



REACH alternative testing strategy for skin sensitization in practice: Fact or fiction?

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ABSTRACT

From October 2016 the REACH Regulation requires an alternative testing strategy for skin sensitization. The current paper describes our experience when putting into practice the REACH alternative testing strategy with a modification for 50 industrial chemicals in total, including mono-constituents, multi-constituents and UVCBs. For mono- and multi-constituents, a tiered approach was followed starting with an *in silico* (Derek Nexus) assessment, DPRA and KeratinoSens™ assay, followed by a weight of evidence conclusion based on the generated data, or further testing using the U-SENS™ assay. For UVCBs testing started with the KeratinoSens™ assay followed by the U-SENS™ assay if additional relevant information could be gained for an overall conclusion. From the 50 substances tested, for 46% a conclusion on skin sensitization potential and potency could be drawn based on the non-animal testing strategy; however, 54% of the substances still needed to be studied *in vivo* due to discordant results from non-animal testing or the need for reliable potency data. Important issues with the currently available, validated non-animal methods are the lack or comparability of skin metabolism and lack of potency indication, which is present in the *in vivo* assays. Skin sensitization testing for UVCBs and multi-constituents is still in a grey area, as neither the *in chemico*, *in vitro* assays, and *in vivo* LLNA have been validated for UVCBs and multi-constituents.

1. Introduction

With the public opinion against animal testing being more demanding than ever, and the legal obligation in the EU to avoid animal testing (EU, 2010), the European Commission introduced obligatory *in chemico* and *in vitro* testing for the endpoint skin sensitization of chemicals by October 2016 (EU Com, 2016). This regulation was repealed by Commission Regulation (2017)/706 of 19 April 2017, in which it was stated that *in vivo* testing had still been possible until 10 May 2017 (EU Com, 2017). In 2017, ECHA introduced a non-animal testing strategy based on the Adverse Outcome Pathway (AOP) for skin sensitization (ECHA, 2017a). The AOP consists of four key events (KE): covalent binding to skin proteins (KE-1 or molecular initiating event), activation of keratinocytes (KE-2), maturation of dendritic cells (KE-3) and activation and proliferation of T-cells (KE-4). Only for the first three key events were one or more validated and OECD-accepted non-animal tests available: the Direct-Peptide Reactivity Assay (DPRA; OECD 442C) for KE-1, the KeratinoSens™ and LuSens™ assay (OECD 442D) for KE-2, the human Cell Line Activation test (h-CLAT), the U937 cell line activation test (U-SENS™) and the Interleukin-8 receptor

gene assay (IL-8 Luc) – all described in OECD 442E – for KE-3. At Charles River Laboratories Den Bosch the DPRA was validated for KE-1, the KeratinoSens™ assay for KE-2 and the U-SENS™ assay for KE-3.

ECHA mentioned explicitly that each single *in chemico/in vitro* assay of the AOP was not considered sufficient to serve as a stand-alone method to discriminate between skin sensitizers and non-sensitizers, especially if negative results are obtained (ECHA, 2017a). Therefore, several testing strategies were developed as presented in Annex I of OECD guidance document 256 (OECD, 2016). According to REACH (EU Com, 2017) an assay for each key event 1, 2 and 3 is required to complete the endpoint skin sensitization. REACH also states: “If information from test method(s) addressing one or two of the key events ... already allows classification and risk assessment according to point 8.3, studies addressing the other key event(s) need not be conducted.” ECHA stated that for the skin sensitization endpoint “the current understanding is that covering different key events by *in in chemico/in vitro/in silico* or other data provides the best predictivity for the endpoint” “taking into consideration the limitations of each assay” (ECHA, 2017a).

According to the REACH update presented in Regulation (2016)/

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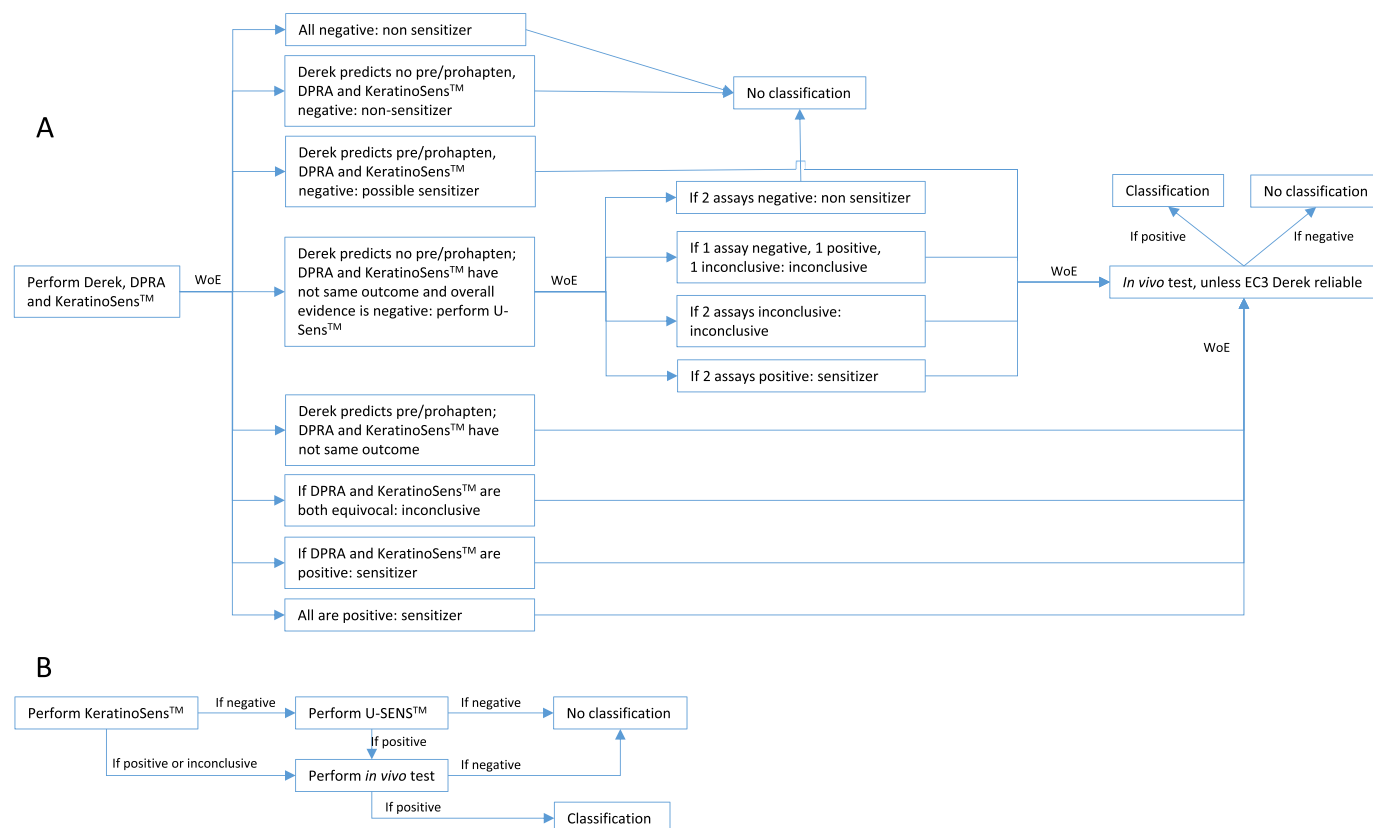


Fig. 1. Proposed test strategy for A) organic mono/multi-constituents and for B) UVCBs/metal containing substances (Weight of Evidence (WoE) indicates a decision to be made based on alternative testing).

