermat.* 54, 73–74.
- Moss, E., Debeuckelaere, C., Berl, V., Elbayed, K., Moussellieh, F.-M., Namer, I.-J., Lepoittevin, J.P., 2016. In situ metabolism of cinnamyl alcohol in reconstructed human epidermis: new insights into the activation of this fragrance skin sensitizer. *Chem. Res. Toxicol.* 29, 1172–1178.
- National Academies of Sciences, 2015. Application of Modern Toxicology Approaches for Predicting Acute Toxicity for Chemical Defense. <http://www.nap.edu/catalog/21775/application-of-modern-toxicology-approaches-for-predicting-acute-toxicity-for-chemical-defense>.
- Natsch, A., Emter, R., 2008. Skin sensitizers induce antioxidant response element dependent genes: application to the in vitro testing of the sensitization potential of chemicals. *Toxicol. Sci.* 102 (1), 110–119.
- OECD, 2012. The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins Part 1: Scientific Evidence. Series on Testing and Assessment No. 168 ENV/JM/MONO(2012)10/PART1.
- OECD, 2015a. Organisation for Economic Cooperation and Development. Test Guideline 442C: in Chemico, Skin Sensitisation, Direct Peptide Reactivity Assay (DPRA). http://www.oecd-ilibrary.org/environment/test-no-442c-in-chemicoskin-sensitisation_9789264229709-en.
- OECD, 2015b. Organisation for Economic Cooperation and Development. Test Guideline 442D: in Vitro Skin Sensitisation, ARE-Nrf2 Luciferase Test Method. <http://www.oecd-ilibrary.org/content/book/9789264229822-en>.
- Patlewicz, G., Dimitrov, S.D., Low, L.K., Kern, P.S., Dimitrova, G.D., Comber, M.I., Aptula, A.O., Phillips, R.D., Niemelä, J., Madsen, C., Wedebye, E.B., Roberts, D.W., Bailey, P.T., Mekenyan, O.G., 2007. TIMES-SS—a promising tool for the assessment of skin sensitization hazard. A characterization with respect to the OECD validation principles for (Q)SARs and an external evaluation for predictivity. *Regul. Toxicol. Pharmacol.* 48, 225–239.
- Patlewicz, G., Roberts, D.W., Uriarte, E., 2008. A comparison of reactivity schemes for the prediction skin sensitization potential. *Chem. Res. Toxicol.* 21 (2), 521–541.
- Patlewicz, G., Kuseva, C., Mehmed, A., Popova, Y., Dimitrova, G., Ellis, G., Hunziker, R., Kern, P., Low, L., Ringeissen, S., Roberts, D.W., Mekenyan, O., 2014. TIMES-SS—recent refinements resulting from an industrial skin sensitisation consortium. *SAR QSAR Environ. Res.* 25, 367–391.
- Roberts, D.W., Aptula, A.O., 2008. Determinants of skin sensitisation potential. *J. Appl. Toxicol.* 28 (3), 377–387.
- Roberts, D.W., Patlewicz, G., Kern, P.S., Gerberick, F., Kimber, I., Dearman, R.J., Ryan, C.A., Basketter, D.A., Aptula, A.O., 2007a. Mechanistic applicability domain classification of a local lymph node assay dataset for skin sensitization. *Chem. Res. Toxicol.* 20 (7), 1019–1030.
- Roberts, D.W., Aptula, A.O., Patlewicz, G., 2007b. Electrophilic chemistry related to skin sensitization. Reaction mechanistic applicability domain classification for a published data set of 106 chemicals tested in the mouse local lymph node assay. *Chem. Res. Toxicol.* 20, 44–60.
- Rudbäck, J., Bergström, M.A., Börje, A., Nilsson, U., Karlberg, A.T., 2012. alpha-Terpinene, an antioxidant in tea tree oil, autoxidizes rapidly to skin allergens on air exposure. *Chem. Res. Toxicol.* 25, 713–721.
- Smith, C.K., Hotchkiss, S.A.M., 2001. Allergic Contact Dermatitis – Chemical and Metabolic Mechanisms. Taylor and Francis, London and New York.
- Urbisch, D., Becker, M., Honarvar, N., Kolle, S.N., Mehling, A., Teubner, W., Wareing, B., Landsiedel, R., 2016. Assessment of pre- and pro-haptens using nonanimal test methods for skin sensitization. *Chem. Res. Toxicol.* 29 (5), 901–913. <http://dx.doi.org/10.1021/acs.chemrestox.6b00055>.
- Wiegand, C., Hewitt, N.J., Merk, H.F., Reisinger, K., 2014. Dermal xenobiotic metabolism: a comparison between native human skin, four in vitro skin test systems and a liver system. *Skin. Pharmacol. Physiol.* 27 (5), 263–275. <http://dx.doi.org/10.1159/000358272>.
- Zuang, V., Desprez, B., Barroso, J., Belz, S., Berggren, E., Bernasconi, C., Bessems, J., Bopp, S., Casati, S., Coecke, S., Corvi, R., Dumont, C., Varvara Gouliarmou, V., Griesinger, C., Halder, M., Janusch-Roi, A., Kienzler, A., Landesmann, B., Madia, F., Milcamps, A., Munn, S., Price, A., Prieto, P., Schäffer, M., Triebe, J., Wittwehr, C., Worth, A., Whelan, M., 2015. EURL ECVAM Status Report on the Development, Validation and Regulatory Acceptance of Alternative Methods and Approaches. EUR 27474 EN Available at: <http://publications.jrc.ec.europa.eu/repository/bitstream/JRC97811/ibna27474enn.pdf>.