

A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation

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ABSTRACT: Skin sensitization is a key endpoint for cosmetic ingredients, with a forthcoming ban for animal testing in Europe. Four alternative tests have so far been submitted to ECVAM prevalidation: (i) MUSST and (ii) h-Clat assess surface markers on dendritic cell lines, (iii) the direct peptide reactivity assay (DPRA) measures reactivity with model peptides and (iv) the KeratinoSensTM assay which is based on detection of Nrf2-induced luciferase. It is anticipated that only an integrated testing strategy (ITS) based on a battery of tests might give a full replacement providing also a sensitization potency assessment, but this concept should be tested with a data-driven analysis. Here we report a database on 145 chemicals reporting the quantitative endpoints measured in a U937- test, the DPRA and KeratinoSensTM. It can serve to develop data-driven ITS approaches as we show in a parallel paper and provides a view as to the current ability to predict with *in vitro* tests as we are entering 2013. It may also serve as reference database when benchmarking new molecules with *in vitro* based read-across and find use as a reference database when evaluating new tests. The tests and combinations thereof were evaluated for predictivity, and overall a similar predictivity was found as before on three-fold smaller datasets. Analysis of the dose-response parameters of the individual tests indicates a correlation to sensitization potency. Detailed analysis of chemicals false-negative and false-positive in two tests helped to define limitations in the tests but also in the database derived from animal studies. Copyright © 2013 John Wiley & Sons, Ltd.

Additional Supporting Information may be found in the online version of this article

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Introduction

European Legislation has set a deadline by 2013 for the replacement of animal tests to determine the safety of cosmetic ingredients. At the same time, the REACH regulation (Registration, Evaluation and Authorization of Chemicals) requires the testing of thousands of not previously tested substances for their safety, which will lead to an increase in the numbers of animal tests needed (Rovida and Hartung, 2009). One of the most relevant endpoints for cosmetic safety assessment is the determination of the skin sensitization potential, which is currently estimated using the local lymph node assay in mice (LLNA) (Basketter *et al.*, 2002), but several non-animal alternatives have been developed in recent years. Some are in the early stages of development, whereas others have reached the final stages of prevalidation in Europe. The tests currently in prevalidation are (i) the direct peptide reactivity assay (DPRA), which measures depletion of a cysteine- and a lysine-containing hepatpeptide in the presence of the test chemical (Gerberick *et al.*, 2004, 2007), (ii) the myeloid U937 cell line test measuring CD86 induction on the cell surface (Ade *et al.*, 2006)(MUSST), (iii) the human cell line activation assay (h-Clat)(Ashikaga *et al.*, 2006) also addressing CD86 induction on the cell surface combined with evaluation of induction of CD54 and (iv) the KeratinoSensTM assay (Emter *et al.*, 2010), which is a reporter cell-based approach based on the finding that the majority of the skin sensitizers induce the Nrf2-Keap1-ARE regulatory pathway (Natsch, 2010). In addition to these assays, a number of

novel assays have been developed by the European Sens-it-iv project (Roggen, 2012; Rovida *et al.*, 2007). A tiered strategy with a cell-based IL-18 assay to discriminate sensitizers from non-sensitizers and an epidermal model to determine sensitizer potency is the first approach from Sens-it-iv undergoing interlaboratory studies with blind-coded chemicals (Teunis *et al.*, 2012). Other assays based on RT-PCR and gene chip analysis of gene expression changes in primary dendritic cells, dendritic cell lines (Hooyberghs *et al.*, 2008; Johansson *et al.*, 2012; Lambrechts *et al.*, 2010), keratinocyte cell lines (van der Veen *et al.*, 2013; Vandebriel *et al.*, 2010) and epidermal models (Miyazawa *et al.*, 2011; Teissier *et al.*, 2012) have also been proposed. Most of these assays include a prediction model for hazard and only two of these studies reported potency prediction (Lambrechts *et al.*, 2010; Teissier *et al.*, 2011).