1688 for the endpoint skin sensitization sufficient information has to be provided to be able to conclude whether a substance is a skin sensitizer, but also whether it can be presumed to have the potential to produce significant sensitization in humans (category 1A of the CLP Regulation (EU, 2008)). As the current OECD-accepted non-animal methods for skin sensitization lack information on potency, the alternative testing cannot provide a reliable conclusion on the classification, i.e. sensitization potency, in case the substance is a skin sensitizer, and further – *in vivo* – testing should be performed. It should be noted that the CLP Regulation also requires that skin sensitizers should be allocated to a subcategory (1A or 1B), but only *whenever possible*. In the case that the data are not sufficient for sub-categorization, the substance can be classified in the *overall category 1* and no *in vivo* testing is needed to determine the potency – contrary to REACH.

This paper describes how a Contract Research Organisation dealt with the immediate implementation of the alternative strategy for skin sensitization under REACH. The aim of our work is to evaluate whether implementation of a non-animal testing strategy for skin sensitization prevented the need for animal-based test results and the applicability of alternative testing for the range of substances (mono-constituent to UVCBs, soluble to non-soluble, etc.) encountered at our Contract Research Organisation. A defined approach is presented, which uses a combination of information obtained from an *in silico* method, Derek Nexus (Derek), and results from *in chemico/in vitro* assays to determine the potential for skin sensitization. As well as a qualitative prediction on whether the substance is a skin sensitizer, Derek may also predict the EC3 (estimated concentration needed to produce a stimulation index of 3). This prediction of potency may replace animal testing needed for determination of potency. Derek also may provide information on the contribution of metabolites to skin sensitization, which contributes to the overall weight of evidence and the final conclusion on skin sensitization potential.

Determination of skin sensitization properties for UVCBs is more challenging than for mono-constituents. For UVCB's Derek assessment is not possible normally due to the sheer number of constituents to be assessed. The DPRA cannot be performed since the ratio between peptide and the constituents is unknown (OECD, 2015a). Therefore, for UVCBs a different strategy is applied in which the compound is first tested in the KeratinoSens™ assay followed by the U-SENS™ assay when relevant.

2. Materials and methods

2.1. Selection of substances

Substances tested using the alternative non-animal testing strategy effective from 11 October 2016 were considered (EU com, 2016) for which Charles River also executed the weight of evidence report. Mono-, multi-constituents and UVCBs were included. A dataset comprising test data from 50 industrial chemicals from various sponsors was used; the results are proprietary to these sponsors. The substances ranged in molecular weight between 135 and 797, and a log Pow (partition coefficient octanol/water) between –3.1 and > 8.

2.2. Defined approach

Based on the new legal requirements under REACH, Charles River Laboratories Den Bosch developed a new defined approach to obtain information on the human hazard endpoint skin sensitization. This approach consists of an integrated testing strategy using tiered non-animal testing followed by a weight of evidence approach (WoE). Before initiating the strategy according to Commission Regulation (2017)/706 (EC Com, 2017), substances were assessed on eligibility for *in silico/in chemico/in vitro* testing by reviewing the available

information on the nature of the substance, i.e. whether the substance was a mono- or multi-constituent or UVCB, organic or inorganic or containing a metal, and physico-chemical properties, such as solubility in water and organic solvents. In case no reliable, quantitative data on solubility was available, a solubility test was performed to determine whether the substance dissolved sufficiently in a solvent appropriate for the test method. The partition coefficient octanol/water was not available at the start of testing for any substance. The tiered testing strategy consisted of an *in silico* assessment using Derek combined with the *in chemico* method, the Direct Peptide Reactivity Assay (DPRA), and the *in vitro* assays, ARE-Nrf2 luciferase KeratinoSens™ and U937 cell line activation test (U-SENS™), each addressing individual key events in the skin sensitization AOP. Dependent on the nature of the substance one of the following tiered testing strategies was followed (see Fig. 1 and paragraph 2.6 for more detail):

1. Organic mono- or multi-constituents were initially assessed in a Derek assessment, the DPRA and KeratinoSens™ assay. If a conclusion on the potential and potency for skin sensitization could be drawn from these studies, no further testing was initiated. In the absence of a definite conclusion on the potential and/or potency for skin sensitization, either the U-SENS™ assay was performed if it was considered to yield reliable additional information, or an animal study was proposed to provide a conclusion on potential or potency.
2. For UVCB's a Derek assessment is not feasible due to the sheer number of constituents to be assessed or the presence of unknown constituents. Also, generally a DPRA assay is not feasible for most UVCB's as no apparent (mean) molecular weight can be determined. Hence, UVCBs were initially tested in a KeratinoSens™ assay, after which the need for a U-SENS™ assay or *in vivo* test was decided upon.

A standard protocol was followed for the *in silico* assessment, which was not performed under GLP (OECD Principles of Good Laboratory Practice). Each *in chemico/in vitro* assay described in the next paragraphs was performed following its dedicated standard protocol under GLP according to the appropriate OECD guideline. Proficiency of all assays was shown prior to application.

2.3. *In silico* assessment

Derek is a knowledge-based Structure-Activity Relationship (SAR) program able to identify potential skin sensitizers and to indicate the sensitizer's potency level by calculating an EC3 value from the Lhasa EC3 dataset containing a mixture of animal and human data (Lhasa, 2019). For the *in silico* evaluation Derek Nexus versions 5.02v to 6.01v (Lhasa Limited, UK) were used. All predictions were run with the following settings: knowledge base = Derek 2015 2.0 and 2018 1.1, species = mammal, endpoint = skin sensitization, reasoning level ≥ equivocal, perceive mixtures = on, perceive tautomers = on, match alerts without rules = off. Derek predictions that yielded one of the 90 alerts for skin sensitization were classified as positive. Derek also contains an expert-derived functionality that can provide a validated negative prediction for skin sensitization (Chilton et al., 2018). This functionality further evaluates those substances which do not yield any skin sensitization alerts in Derek by comparing the query substance to a Lhasa reference set of skin sensitization data. The model is able to identify structural features that resulted in a lower accuracy or a higher uncertainty in the negative prediction, termed misclassified and unclassified, respectively.

If a substance is predicted to be a skin sensitizer, its potency is predicted by calculating an EC3 value based on experimental data from the closest structurally-related substances (Canipa et al., 2017).

A Derek prediction was generally not applied to UVCB substances or inorganic substances. UVCB substances have a variable or unknown molecular structure with too many constituents to make a Derek

assessment of all constituents workable. For inorganic substances, Derek has alerts, but not all metals are present in its negative reference data set. In a number of Derek alerts metabolism or auto-oxidation is taken into account.

2.4. *In chemico* assessment

The Direct Reactivity Peptide Assay (DPRA) addresses key event 1 of the AOP and models the reactivity of a substance towards endogenous skin proteins. In the DPRA the formation of covalent interactions between the chemical and a cysteine or lysine situated in the heptapeptide Ac-RFAA(C/K)AA-COOH is monitored. The DPRA was performed according to OECD test guideline 442C applicable at the time of testing (OECD, 2015a) and OECD Principles of Good Laboratory Practice (GLP). Solubility of the test substance in an appropriate solvent was assessed before performing the DPRA. Test substances were incubated with peptide in triplicate. After a 24-h incubation, depletion of the peptide was measured with an HPLC-PDA system equipped with a Zorbax SB-C18, 100 mm × 2.1 mm, df = 3.5 µm column (Agilent Technologies, Santa Clara, CA, USA) and a Surveyor PDA detector (Thermo Scientific). Peptides were detected at 220 nm and 258 nm. Peptide depletion values were calculated in percentages and either used in the cysteine 1:10/lysine 1:50 prediction model or, if co-elution of the test substance with lysine occurred, the cysteine 1:10 model. These models allow assigning the test item to one out of four reactivity classes (no or minimal, low, moderate or high reactivity) to discriminate between sensitizers and non-sensitizers. The test results are positive if the mean percentage of cysteine and lysine depletion is higher than 6.38% (higher than 13.89% for the cysteine model). Prior to and after HPLC analysis, the test substance and co-elution control samples were visually inspected for precipitation, turbidity or phase separation. If precipitation or phase separation occurred, a negative result was considered to be less reliable as it was not clear which amount of test substance had gone into solution and was able to react with the peptide. However, the result was still considered negative. The DPRA was not performed with substances for which no accurate molecular weight could be defined (i.e. UVCBs), as no defined molecular ratio of test chemical and peptide can be determined. The DPRA is also not applicable to inorganic substances or substances containing metals, as metals are known to react with proteins by mechanisms other than covalent binding. The outcome of the DPRA may be positive, negative or inconclusive.

2.5. *In vitro* assessment

2.5.1. KeratinoSens™

The KeratinoSens™ assay can identify activation of the Nrf2 pathway, central to key event 2, resulting in inflammation of keratinocytes. Nrf2 pathway activation in the KeratinoSens™ cell line results in luciferase expression that can be easily quantified and indicates potential skin sensitizers. The KeratinoSens™ assay was performed according to OECD test guideline 442D applicable at the time of testing (OECD, 2015b) and GLP. The substances were incubated for 48 h with the KeratinoSens™ cell line (Givaudan Duebendorf, Switzerland) in two independent experiments, or in the case of discordant results in three independent experiments. Luciferase expression (Steady-Glo Luciferase, Promega, The Netherlands) was measured with a plate reader (TECAN Infinite® M200 Pro). Cell viability was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CAS 298-93-1). The KeratinoSens™ assay was classified as positive if for 2 out of 2 or 2 out of 3 repetitions the luciferase expression increased > 1.5 fold at a cellular viability > 70% at concentrations lower than 1000 µM (or < 200 µg/mL for test substances with no defined molecular weight) with an apparent overall dose-response for luciferase induction. The KeratinoSens™ assay was deemed inconclusive if negative results were obtained with test substance concentrations below 1000 µM. Otherwise the KeratinoSens™ prediction is considered negative.

Table 1
Results of individual assays, the overall outcome based on *in silico/in chemico/in vitro* testing and the outcome of the *in vivo* study if considered needed.

Substance number	Class	Substance type	MW range ^a	Log Pow range ^b	Derek ^c	DPRA ^d	KeratinoSens nd		U-SENS TM		Overall outcome ^m	LLNA ^e
							Individual runs	Result	Individual runs	Result		
1	organic	Mono-constituent	50–500	1 < x ≤ 4	Negative for fluorinated substance; non-fluorinated: alkyl aldehyde precursor; EC3 > 2%; pre/prohaptent	Positive; high	P,N,P	Positive	–	–	Positive	–
2	organic	Mono-constituent	> 700	x > 1	Positive; quaternary ammonium salt, EC3 na	Negative	I,I (cytotox)	Inconclusive	–	–	Negative	- (corrosive)
3	organic	Mono-constituent	50–500	x ≤ 0	Negative	Negative	N,N	Negative	–	–	Negative	–
4	organic	Mono-constituent	50–500	x > 6	Negative	Negative	P,N,P	Positive	P,P	Positive	Positive	Positive
5	organic	Mono-constituent	50–500	x ≤ 0	Negative	Negative	N,N	Negative	–	–	Negative	–
6	organic	Mono-constituent	50–500	1 < x ≤ 4	Positive; thiourea, EC3 na; thiol/thiol exchange agent, EC3 > 2%; pre/prohaptent	Negative	I,P,I (cytotox)	Inconclusive	–	^h	Inconclusive	Negative ^G
7	organic	Mono-constituent	50–500	1 < x ≤ 4	Positive; 1,2-dihydroxybenzene or derivative, EC3 ≤ 2%; pre/prohaptent	Negative	N,N	Negative	P,P	Positive ⁱ	Positive	-(EC3 DEREK used)
8	organic	Mono-constituent	50–500	x ≤ 0	Positive; Thiol/thiol exchange agent, EC3 > 2%	Positive; high	I,P,P	Positive	–	–	Positive	-(EC3 DEREK used)
9	metal-salt of organic	UVCB	50–500	4 < x ≤ 6	–	–	P,P	Positive	–	–	Inconclusive	Negative
10	metal-salt of organic	UVCB	> 700	4 < x ≤ 6	–	–	I,I (cytotox)	Inconclusive	–	–	Inconclusive	Positive
11	organic (sodium salt)	Mono-constituent	50–500	x ≤ 0	Positive; aromatic azo compound, EC ≤ 2%	Positive; high	P,I,I (cytotox)	Inconclusive	–	–	Inconclusive	Negative
12	metal-salt of organic	Mono-constituent	50–500	x ≤ 0	^f	^f	I,I (cytotox)	Inconclusive	–	–	Inconclusive	Negative
13	organic	UVCB	50–700	0 < x ≤ 6	–	–	N,P,I (cytotox)	Inconclusive	–	–	Inconclusive	Negative ^G
14	organic	Mono-constituent	50–500	0 < x ≤ 1	Negative	Negative	P,P	Positive	P,P	Positive	Positive	Negative
15	organic	Mono-constituent	50–500	1 < x ≤ 4	Negative	Positive; low (cysteine)	N,P,N (–cytotox)	Negative	I,P,P	Positive	Positive	Positive
16	organic	Mono-constituent	50–500	1 < x ≤ 4	Negative	Negative	P,P	Positive	P,I,P	Positive	Positive	Negative
17	organic	Mono-constituent	50–500	x ≤ 0	Negative	Negative	N,P,P	Positive	I,P,N,P	Positive	Positive	Negative
18	organic (sodium salt)	Mono-constituent	50–500	0 < x ≤ 1	Negative	Inconclusive (cysteine co-elution)	N,N	Negative	N,N (precipitate)	Positive	Negative	–
19	organic	Mono-constituent	50–500	x ≤ 0	Positive; aromatic primary/secondary amine, EC3 > 2%; pre/prohaptent	Negative	N,N	Negative	–	^h	Negative	Negative
20	organic	Mono-constituent	50–500	0 < x ≤ 4	Negative	Negative	N,N	Negative	–	–	Negative	–
21	organic	Mono-constituent	50–500	x ≤ 1	Negative	Positive; moderate	N,N	Negative	N,P,N	Negative	Negative	–
22	organic	Mono-constituent	> 700	4 < x ≤ 6	Positive; α,β-unsaturated amide, EC3 na; enol ether, EC3 > 2%; pre/prohaptent	Positive; high	P,P	Positive	–	–	Positive	Negative

(continued on next page)

Table 1 (continued)