The assays in ECVAM prevalidation all comprise a prediction model to discriminate sensitizers from non-sensitizers, and they therefore could be used as stand-alone assays for hazard

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assessment. However, they are not sufficiently able to predict sensitization potency of the chemicals, and if used on their own, they all have some limitations to their applicability domain. For example, the assays have no or only a limited metabolic capacity and thus a weakness to detect some prohaptens, i.e. chemicals which need to undergo metabolic activation to become the true sensitizing hapten (Gerberick *et al.*, 2009). Furthermore, some assays preferentially detect sensitizers specifically reacting with cysteine in proteins (Natsch, 2010). Part of these limitations, especially the limitations in potency predictions, may be a result of the fact that each of these assays only addresses a specific aspect of a complex chain of chemical and biological events leading to sensitization *in vivo*. A consensus has thus arisen in the scientific community, that only a 'battery of tests' applied in an integrated testing strategy (ITS) will inform us on the true sensitizing potency of chemicals. This has been formalized in a theoretical scheme (Jowsey *et al.*, 2006) which was later updated (Basketter and Kimber, 2009). More recently, the theory underlying this approach has been formalized in the 'adverse outcome pathway' (AOP) paradigm, a concept that links the individual events starting from the molecular initiating event (MIE) to the result at the level of the whole organism. A detailed analysis of the AOP for skin sensitization has been presented and widely agreed upon by the OECD (2012).

The MIE in skin sensitization is the formation of a hapten-protein complex, which can be mimicked *in vitro* with reactivity assays such as the DPRA (Gerberick *et al.*, 2008). Other events listed in the OECD analysis include: skin penetration and metabolism, keratinocyte inflammatory responses, gene expression of antioxidant response element dependent genes in keratinocytes, activation of dendritic cells and T-cell proliferation. Some, but not all, of these individual events are modeled by the *in vitro/in chemico* assays now undergoing prevalidation.

The proposals of creating a testing strategy based on the known biological pathways leading to skin sensitization are theoretical in nature with the exception of a proof of concept study based on a small dataset developed by Jaworska *et al.* (2011). To calibrate and parameterize models for integrating *in vitro* and other alternative data, a data-driven approach is needed. In order that this can be done, a significant number of chemicals need to be tested in all tests in parallel. Only with this data-driven approach we can identify weaknesses and gaps in the currently available test battery which may inform us of future research needs.

A simple integration of data would be to just count the pluses and minuses from the prediction models of the different tests, and this has recently been done on a set of 54 chemicals (Bauch *et al.*, 2012). However, the tests also yield a quantitative dose-response output, which may contain partial information on the sensitization potency and subsequent *in vivo* risk. Integrating these dose-response data rather than just the yes/no answers from the individual prediction models is a more informative way forward. Thus the comparative database should not just collect the yes/no assessments but also the quantitative read-outs measured with each test.

Few attempts to assemble dose-response data and integrate them have been made so far. McKim *et al.* (2010) tested chemicals for induction of Nrf2-dependent genes in HaCaT keratinocytes, in parallel determined reactivity with glutathione and integrated the data in a patent pending and thus a non-transparent algorithm. Natsch *et al.* (2009) compiled data from

an Nrf2-reporter assay, DPRA-Cys data and the TIMES SS *in silico* prediction in a first attempt to explore the ITS approach. A modification of the DPRA including dose-response curve measurements has been used for potency correlations within specific applicability domains (Natsch *et al.*, 2011; Roberts and Natsch, 2009).

Here we report a database on 145 chemicals with data from a U937-CD86 test, the KeratinoSens™ assay and the DPRA. The U937-CD86 test, and not h-Clat was selected, as this test was already established in one of our laboratories and at the start of this project it was not known that the h-Clat would progress through prevalidation faster. This comprehensive database may serve to (i) develop data-driven ITS approaches as we show in a parallel paper, (ii) give a *current status* of what we can and what we cannot predict with the *in vitro/in chemico* tests in the prevalidation pipeline as we are entering 2013 and (iii) serve as reference database when benchmarking other molecules with *in vitro*-based read across. As all the test chemicals are commercially available, this dataset may also be used to assess future methods for their usefulness in complementing the testing strategy.

Materials and Methods

Rationale for selection of chemicals and sources of *in vivo* data

Selection of chemicals for this dataset started using the LLNA database papers published in 2005 (Gerberick *et al.*, 2005) and 2010 (Kern *et al.*, 2010). Chemicals in those papers were considered to have quality LLNA *in vivo* data, all conducted according to the OECD 429 protocol with a clear dose-response curve or a negative result. The LLNA data in those publications were either generated in house by the authors or collected from literature. Considering chemical availability, compatibility with assay conditions (e.g. suitable solubility in testing medium), no interference with read out in assay (e.g. no co-elution in HPLC for the DPRA), and a good coverage of non-sensitizer and sensitizers of different sensitization potencies, chemicals were chosen and tested in all selected alternative assays. As a result a complete set of *in vivo* and alternative data was compiled for 145 different chemicals with only three data gaps for the U937-CD86 test.

DPRA

The DPRA has been described previously in full detail (Gerberick *et al.*, 2004, 2007). The assay is based on the correlation between skin protein reactivity and skin sensitization. In short, test chemicals were incubated in triplicate with synthetic cysteine- and lysine-containing peptides. After a 24-h reaction period, the samples were analyzed by HPLC. UV detection was used to measure the concentration of the peptides after the reaction. Peptide depletion and per cent remaining peptide was calculated for each sample by comparing the amount of peptide in the reaction sample to the amount of peptide in the peptide-alone control samples. For each test chemical, the mean peptide depletion of the triplicates for both peptides was calculated. Using the means of cysteine and lysine depletion, an overall average peptide depletion was calculated and a determination of a sensitizer or non-sensitizer based on the decision tree model (Gerberick *et al.*, 2007) was conducted. Chemicals with an average cysteine and lysine depletion < 6.37% are considered to have minimal reactivity. Meanwhile, chemicals with average depletion between 6.37% and 22.62% are considered to have low reactivity, between 22.62% and 42.27% they are

attributed to moderate and above 42.47% to the high reactivity class. Generally, chemicals with moderate to high reactivity are associated with moderate to strong allergenicity, whereas those categorized as having minimal to low reactivity include weak and non-sensitizers. For hazard identification, chemicals with high, moderate and low reactivity are grouped as sensitizers, whereas chemicals with minimal reactivity are considered non-sensitizers.

U937-CD86 assay

For the U937-CD86 assay, cells were cultured and treated in a manner similar to that described by Ade *et al.* (2006). Briefly, U937 cells, a human myelomonocytic cell line purchased from the American Type Culture Collection (Rockville, MD, USA), were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin (Gibco), 2 mM L-glutamine (Gibco) and 10% fetal bovine serum (FBS; Hyclone). Cells were counted, resuspended to a concentration of 0.5×10^6 viable cells per ml and 100 µL of the cell suspension per well was plated into 96-well flat bottom plates. Then either 100 µL of the test chemical solution (at $2 \times$ desired final in-well concentration) or 100 µL of the vehicle [complete culture medium or complete medium with 0.8% DMSO (0.4% DMSO final in-well)] was added and the plates were incubated for 48 h at 37 °C under humidified 5% CO₂. Four to six concentrations of each chemical were tested based on solubility and resulting cell viability as determined by propidium iodide (PI; Invitrogen) staining. Each chemical was tested in at least two independent experiments. After incubation the cells were harvested and stained on ice with fluorescein isothiocyanate (FITC)-conjugated anti-human CD86 monoclonal antibody (Clone FUN-1; BD Pharmingen) and FITC-isotype control IgG1 (Clone MOPC-21; BD Pharmingen). After staining, the cells were washed, propidium iodide was added to the cell suspension and CD86 expression and cell viability was analyzed by flow cytometry using a Beckman Coulter FC-500-MPL Flow Cytometer (Beckman Coulter, Miami, FL, USA). For each sample with viability $\geq 70\%$, the per cent of IgG1 isotype positive cells was subtracted from the per cent of CD86 positive cells. Results were expressed as stimulation indexes (SI) calculated as the isotype corrected per cent CD86+ test/ isotype corrected per cent CD86+ control $\times 100$. A test chemical was considered to be positive if the SI value was ≥ 150 . From the dose-response curve, the theoretical concentration at which the chemical induces a SI of 150 was calculated by linear regression using the equation $EC150 = C1 + [(150 - SI.1)/(SI.2 - SI.1) \times (C2 - C1)]$ where C1 and SI 1 are the concentration and SI immediately below SI = 150 and C2 and SI 2 are the data points immediately above SI = 150.

KeratiSens™ assay

The KeratiSens™ assay was performed as previously described in detail (Emter *et al.*, 2010). Briefly, cells were grown for 24 h in 96-well plates. The medium was then replaced with medium containing the test chemical and a final level of 1% DMSO. Each compound was tested at 12 binary dilutions in the range from 0.98 to 2000 µM. In each repetition, three parallel replicate plates were run with this same set-up and a fourth parallel plate was prepared for cytotoxicity determination. Cells were incubated for 48 h with the test agents, after which luciferase activity and cytotoxicity (with the MTT-assay (Mosmann, 1983)) were

determined. This full procedure was repeated at least two times for each chemical and in most cases 3 to 4 times. For each chemical in each repetition and at each concentration, the gene induction was compared with DMSO controls and the wells with statistically significant induction over the threshold of 1.5 (i.e. 50% enhanced gene activity) were determined. Furthermore, the EC1.5 and the EC3 values (concentration in µM for induction above these thresholds) based on linear extrapolation as done in the LLNA and the IC50 for the concentration with 50% reduction in viability were calculated. In the prediction model, chemicals with significant gene induction > 1.5 -fold below 1000 µM and at a concentration at which the cells retain 70% viability in at least 2 of 3 repetitions are rated positive.