Substance number	Class	Substance type	MW range ^a	Log Pow range ^b	Derek ^c	DPRA ^d	KeratinSens nd		U-SENS TM		Overall outcome ^m	LLNA ^e
							Individual runs	Result	Individual runs	Result		
23	organic	Mono-constituent	> 700	1 < x ≤ 4	Positive; α,β-unsaturated amide, EC3 na; enol ether, EC3 > 2%; pre/prohaptan	Positive; high	P,P	Positive	–	–	Positive	Negative
24	organic	UVCB	50–500	x ≤ 0	–	Negative ^c	N,N	Negative	–	–	Negative	–
25	organic	Multi-constituent	500–700	x ≤ 0	–	Negative	N,N	Negative	–	–	Negative	–
26	organic	Multi-constituent	50–500	x ≤ 0	–	Negative	N,N	Negative	–	–	Negative	–
27	organic	UVCB	> 700	x > 6	–	Negative ^c	–	– ^d	–	–	Inconclusive	Negative
28	organic	UVCB	50–500	4 < x ≤ 6	–	–	P,N,P	Positive	–	–	Inconclusive	Positive
29	organic	Mono-constituent	50–500	1 < x ≤ 4	Negative	Positive; moderate	P,P	Positive	–	–	Positive	–(EC3 DEREK used)
30	organic	Mono-constituent	50–500	4 < x ≤ 6	Negative	Negative	P,P	Positive	–	– ^k	Inconclusive	Positive
31	organic	UVCB	50–700	x > 1	–	Positive ^c ; high	P,P	Positive	–	–	Positive	Negative
32	organic	UVCB	50–700	na	–	– ^{c,d}	–	– ^d	–	–	Inconclusive	Negative
33	organic	Mono-constituent	50–500	0 < x ≤ 4	Positive; thiol/thiol exchange agent, EC3 ≤ 2%	Positive; high	P,P	Positive	–	–	Positive	–(EC3 DEREK used)
34	organic (sodium salt)	Mono-constituent	50–500	x ≤ 6	Negative	Negative	N,N	Negative	–	–	Negative	–
35	organic	UVCB	500–700	x > 6	–	–	–	– ^d	–	–	Inconclusive	Negative
36	organic (sodium salt)	Mono-constituent	50–500	x ≤ 0	Negative	Negative	N,N	Negative	–	–	Negative	–
37	organic (sodium salt)	Mono-constituent	50–500	x ≤ 0	Negative	Negative	N,N	Negative	–	–	Negative	–
38	organic	Mono-constituent	50–500	0 < x ≤ 1	Negative	Inconclusive (cysteine co-elution)	N,P,N	Negative	N,N (precipitate)	Positive	Negative	–
39	organic	Mono-constituent	50–500	1 < x ≤ 4	Positive (equivocal); substituted phenol, EC3 ≤ 2%; pre/prohaptan	Negative	P,P	Positive	–	–	Inconclusive	Negative
40	organic	Mono-constituent	50–500	x ≤ 0	Negative	Positive; no class (co-elution with cysteine (minor) and lysine)	N,N	Negative	P,P	Positive	Positive	Negative
41	organic	Mono-constituent	50–500	1 < x ≤ 4	Positive (equivocal); substituted phenol, EC3 ≤ 2%; pre/prohaptan	Inconclusive; not tested up to 100 mM due to insolubility	P,P	Positive	–	–	Inconclusive	Negative
42	organic (sodium salt)	Mono-constituent	50–500	x ≤ 0	Negative	Negative	N,N	Negative	–	–	Negative	–
43	organic (sodium salt)	UVCB	50–500	x ≤ 4	–	Negative ^c	N,N	Negative	–	–	Negative	–
44	organic	Mono-constituent	> 700	4 < x ≤ 6	Positive; phenyl ester, EC3 > 2%; α,β-unsaturated ester, EC3 > 2%	Positive; high	P,P	Positive	–	–	Positive	Negative
45	organic	Multi-constituent	50–500	x > 6	Negative	Negative	N,P,P	Positive ⁿ	–	–	Negative	–
46	organic	UVCB	> 700	0 < x ≤ 6	–	–	P,P	Positive	–	–	Inconclusive	Needed, but not performed

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Table 1 (continued)

Substance number	Class	Substance type	MW range ^a	Log Pow range ^b	Derek ^c	KeratoSens nd		U-SENS TM		Overall outcome ^m	LLNA ^e
						Individual runs	Result	Individual runs	Result		
47	inorganic	Mono-constituent	50–500	na	–	N,P,P	Positive	–	–	Positive	– (high reactivity DPRA used)
48	organic	Mono-constituent	50–500	na	Negative (substructure outside domain)	P,P	Positive	–	–	Positive	Negative
49	organic	Multi-constituent	50–500	4 < x ≤ 6	Positive; isocyanate, EC3 ≤ 2%	P,P	Positive	–	–	Positive	– (EC3 DEREK used)
50	organic	Mono-constituent	50–500	1 < x ≤ 4	Negative	N,N	Negative	–	–	Negative	–

N = negative; P = positive; I = inconclusive; – = not performed.

^a Molecular weight was within one of the following ranges: 50–500, 501–700 or > 700 g/mol; if molecular weights for a UVCB were part of two ranges, the combined range is presented.

^b Log Pow was not available at start of testing, but included afterwards; Log Pow was within one of the following ranges: x ≤ 0; 0 < x ≤ 1; 1 < x ≤ 4; 4 < x ≤ 6; x > 6; if log Pow of a UVCB were part of two ranges, the combined range is presented.

^c Although the owner of the substance indicated the substance to be a UVCB, an apparent molecular weight was available.

^d Insoluble in standard solvent from OECD guideline and therefore assay not performed.

^e The standard *in vivo* study executed was an LLNA, except for the entries with “G” a GPMT was performed as the LLNA was not considered appropriate for that substance; in case a reliable (expert judgment) EC3 value was predicted by DEREK, the classification was based on this value.

^f Metal and therefore not suited for the Derek (specific metals not included in negative reference set) or DPRA assay.

^g If Derek was positive, the alert is presented here; the EC3 predicted by Derek is given if available; na = not available.

^h According to Derek metabolism is needed before skin sensitization can take place, so DPRA and KeratoSensTM may be false negative.

ⁱ U-SENSTM assay should not have been performed as the DPRA and KeratoSensTM assay may have been false negative and one positive assay is not conclusive for skin sensitization potential and potency.

^j Negative/positive according to OECD 442C cysteine/lysine model; cysteine model was used, if stated “cysteine”; for positive results the low, moderate and high reactivity according to OECD 442C has been included.

^k Although Derek was negative, the substance was not stable in water forming a precursor of an aldehyde; consequently a U-SENSTM assay, irrespective if negative or positive, would not have been conclusive on skin sensitization potential and potency.

^l KeratoSensTM assay was inconclusive if not tested up to required concentration according to the old OECD 442D (2015); cytotox = cytotoxicity was observed; @cytotox = only positive at cytotoxic concentration.

^m If only one assay performed, outcome is inconclusive.

ⁿ Only next to cytotoxic concentration.

2.5.2. U-SENS™

The U-SENS™ assay quantifies the change in expression levels of cell surface markers CD86. An increase in CD86 is associated with activation of dendritic cells thus addressing the third key event in the AOP. The U-SENS™ was performed according to OECD test guideline 442E (OECD, 2017) and GLP. U937 human monocytes acquired from ATCC (American Type Culture Collection, Virginia, USA) were exposed to the test substance for 45 ± 3 h in two or three independent runs. Fluorescein isothiocyanate-conjugated antibodies were used for staining of the cells. The acquisition was performed with a BD FACSCanto flow cytometer and further analyzed with BD FACSDiva software. The U-SENS™ assay is regarded as negative if two independent runs result in a stimulation index (S.I.) of CD86 lower than 150% with a cell viability $\geq 70\%$. The U-SENS™ assay is regarded as positive if two independent runs result in a S.I. of CD86 higher or equal to 150% with a cell viability $\geq 70\%$. However, if, in the first run, the S.I. of CD86 is higher or equal to 150% at the highest non-cytotoxic concentration only, the run is not conclusive, and additional concentrations (between the highest non cytotoxic concentration and the lowest cytotoxic concentration) were tested in at least two additional runs. If for a substance tested in the U-SENS™ assay precipitation or color interference was observed - but no induction -, the assay was also classified as positive following the guideline.