Results

The distribution of chemicals in the database according to LLNA EC3 values, cLogP and molecular weight

The database contains 43 non-sensitizers according to the LLNA, 33 weak, 39 moderate, 19 strong and 11 extreme sensitizers according to the classification proposed by Kimber *et al.* (2003). The distribution of the EC3 values for all the test chemicals is summarized in Fig. 1. The chemicals were then separated for sensitizers and non-sensitizers, grouped according to molecular weight and cLogP (Fig. 2). This analysis shows that based on molecular weight the majority of test chemicals, both sensitizers and non-sensitizers, fall in the range between 100 and 200 Da. The database contains a low number of molecules below 50 Da or above 350 Da. Currently there is little experience in testing molecules above a MW of 500 in both the LLNA and the alternative tests, mainly because only around 14% of registered chemicals have a MW > 500 (Roberts *et al.*, 2013). These larger molecules are also clearly underrepresented in the current database. The cLogP for the majority of the tested molecules falls in the range between 0 and 4, but the molecules cover a range from -2 up to > 6 . Overall the distribution for the non-sensitizers (median cLogP = 1.82) is similar as compared with the sensitizers (median = 1.8). The test chemicals cover a broad chemical diversity as becomes obvious from inspection of the structures displayed in Supplementary Table S1.

The *in vitro* database and the yes/no predictions according to individual prediction models

Table 1 lists the different read-outs from the three tests for all chemicals, sorted according to their sensitization potential in

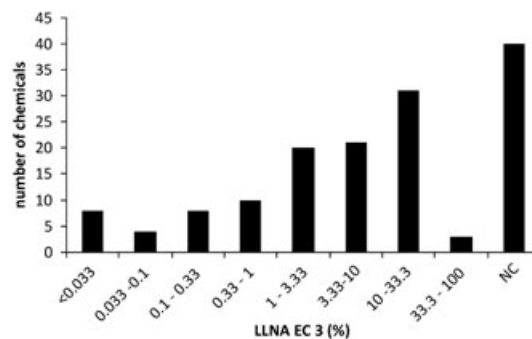


Figure 1. Distribution of the chemicals according to the LLNA EC3 values.

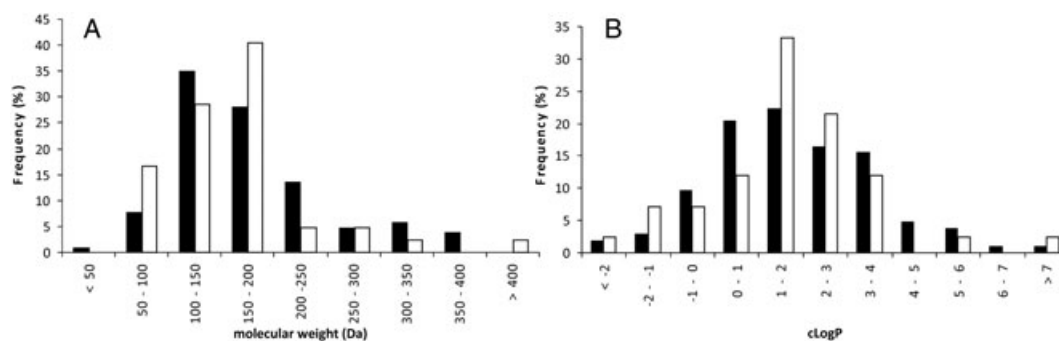


Figure 2. Distribution of the chemicals in different ranges of molecular weight and different hydrophobicity as determined by cLogP. Solid bars: sensitizers according the LLNA. Open bars: non-sensitizers according to LLNA.

the LLNA. For the cell-based assays the key read-out from the full dose response analysis, which covers a dynamic range of around three orders of magnitude (1 – > 1000 μ M), is reported. While the read-out of the U937-CD86 test is the concentration for 1.5-fold induction of CD86 expression (EC 150 value), the dose–response analysis in KeratinoSens™ yields the IC50 value for cytotoxicity and the EC1.5 and EC3 values for 1.5- and 3-fold gene induction. The DPRA reports per cent of peptide remaining after 24 h incubation with test chemicals. In Table 2, these data are reduced to the yes/no rating according to the prediction model of the individual tests. In Table 2, also the yes/no rating according to a simple WoE is given, with a positive prediction attributed to each chemical which is rated positive in at least two *in vitro* tests as has recently been suggested based on the analysis of a smaller dataset (Bauch *et al.*, 2012). Both Tables are also available online as Table S1 in supporting information for further analysis and ITS building. The predictions in Table 2 were then used to calculate Cooper statistics vs. the LLNA prediction and these are summarized in Table 3. The sensitivity of the individual assays are at 71% (U937-CD86), 82% (DPRA) and 79% (KeratinoSens™), the specificities are at 70% (U937-CD86), 74% (DPRA) and 72% (KeratinoSens™). The accuracies are at 71% (U937-CD86), 77% (KeratinoSens™) and 80% (DPRA). An only slightly enhanced accuracy was found for the simple WoE prediction as compared with the individual tests, with an overall sensitivity of 82%, specificity of 77% and an accuracy of 81%. The sensitivity of this WoE analysis is highest for the strong and extreme sensitizers (93%), in this class only 2 of 30 chemicals were not positive in at least two assays (Squaric acid diethyl ester and 7,12-Dimethylbenz[α]anthracene).

It is beyond the scope of this compilation to discuss all the individual wrong predictions of the three tests, but it is instructive to study the cases, in which two of the three *in vitro* tests did predict the LLNA outcome wrongly, or, in other words, the cases in which the simple WoE assessment led to a false call. These cases are compiled in Table 4 for the ‘double false-positives’ and in Table 5 for the ‘double false-negatives’. A detailed analysis of the chemicals in these two Tables is presented in the discussion section. It is worth noting, that among the false-positives in the U937-CD86 test the majority of the chemicals induce surface marker expression only at a relatively high concentration (10 of 12 have an EC150 > 300 μ M), and also in the KeratinoSens™ assay 5 of the 11 false positives induced the luciferase gene above 300 μ M. As discussed below, it is therefore not just the yes/no rating which may be of interest in an ITS for hazard assessment, but also the observation on how strongly positive the chemicals are in the individual tests.

Distribution of the dose–response data in the different LLNA potency classes

We next analyzed the quantitative data of the individual tests to obtain a first indication on how the different read-outs correlate with potency when analyzed in this larger dataset. First, the distributions for the different endpoints in the different LLNA potency classes were visualized (Fig. 3). For this analysis, we pooled the strong and extreme classes. The results indicate that all six read-outs contain potency information, although to a varying degree: in each case the median value decreases with increasing potency class. The difference is relatively small for the cytotoxicity, in which case we note a quite similar distribution for the weak and moderate classes, whereas, just to note an example, the difference between these two classes is more pronounced for the Cys-DPRA read-out. In the U937-CD86 assay, weak sensitizers tend to induce CD86 between 100 and 1000 μ M (median 277 μ M), whereas for strong and extreme sensitizers the median is at 20 μ M. The Median for the KeratinoSens™ EC1.5 value is at 122 μ M for weak, 48 μ M for moderate and 7.1 μ M for strong/extreme sensitizers, whereas the median of the EC3 is at 2343 μ M for weak, 133.3 μ M for moderate and 39.8 μ M for strong/extreme sensitizers. At the same time, this analysis also indicates that the data are quite widely spread. Individual read-outs clearly cannot sufficiently discriminate the LLNA-potency classes when all chemical classes are viewed together. However, this does not exclude the possibility that individual tests can, in certain cases, predict potency within chemical classes or mechanistic applicability domains if they are used for *in vitro* based read across (see for example (Delaine *et al.*, 2011)).

Spearman’s rank correlation for the results vs. LLNA potency classes and LLNA values

In a next step, we performed Spearman’s rank correlation for each alternative endpoint either against the LLNA class or against the LLNA value (LLNA negatives were set to an arbitrary value of 100 for this latter analysis to calculate the ranks, similarly for the *in vitro* data a numerical value above the maximal test concentration was chosen for the negatives). Table 6 lists the results: the highest and similar rank coefficients for the *in vitro* data with the LLNA results were found for DPRA-Cys and KeratinoSens™ EC1.5. The KeratinoSens™ EC3, IC50 and the U937-CD86 EC150 gave somewhat lower but also highly significant correlations. The lowest value was found for DPRA-Lys, which may be related to the fact that Lys-residues in proteins are an important molecular target, but for a minority of