2.6. Data interpretation procedure (DIP)

According to ECHA, none of the *in chemico/in vitro* assays was considered to serve as a stand-alone method to predict skin sensitization. The DIP applied here used the outcome of each of the assessment/assays performed as defined by the testing strategy developed, which is presented in Fig. 1. The outcomes were combined according to the following principles:

2.6.1. For mono-/multi-constituent substances

- In case of a concordant prediction from the DPRA and KeratinoSens™ assay, it was deemed sufficient to classify the substance accordingly, i.e. in the case of two positive results the substance was considered a sensitizer and two negative results indicated a non-sensitizer. If Derek predicted the substance to be a pre- or prohaptan, and the KeratinoSens™ (and DPRA) was negative then no U-SENS™ was performed, as the U-SENS™ cannot give decisive information and *in vivo* testing is needed: a negative outcome of the U-SENS™ may be a false negative and a positive outcome will need data on potency. In the absence of a reliable and acceptable *in vitro* assay for potency prediction, an *in vivo* study was performed for classification purposes according to REACH, if the assays indicated the substance to be a skin sensitizer or were inconclusive and Derek predicted no reliable EC3 value.

- Discordant results between the DPRA and the KeratinoSens™ assay initiated testing in the U-SENS™ assay, unless problems were foreseen (i.e. solubility thresholds, the assay could be expected to be false negative or false positive based on results with the KeratinoSens™) or indications that further testing would not yield a conclusion on skin sensitization properties (see below), which in turn would trigger the need for *in vivo* testing.

2.6.2. For UVCBs and metal-containing substances

Initially, the KeratinoSens™ assay was performed. If the KeratinoSens™ assay was negative, a U-SENS™ assay was performed; if the U-SENS™ assay result was also negative the substance was considered to be a non-sensitizer; if the U-SENS™ assay was positive, *in vivo* testing was initiated. If the KeratinoSens™ was positive, *in vivo* testing was initiated as the U-SENS™ assay could not yield additional, decisive information, and determination of potency was required by REACH.

2.7. In vivo testing

In vivo testing was performed as a last resort. The standard test is the Local Lymph Node Assay (LLNA), which was performed according to OECD 429 (OECD, 2010). In case information was available that the LLNA was not appropriate for the substance, e.g. class of substances is known to yield a false positive result (Kreiling et al., 2008), the guinea pig maximization test (GPMT) was performed according to OECD 406 (OECD, 1992). *In vivo* testing was performed after approval of the Animal Welfare Body of Charles River Laboratories Den Bosch B.V. within the framework project license AVD2360020172866 approved by the Central Authority for Scientific Procedures on Animals (CCD) as required by the Dutch Act on Animal Experimentation (Dec 2014; <https://www.ivd-utrecht.nl/en/legislation-regulations-and-guidelines/experiments-on-animals-act/>).

3. Results

The results from the individual tests and the overall prediction for the 50 substances that were assessed according to our testing strategy are presented in Table 1. Substances are identified by substance class: organic or metal-salt of an organic substance or inorganic substance.

The overall non-animal result was more or less equally distributed between positive (36%), negative (36%) and inconclusive (28%) outcomes (Fig. 2a). For 54% of the substances under investigation *in vivo* testing was still needed to determine the potential and/or potency of skin sensitization (Fig. 2b). Of the 38 mono- and multi-constituent substances tested *in vitro* 17 were positive, 16 negative, and 5 inconclusive due to discordant results of the DPRA and KeratinoSens™ assay or insoluble in solvents indicated in the test guideline of each assay. Ten of the positive mono- and multi-constituents and all inconclusive outcomes had to be tested further *in vivo* to determine potency as no reliable information on potency was available. Of the 11 UVCBs tested, one was predicted positive, two negative and eight inconclusive due to an inconclusive or positive outcome of the KeratinoSens™ assay or the KeratinoSens™ could not be performed due to insolubility in the solvents indicated in the test guideline. The positive and inconclusive UVCBs had to be tested further *in vivo* to determine the potential and/or potency. The only metal-containing substance evaluated was inconclusive due to an inconclusive KeratinoSens™ outcome and also had to be tested *in vivo* to be able to conclude on skin sensitization potential or potency.

4. Discussion

Normally, very little detail on the properties of the substance is available for industrial chemicals when it arrives at a CRO such as Charles River Laboratories, except, for example, the molecular weight or that the substance is a UVCB, multi-constituent or mono-constituent, or the solubility in water and organic solvents (mostly no quantitative data) and/or pH of an aqueous solution (if extreme). If no quantitative data were available on the solubility of the substance in the standard solvents of the assays, a solubility test was performed to reduce the chance of a result from the assay that would not contribute to the conclusion on skin sensitization (e.g. inconclusive or false negative result).

As demonstrated in the results, of the 50 substances tested, 36% (18) were predicted to be negative, 36% (18) positive and 28% (14) were inconclusive following our DIP. Based on non-animal testing for 46% (23 out of 50) of the substances an overall conclusion could be drawn for skin sensitization potential and potency. Unfortunately, for 54% of substances (27 out of 50), the alternative testing strategy did not yield a definite conclusion on the potential and/or potency of the substance, and had to be tested *in vivo* subsequently. For 12 substances determination of the skin sensitization potential was the driver for *in vivo* testing (with inclusion of potency testing), and for 15 substances

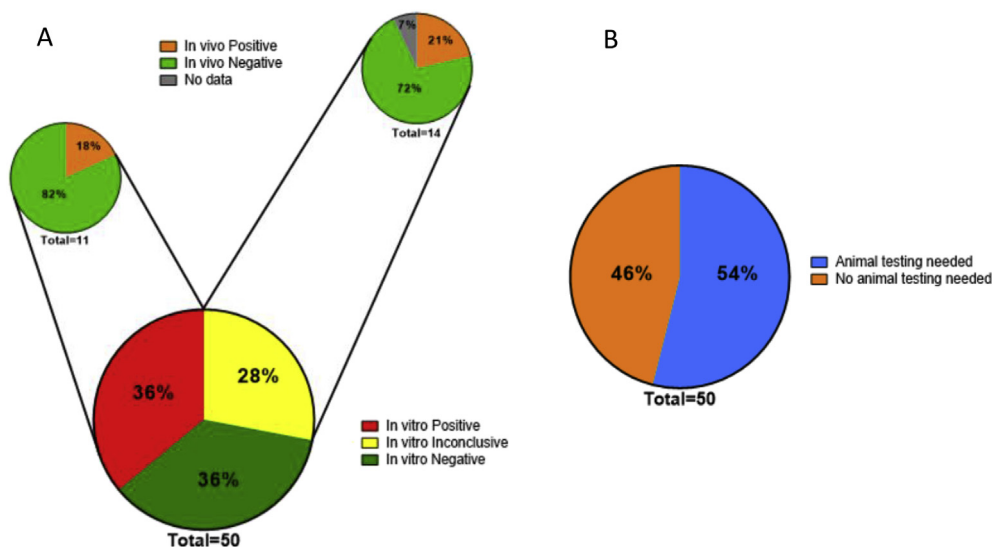


Fig. 2. Overall result from *in silico/in chemico/in vitro* assays and the results from the *in vivo* tested substances (A) and percentage of animal tests needed (B).

solely information on potency was needed. Nine of the twenty seven substances were UVCBs, while seventeen were mono- or multi-constituents and one a metal-containing substance. For UVCBs, in most cases only the KeratinoSens™ assay was executed. As this assay was either positive or inconclusive for most UVCBs, *in vivo* testing was needed for these substances. For mono- or multi-constituents, the general reason for *in vivo* testing was discordant results of the assays performed and, of course, the need for potency data in the case of a positive result.