Table 1. Quantitative data from the three *in vitro* tests

Chemical	Case no.	LLNA U937-CD86 Test		Direct Peptide Reactivity Assay		KeratinoSens™ Assay		
		EC3 %	EC 150 [μM]	Cysteine [% pept. remain.]	Lysine [% pept. remain.]	EC1.5 [μM]	EC3 [μM]	IC50 [μM]
Non-sensitizers according to LLNA								
1-Bromobutane	109-65-9	NC	>7000	86.2	98.8	1842.4	>2000	>2000
1-Butanol	71-36-3	NC	>7000	100.0	98.8	>2000	>2000	>2000
4-Hydroxybenzoic acid	99-96-7	NC	>7000	100.0	97.8	>2000	>2000	>2000
Diethyl phthalate	84-66-2	NC	>7000	99.2	100.0	>2000	>2000	>2000
Glycerol	56-81-5	NC	>7000	100.0	97.9	>2000	>2000	>2000
Hexane	110-54-3	NC	>7000	100.0	100.0	>2000	>2000	>2000
Methylparaben	99-76-3	NC	341.8	96.4	100.0	805.35	>2000	>2000
Octanoic acid	124-07-2	NC	37.5	100.0	99.1	>2000	>2000	>2000
Propylene glycol	57-55-6	NC	>7000	100.0	99.4	>2000	>2000	>2000
Sulphanilic acid	121-57-3	NC	>7000	94.7	99.5	>2000	>2000	>2000
Benzaldehyde	100-52-7	NC	556.0	92.8	100.0	443.13	>2000	>2000
Coumarin	91-64-5	NC	>7000	99.0	100.0	60.03	479.96	1844.2
Propyl paraben	94-13-3	NC	299.7	91.8	100.0	14.53	>2000	813.1
Streptomycin sulfate	3810-74-0	NC	>7000	100.0	95.5	>2000	>2000	>2000
Sulphanilamide	63-74-1	NC	>7000	100.0	99.2	>2000	>2000	>2000
4-Methoxyacetophenone	100-06-1	NC	779.1	95.3	99.9	377.61	1192.39	>2000
6-Methylcoumarin	92-48-8	NC	>7000	98.6	96.0	239.27	1167.40	>2000
Benzalkonium chloride	8001-54-5	NC	0.8	97.2	100.0	>2000	>2000	4.0
Vanillin	121-33-5	NC	690.1	96.8	100.0	>2000	>2000	>2000
Ethyl vanillin	121-32-4	NC	>7000	98.9	90.3	161.73	700.60	>2000
Nonanoic acid	112-05-0	NC	306.5	100.0	100.0	>2000	>2000	>2000
Tartaric acid	87-69-4	NC	>7000	96.2	98.8	>2000	>2000	>2000
4-Carboxyphenylacetate	2345-34-8	NC	>7000	9.4	78.4	>2000	>2000	>2000
Benzoic acid	65-85-0	NC	>7000	83.8	31.9	>2000	>2000	>2000
3-Chloro-4-methoxybenzaldehyde	4903-09-7	NC	89.1	93.5	31.3	>2000	>2000	785.6
Octanenitrile	124-12-9	NC	>7000	100	96.9	>2000	>2000	>2000
3-Phenoxypropiononitrile	3055-86-5	NC	1178	100	70.3	>2000	>2000	1753.8
Benzocaine	94-09-7	NC	720.4	70.8	100.0	18.20	>2000	>2000
2-Acetyl-cyclohexanone	874-23-7	NC	891.7	81.8	87.5	756.61	825.46	>2000
Clofibrate	637-07-0	NC	>7000	97.7	98.1	>2000	>2000	195.0
Ethyl benzoylacetate	94-02-0	NC	>7000	88.8	99.1	63.37	689.02	>2000
Saccharin	81-07-2	NC	>7000	97.6	100.0	>2000	>2000	>2000
Vinylidene dichloride	75-35-4	NC	>7000	99.2	100.0	>2000	>2000	>2000
Isopropyl myristate ^a	110-27-0	44	>7000	94.1	100.0	>2000	>2000	384.8
Ethanol-2-butoxy-, acetate	112-07-2	NC	>7000	86.9	32	>2000	>2000	>2000
Sodium lauryl sulphate	151-21-3	14.0	>7000	100	100	>2000	>2000	44.7
Chlorobenzene	108-90-7	NC	>7000	99.6	98.7	>2000	>2000	>2000
Isopropanol	67-63-0	NC	>7000	100.0	99.5	>2000	>2000	>2000
Lactic acid	50-21-5	NC	>7000	100.0	99.2	>2000	>2000	>2000
Methyl salicylate	119-36-8	NC	>7000	99.7	98.4	>2000	>2000	>2000
Salicylic acid	69-72-7	NC	>7000	96.5	78.9	>2000	>2000	>2000
1-Iodohexane	638-45-9	NC	>7000	67.8	98.6	25.6	>2000	222.3
2-Hydroxypropyl methacrylate	923-26-2	NC	402.3	41.6	100.0	866	>2000	>2000
Weak sensitizers according to LLNA								
Aniline	62-53-3	89	332.9	100	90.3	>2000	>2000	>2000
Diethyl acetaldehyde	97-96-1	76	>7000	45.5	39.8	238.26	520.37	>2000
Hydroxycitronellal	107-75-5	33	27.9	82.5	93.5	79.40	142.91	>2000
Butyl glycidyl ether	2426-08-6	31	291.9	32.7	88.5	218.53	381.58	>2000
Penicillin G	61-33-6	30	6730	85.7	100	1308.7	>2000	>2000
Linalool	78-70-6	30	614.6	100	92.1	>2000	>2000	>2000
Ethyl acrylate	140-88-5	28	289.7	3.6	6.3	45.49	231.19	646.0