4.1. Limitations of assays

Although the DPRA, the KeratinoSens™ and U-SENS™ assays have not been validated for multi-constituents and UVCBs (as is the LLNA), the OECD guidelines for these tests do not exclude testing of chemicals that are multi-constituent or UVCB. The DPRA can be performed for multi-constituents of known composition in case an apparent molecular weight can be determined based on the individual molecular weights of each component and their individual proportions, but is not appropriate for UVCBs (OECD, 2015a). In the OECD 442D (OECD, 2015b) and 442E (OECD, 2017) guidelines it is stated what should be reported in case the chemical is a multi-constituent or UVCB, which would imply testing of such a chemical. Therefore, these assays were used if considered appropriate. In a few cases the assays, DPRA, KeratinoSens™ and/or U-SENS™ could not be performed as the substance did not dissolve in any of the standard solvents indicated in the OECD guideline.

The KeratinoSens™ assay yielded an inconclusive result for six substances following the old OECD 442D guideline (OECD, 2015a), as the substances could not be tested up to the required 1000 µM or 200 µg/mL due to cytotoxicity. Following the revised version of OECD 442D (OECD, 2018), these KeratinoSens™ assays with cytotoxicity below 1000 µM or 200 µg/mL and no evidence of a biologically relevant induction are considered negative.

According to REACH the non-animal testing strategy for skin sensitization does not have to be initiated if the substance is corrosive. However, the ECHA guidance R7.a (ECHA, 2017a,b) states on page 304 that “extreme pH values/corrosive properties do not preclude performing *in chemico/in vitro/in vivo* test(s) at suitable concentrations and, therefore, it is possible to assess skin sensitization hazard at sub-corrosive concentrations, if considered necessary”. For one substance (number 2) the alternative strategy was started simultaneously with the skin corrosion test, while the substance turned out to be corrosive. The skin sensitization testing yielded a positive Derek, negative DPRA and

inconclusive KeratinoSens™ outcome, from which no overall conclusion could be drawn. Subsequent performance of the U-SENS™ assay was not considered scientifically justified as a concentration up to 200 µg/mL should be tested and cell viability should be more than 50% in at least four tested concentrations per run. Assuming that the cell viability in the U-SENS™ assay will not differ significantly from that in the KeratinoSens™ assay ($IC_{30} \geq 2.4 \mu M = > 1 \mu g/mL$), the tested concentrations should be lower than 1.2 µg/mL. Since, this is far lower than the required 200 µg/mL, the U-SENS™ would have been considered to be automatically positive due to cytotoxicity. Likewise, it was possible to reject performance of the U-SENS™ right away for some substances in which the DPRA and KeratinoSens™ assay were discordant, as the solvents and medium are exactly the same for the KeratinoSens™ and U-SENS™ assay, and the U-SENS™ assay has to be regarded as positive if a concentration of 200 µg/mL cannot be tested. Hence, performance of the U-SENS™ assay was not considered scientifically justified if precipitation was expected below 200 µg/mL or cytotoxicity well below 200 µg/mL (as cytotoxicity may differ between the cell lines), as results from the U-SENS™ assay would not result in a different scientific overall conclusion. It should be stressed that, in line with the REACH Regulation and the obligation to reduce animal testing for a corrosive substance, in general, an LLNA was not initiated. Moreover, similar risk mitigation measures apply for an industrial-used substance whether it is corrosive (H314) or has skin sensitization properties (H317) according to the CLP Regulation (EU, 2008): such as chemically resistant gloves, chemical goggles and a protective suit (long sleeved clothing) and closed shoes/boots.

For 9 out of the 50 substances it was decided that a U-SENS™ assay could possibly help to determine the skin sensitizing potential. One of these substances was found negative. Of the eight positive results, two were positive as the substance could not be tested at a high enough concentration because of precipitation interference, while the other positive results were all caused by a biologically relevant induction of CD86 and, hence, real positive U-SENS™ results.

4.2. Use of Derek EC3 to avoid animal testing

Use of a reliable EC3 value predicted by Derek could prevent an *in vivo* study in six cases (substance 1, 7, 8, 29, 33, and 49). For the other positive cases for which a Derek assessment was performed, no reliable EC3 was available. Either the EC3 could not be predicted due to an insufficient number of analogues in the database to calculate an EC3 or the prediction of the EC3 was considered unreliable, i.e. data on

analogues consisted of a wide range of potencies for skin sensitization, and the potency and similarity of the analogues did not show a clear correlation resulting in a less reliable EC3 for the substance under investigation.

If Derek predicted the substance to be a pre- or prohapten, the U-SENS™ assay was skipped if results from DPRA and KeratinoSens were negative or did not match. In this case, a negative U-SENS™ could be false negative and a positive U-SENS™ would not yield potency information. Therefore, an *in vivo* assay was initiated.

4.3. Discordance between *in vitro* and *in vivo*

A number of substances showed quite clearly a difference in skin sensitization results between the currently validated *in vitro* assays and *in vivo* testing. The coherence between non-animal testing and *in vivo* testing was not high. Substances 14, 16, 17, 22, 23, 31, 40, 44 and 48 all had a positive outcome based on the alternative strategy, but a negative outcome in the LLNA. Reactivity of the chemical and uptake into/through the stratum corneum, i.e. access to the protein to react with in the molecular initiating event, are both factors that are at the moment regarded to be able to influence skin sensitization (OECD, 2016; ECHA, 2017b; Roberts and Aptula, 2008). Some papers refute the latter by giving examples of chemicals with a MW > 500 or log Pow < 0, which may be indicators for poor uptake into/through the stratum corneum, that are skin sensitizers (Roberts and Patlewicz, 2017; Fitzpatrick et al., 2017a and b).

For substances 14, 16 (methylated 14) and 17 the DPRA was negative. As they contained an alkyl alcohol or amine, which may be oxidized to an aldehyde, the DPRA did not take this into account, while the KeratinoSens™ and/or U-SENS™ assay showed the potential for skin sensitization for these substances. However, apparently insufficient reactivity of the substance was achieved in the LLNA.

For substances 22, 23 and 31 sufficient reactivity seemed to be present in the molecule as Derek, DPRA and KeratinoSens™ were positive; however, absence of reactivity or insufficient uptake into/through the stratum corneum may be the issue in the LLNA, or the LLNA may be false negative. The skin sensitization prediction from the alternative strategy for substance 40 is inconsequent: a positive DPRA indicates sufficient reactivity is present in the molecule, but the KeratinoSens™ assay was negative (detoxification of the substance?), while the U-SENS™ assay confirmed the DPRA. The LLNA was again found to be negative. Although substance 44 contains reactive groups, which were shown to be reactive *in chemico* and *in vitro*, the *in vivo* LLNA was again negative. Substance 48 does have a borole group, which may be reactive enough as shown in the DPRA and KeratinoSens™ assay. However, the substance did not react with/reach the proteins in the LLNA. The following question remains: is the right prediction of human skin sensitization given by the *in vitro* assays or the LLNA? Or in short, which assay is false/true? Human skin metabolism may differ from that of mice and consequently, the LLNA might give an inaccurate result and the *in vitro* data may be correct. However, in the absence of human results no conclusion can be drawn on the relevance of these results for human skin sensitization potential.