(Continues)

Table 1. (Continued)

Chemical	Case no.	LLNA U937-CD86 Test		Direct Peptide Reactivity Assay		KeratoSens™ Assay		
		EC3 %	EC 150 [μM]	Cysteine [% pept. remain.]	Lysine [% pept. remain.]	EC1.5 [μM]	EC3 [μM]	IC50 [μM]
Ethylene glycol dimethacrylate	97-90-5	28	398.6	12.7	87.6	57.41	253.37	1655.8
2,2,6,6-Tetramethyl-3,5-heptanedione	1118-71-4	27	>7000	98.6	100.0	>2000	>2000	384.9
5-Methyl-2,3-hexanedione	13706-86-0	26	265.3	74.2	92.5	49.79	332.21	1431.9
Geraniol	106-24-1	26	302.8	100	90	209.79	>2000	722.0
Imidazolidinyl urea	39236-46-9	24	28.8	96.2	99.1	45.43	41.95	90.4
cis-6-nonenal	2277-19-2	23	36.4	92.0	97.3	121.93	687.67	381.6
Cyclamen aldehyde	103-95-7	22	31.0	81.1	99.0	111.86	>2000	190.8
Cinnamyl Alcohol	104-54-1	21	572.4	100.0	84.9	123.56	>2000	774.7
Methyl acrylate	96-33-3	20	112.7	0.7	17.9	124.00	252.46	763.7
Pentachlorophenol	87-86-5	20	>7000	100	85.5	>2000	>2000	27.7
Lilial	80-54-6	19	29.9	86.0	99.3	>2000	>2000	94.5
1-Iodohexadecane	544-77-4	19	249.8	14.5	98.7	230.28	>2000	>2000
4-Allylanisole	140-67-0	18	>7000	79.4	100.0	>2000	>2000	419.0
Lyrar	31906-04-4	17	29.5	60.6	96.6	79.63	197.12	355.4
Benzyl benzoate	120-51-4	17	>7000	99.8	97.0	72.49	142.47	767.2
Abietic acid	514-10-3	15	43.3	0.1	83.7	16.56	34.74	104.6
Oxalic acid anhydrous	144-62-7	15	>7000	99.1	100.0	717.20	>2000	>2000
Eugenol	97-53-0	13	152.3	90.8	80.8	>2000	>2000	1505.7
Farnesal	502-67-0	12	>7000	83.6	91.5	10.37	>2000	24.0
2,3-Butanedione	431-03-8	11	255.5	21.0	73.0	141.92	253.40	859.9
Butyl acrylate	141-32-2	11	249.7	0.0	14.9	37.74	76.91	200.9
α-Amyl cinnamic aldehyde	122-40-7	11	123.1	99.4	96.1	>2000	>2000	51.0
Hexyl cinnamic aldehyde	101-86-0	11	29.1	100.0	100.0	17.27	>2000	26.34
2-Ethylhexyl acrylate	103-11-7	10	>7000	1.2	65.9	35.02	63.85	101.0
2-Methylundecanal	110-41-8	10	187.2	0.0	100.0	24.24	50.47	178.0
Moderate sensitizers according to LLNA								
1-(p-Methoxyphenyl)-1-penten-3-one	104-27-8	9.3	46.3	70.1	85.7	14.78	31.27	159.3
Citral	5392-40-5	9.2	14.5	14.3	83.1	23.16	67.36	182.8
Trimellitic anhydride	552-30-7	9.2	>7000	100.0	56.3	>2000	>2000	>2000
Perillaldehyde	2111-75-3	8.1	47.9	68.1	86.2	16.64	61.85	355.62
Methyl methanesulphonate	66-27-3	8.1	29.1	7.0	89.7	48.03	122.33	652.16
1,1,3-Trimethyl-2-formylcyclohexa-2,4-diene	116-26-7	7.5	82.5	8.2	100.0	5.39	33.49	337.27
Dihydroeugenol	2785-87-7	6.8		100	87.7	462.01	>2000	759.17
2-Phenylpropionaldehyde	93-53-8	6.3	29.8	51.8	78.8	64.76	76.67	195.06
Diethyl maleate	141-05-9	5.8	12.2	0.0	14.5	9.37	82.85	361.1
2-methoxy-4-methylphenol	93-51-6	5.8	338.0	100	88.5	>2000	>2000	>2000
3,4-Dihydrocoumarin	119-84-6	5.6	>7000	100	60.3	>2000	>2000	>2000
trans-2-Hexenal	6728-26-3	5.5	>7000	2.1	96.4	83.36	374.57	802.8
Resorcinol	108-46-3	5.5	268.8	98.4	100.0	>2000	>2000	>2000
Tetramethylthiuram disulfide	137-26-8	5.2	0.25	0.5	93.1	0.83	10.68	39.1
α-methyl-trans-cinnamaldehyde	101-39-3	4.5	273.6	89.6	71.2	41.27	139.93	1190.8
Squaric acid	2892-51-5	4.3	>7000	53.1	95.2	>2000	>2000	>2000
2,4-Heptadienal	5910-85-0	4		2.7	76.1	4.74	21.91	55.4
Benzylidene acetone	122-57-6	3.7	19.2	5.3	98.5	9.66	31.48	174.5
3-Propylidenephthalide	17369-59-4	3.7	>7000	85.7	69.4	>2000	>2000	717.4
5-Amino-2-methylphenol	2835-95-2	3.4	20.3	10.8	87.5	325.28	1184.07	1800.0
3-Aminophenol	591-27-5	3.2	47.6	93.4	98.8	>2000	>2000	>2000
Cinnamic aldehyde	104-55-2	3.0	3.8	29.4	56.8	16.13	63.94	194.4
Phenylacetaldehyde	122-78-1	3	>7000	39.3	77.4	28.54	69.03	116.2
Methyl 2-nonynoate	111-80-8	2.5	>7000	0.0	96.8	1.80	26.70	121.9
trans-2-decenal	3913-71-1	2.5	18.2	5.1	90.3	11.30	21.59	37.8