4.4. Considerations for the future of non-animal testing for skin sensitization

In public literature as well as in the present study a combination of *in chemico/in vitro* assays has been used to determine whether a substance is a skin sensitizer in humans (Kreiling et al., 2017; Otsubo et al., 2017; Annex I of OECD, 2016; Takenouchi et al., 2015). Most strategies in the literature compare the prediction from *in vitro* data with the result from the LLNA, only a few compare it to human data for the simple reason that no reliable human data is available for most substances. However, human skin sensitization is the endpoint to predict and a number of strategies have reported comparability to human data. Most

strategies (in the literature and in the present study) include performance of the DPRA, which is the first key event in the AOP, provided the substance can penetrate the skin. Following the principle of the AOP the DPRA is crucial for skin sensitization; if no binding to proteins occurs, further key events, i.e. activation of keratinocytes and dendritic cells cannot take place. Compared to human data, the DPRA can predict sensitizers with an accuracy of about 84% (Otsubo et al., 2017; Urbisch et al., 2016), and the LLNA is able to predict human data with a comparable accuracy of about 80–90% (Anderson et al., 2011; Roberts, 2018a). The KeratinoSens™ or the h-CLAT assay predict human data with about 79% and 83.5%, respectively (Otsubo et al., 2017), which indicates they have a similar predictive capacity as the DPRA. A binary test battery of DPRA and h-CLAT or DPRA and KeratinoSens™ did not increase the accuracy significantly compared to the DPRA alone, nor did a 3 out of 3 approach for prediction of human skin sensitization (Otsubo et al., 2017). Roberts and Patlewicz (2017, 2018b) came to the same conclusion with regard to the DPRA, h-CLAT and KeratinoSens™ assay and any combination of these (including 2 out of 3) against the LLNA assay. It should be mentioned that the substances tested in the studies present in the literature are all mono-constituents, while in the current paper also multi-constituents and UVCBs were tested.

Both *in chemico/in vitro* assays and the LLNA assay are models for human exposure and both have their limitations. Although the *in chemico/in vitro* assays each have a lower accuracy than the LLNA for prediction of human skin sensitization, confirmation of the first result by a second non-animal assay does not lead to a significantly more reliable result (accuracies do not increase). Hence, it may be concluded that other factors may play a significant role in the prediction of human skin sensitization. As already mentioned above, metabolism may occur in the skin and for reaction with proteins to occur reactivity has to be sufficient as well as the access to the protein before the skin sensitization process can start.

At the end of 2016 the European Commission (EU Com, 2016) and in July 2017 ECHA (ECHA, 2017a) introduced an alternative strategy in the form of an Integrated Approach to Testing and Assessment (IATA), which was highly welcomed, but the question was whether the point in time and the high speed of introduction were workable? It is highly appreciated that the lives of about 50% of animals used in the LLNA are saved - as available validated tests and adopted as OECD guideline at the moment of testing could not predict the potency for skin sensitization of a substance. In the past the LLNA was performed and no questions on human relevance were asked in most cases. Nowadays a number of non-animal tests have to be performed followed by a weight of evidence, which is often complicated due to discordant results between the non-animal assays. REACH (EU Com, 2017) states at point 8.3 “skin sensitization” in column 2 that “if information from test method(s) addressing one or two of the key events in column 1 already allows classification and risk assessment according to point 8.3, studies addressing the other key event(s) need not be conducted”. Currently, no validated and adopted OECD *in vitro* tests that can be used for classification are available, in case the alternative testing strategy shows indications of the substance being a skin sensitizer. According to REACH you then have to determine potency by *in vivo* testing. To further reduce the need for *in vivo* testing for skin sensitizing substances, information on potency is essential. Currently, there are several assays in the process of OECD validation that can predict potency, such as the GARDskin assay and the SENS-IS assay. At the moment, the GARDskin assay is under validation in the OECD Test Guideline Program (TGP no. 4.106). GARDskin is an assay with dendritic cells using genetic biomarkers for more than 200 genes to determine skin sensitizing potential which cover the entire immune reaction and can predict the potency of a skin sensitizer based on 50 complementary genes (www.senzagen.com). This assay has an accuracy of almost 90% against the LLNA assay for prediction of human skin sensitization, whereas the LLNA predicts human skin sensitization with an accuracy of approximately 80–90% (Anderson et al., 2011; Roberts, 2018a) when tested on mono-

constituent substances. Moreover, a more elaborate range of solvents compared to the KeratinoSens™ and U-SENS™ assay may be used, including DMSO, water, DMF, glycerol, ethanol and sesame oil, although the GARDskin assay is still a cellular assay, which implies limitations of the concentration of solvent in the cell medium.

The SENS-IS protocol is also under validation in the OECD Test Guideline Program (TGP no. 4.107) and analyzes the expression of a group of 38 genes to predict skin sensitization potency (Cottrez et al., 2016). This model is based on a reconstructed human skin model (Episkin) – and thus takes uptake into/through the stratum corneum into consideration. The lowest of 4 dilutions of the substance applied that induces a positive response is used to classify the chemical as a weak, moderate, strong or extreme sensitizer. The accuracy of the SENS-IS assay is 96% compared to human testing as determined from a test set of 150 chemicals. It must be said these were all mono-constituents. Using a set of 40 proprietary and public domain chemicals from L'Oréal an overall performance of sensitizer/non-sensitizer prediction of 87% was shown, while the predictive capacity of the potency category was less promising (63% concordance) with 7 chemicals being overestimated and 8 chemicals being underestimated (<http://www.episkin.com/en/Scientific%20References/4162>). Also for this assay a more elaborate range of solvents is possible, i.e. DMSO, water, phosphate-buffered saline and olive oil and the substance may even be applied undiluted.

The currently validated *in chemico/in vitro* assays for skin sensitization all lack the actual first initiating event of skin sensitization, uptake into/through the stratum corneum. Therefore, if both the GARDskin and the SENS-IS assay would predict potency with comparable accuracy, sensitivity and specificity, the SENS-IS assay would seem most promising as it includes penetration of the skin and therefore, resembles the human situation more closely than other available non-animal assays.

It is at the moment not clear whether such an assay, that can predict potency next to the potential for skin sensitization, will replace other *in chemico/in vitro* assays as a stand-alone, or will also need to be used in a weight of evidence. Until then acceptability of both assays in a regulatory framework is uncertain, since neither the GARDskin assay nor the SENS-IS assay has been implemented yet in an OECD test guideline.

5. Conclusions

In this study, a defined non-animal testing approach to predict the skin sensitization potential for humans of chemicals for registration under REACH was evaluated. A wide range of mono- and multi-constituent substances as well as UVCBs were tested. For 46% of the substances the alternative approach could determine the skin sensitization potential – and potency, if positive, and an animal test was prevented. In 54% of the cases additional *in vivo* testing was still needed to determine the skin sensitization potential and/or predict potency. For 12 substances the determination of skin sensitization potential was the driver for *in vivo* testing – and thus also potency –, and for 15 substances information on potency was only needed. Almost all UVCBs needed further testing on animals.

There are a number of issues that complicate the non-animal testing strategy for skin sensitization, such as cytotoxicity of the substance, whether the substance is a UVCB or metal-containing substance, the limited solubility of the substance, the absence or limited presence of metabolic capacity in the assays, and whether the metabolic capacity present is comparable/part of the metabolism present in the skin. Moreover, no potency prediction is possible or accepted with the assays at the moment. An *in vitro* assay with a dermal barrier and skin metabolism present and the ability to predict potency may overcome these issues.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conflict of interest

The authors are not aware of any conflicts of interest.

References

- Anderson, S.E., Siegel, P.D., Meade, B.J., 2011. The LLNA: a brief review of recent advances and limitations. *J. Allergy* 2011, 1–10.
- Canipa, S.J., Chilton, M.L., Hemingway, R., Macmillan, D.S., Myden, A., Plante, J.P., Tennant, R.E., Vessey, J.D., Steger-Hartmann, T., Gould, J., Hillegass, J., Etter, S., Smith, B.P.C., White, A., Sterchele, P., De Smedt, A., O'Brien, D., Parakhia, R., 2017. A quantitative *in silico* model for predicting skin sensitization using a nearest neighbours approach within expert-derived structure-activity alert spaces. *J. Appl. Toxicol.* 37, 985–995.
- Chilton, M.L., Macmillan, D.S., Steger-Hartmann, T., Hillegass, J., Bellion, P., Vuorinen, A., Etter, S., Smith, B.P.C., White, A., Sterchele, P., De Smedt, A., Glogovac, M., Glowienke, S., O'Brien, D., Parakhia, R., 2018. Making reliable negative predictions of human skin sensitization using an *in silico* fragmentation approach. *Regul. Toxicol. Pharmacol.* 95, 227–235.
- Cottrez, F., Boitel, E., Ourlin, J.-C., Peiffer, J.-L., Fabre, I., Henaoui, I.-S., Mari, B., Vallauri, A., Paquet, A., Barbry, P., Aurialt, C., Aeby, P., Groux, H., 2016. SENS-IS, a 3D reconstituted epidermis based model for quantifying chemical sensitization potency: reproducibility and predictivity results from an inter-laboratory study. *Toxicol. Vitro* 32, 248–260.
- ECHA, 2017a. Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.7a: Endpoint Specific Guidance. Version 6.0, July 2017. Retrieved from: <https://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment>.
- ECHA, 2017b. Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.7a: Endpoint Specific Guidance. Version 6.0, July 2017. Retrieved from: <https://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment>.
- EU, 2008. Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labeling, and packaging of substances and mixtures. Official Journal of the European Union Retrieved from: <http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02008R1272-20170101&from=EN>.
- EU, 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes, 20.10.2010, O.J. L 276, pp. 33–79.
- EU Com, 2016. Commission Regulation (EU) 2016/1688 of 20 September 2016 Amending Annex VII to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as Regards Skin Sensitisation, 21.9.2016, O.J. L 255, pp. 14–16.
- EU Com, 2017. Commission Regulation (EU) 2017/706 of 19 April 2017 Amending Annex VII to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as Regards Skin Sensitisation and Repealing Commission Regulation (EU) 2016/1688. C/2017/2369, 20.4.2017, O.J. L 104, pp. 8–11.
- Fitzpatrick, J.M., Roberts, D.W., Patlewicz, G., 2017a. What determines skin sensitization potency: myths, maybes and realities. The 500 molecular weight cut-off: an updated analysis. *J. Appl. Toxicol.* 37, 105–116 (Epub 2016).
- Fitzpatrick, J.M., Roberts, D.W., Patlewicz, G., 2017b. Is skin penetration a determining factor in skin sensitization potential and potency? Refuting the notion of a logKow threshold for skin sensitization. *J. Appl. Toxicol.* 37, 117–127 (Epub 2016).
- Kreiling, R., Gehrke, H., Broschard, T.H., Dreeßen, B., Eigler, D., Hart, D., Höpflinger, V., Kleber, M., Kupny, J., Li, Q., Ungeheuer, P., Sauer, U.G., 2017. *In chemico, in vitro* and *in vivo* comparison of the skin sensitizing potential of eight unsaturated and one saturated lipid compounds. *Regul. Toxicol. Pharmacol.* 90, 262–276.

- Kreiling, R., Hollnagel, H.M., Hareng, L., Eigler, D., Lee, M.S., Griem, P., Dreessen, B., Kleber, M., Albrecht, A., Garcia, C., Wendel, A., 2008. Comparison of the skin sensitizing potential of unsaturated compounds as assessed by the murine local lymph node assay (LLNA) and the Guinea pig maximization test (GPMT). *Food Chem. Toxicol.* 46, 1896–1904.
- Lhasa, 2019. Skin Sensitisation Assessment Using Derek Nexus. Retrieved from. <https://www.lhasalimited.org/products/skin-sensitisation-assessment-using-derek-nexus.htm>.
- OECD, 1992. Skin Sensitisation. OECD Guideline for the Testing of Chemicals No 406. OECD, Paris Retrieved from. <http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm>.
- OECD, 2010. Skin Sensitisation: Local Lymph Node Assay, OECD Guideline for the Testing of Chemicals No 429. OECD, Paris Retrieved from. <http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm>.
- OECD, 2015a. *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA). OECD Guideline for the Testing of Chemicals No 422C. OECD, Paris Retrieved from. <http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm>.
- OECD, 2015b. *In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method. OECD Guideline for the Testing of Chemicals No 422D. OECD, Paris Retrieved from. <http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm>.
- OECD, 2016. Organisation for Economic Co-operation and Development. Series on Testing and Assessment No. 256. Guidance Document on the Reporting of Defined Approaches and Individual Information Sources to Be Used within Integrated Approaches for Testing and Assessment (IATA) for Skin Sensitisation. ENV/JM/MONO(2016)29. OECD, Paris, France 27.10.2016.
- OECD, 2017. Skin sensitization. In: *Vitro Skin Sensitisation Assays Addressing the Key Event on Activation of Dendritic Cells on the Adverse Outcome Pathway for Skin Sensitisation*. OECD Guideline for the Testing of Chemicals No 422E. OECD, Paris Retrieved from. <http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm>.
- OECD, 2018. Key event based test guideline 442D. In: *Vitro Skin Sensitisation Assays Addressing the AOP Key Event on Keratinocyte Activation*. OECD Guideline for the Testing of Chemicals No 422D. OECD, Paris Retrieved from. <http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm>.
- Otsubo, Y., Nishijo, T., Miyazawa, M., Saito, K., Mizumachi, H., 2017. Binary test battery with KeratinoSensTM and h-CLAT as part of a bottom-up approach for skin sensitization hazard prediction. *Regul. Toxicol. Pharmacol.* 88, 118–124.
- Roberts, D.W., Aptula, A.O., 2008. Determinants of skin sensitization potential. *J. Appl. Toxicol.* 28, 377–387.
- Roberts, D.W., Patlewicz, G., 2017. Non-animal assessment of skin sensitization hazard: is an integrated testing strategy needed, and if so what should be integrated? *J. Appl. Toxicol.* 38, 41–50.
- Roberts, D.W., Api, A., 2018a. Chemical applicability domain of the local lymph node assay (LLNA) for skin sensitization potency. Part 4. Quantitative correlation of LLNA potency with human potency. *Regul. Toxicol. Pharmacol.* 96, 76–84.
- Roberts, D.W., 2018b. Is a combination of assays really needed for non-animal prediction of skin sensitization potential? Performance of the GARDTM (Genomic Allergen Rapid Detection) assay in comparison with OECD guideline assays alone and in combination. *Regul. Toxicol. Pharmacol.* 98, 155–160.
- Takenouchi, O., Fukui, S., Okamoto, K., Kurotani, S., Imai, N., Fujishiro, M., Kyotani, D., Kato, Y., Kasahara, T., Fujita, M., Toyoda, A., Sekiya, D., Watanabe, S., Seto, H., Hirota, M., Ashikaga, Miyazawa, M., 2015. Test battery with the human cell line activation test, direct peptide reactivity assay and DEREK based on a 139 chemical data set for predicting skin sensitization potential and potency of chemicals. *J. Appl. Toxicol.* 35, 1318–1332.
- Urbisch, D., Honarvar, N., Kolle, S.N., Mehling, A., Ramirez, T., Teubner, W., Landsiedel, R., 2016. Peptide reactivity associated with skin sensitization: the QSAR Toolbox and TIMES compared to the DPRA. *Toxicol. Vitro* 34, 194–203.