(Continues)

Table 1. (Continued)

Chemical	Case no.	LLNA U937-CD86 Test		Direct Peptide Reactivity Assay		KeratinoSens™ Assay		
		EC3 %	EC 150 [μM]	Cysteine [% pept. remain.]	Lysine [% pept. remain.]	EC1.5 [μM]	EC3 [μM]	IC50 [μM]
1,2-Benzisothiazolin-3-one	2634-33-5	2.3	3.6	2.3	90.3	3.16	14.77	50.9
1-Bromohexane	111-25-1	2.3	>7000	85.9	31.8	128.10	>2000	391.90
3-Dimethylamino propylamine	109-55-7	2.2	>7000	89.8	100.0	85.76	472.42	1337.9
Ethylenediamine free base	107-15-3	2.2	782.0	96.6	100.0	99.88	453.36	>2000
2,4-Dinitrobenzenesulfonic acid, sodium salt	885-62-1	2	41.5	5.8	72.6	70.23	133.29	311.0
2-Methyl-2H-Isothiazol-3-one	2682-20-4	1.9	39.9	2.1	100.0	11.80	29.56	139.0
2-Mercaptobenzothiazole	149-30-4	1.7	37.4	2.5	100.0	48.12	340.15	1003.1
4-Vinyl pyridine	100-43-6	1.6	21.9	7.9	100.0	9.73	25.37	58.5
Bisphenol A-diglycidyl ether	1675-54-3	1.5	26.4	57.5	98.9	5.20	8.70	21.9
4-Amino-m-cresol	2835-99-6	1.5	8.4	10	71.2	5.28	17.92	77.7
2-Hydroxyethyl acrylate	818-61-1	1.4	20.7	7.4	11.1	32.27	92.92	207.2
Glyoxal	107-22-2	1.4	482.4	43.5	32.2	89.12	307.80	677.9
1-Phenyl-1,2-propanedione	579-07-7	1.3	194.4	52.9	84.6	121.21	238.94	759.0
1-Naphthol	90-15-3	1.3	9.7	31.8	87.6	129.62	>2000	541.0
Isoeugenol	97-54-1	1.2	91.4	10.7	89.3	16.06	259.43	731.4
Strong sensitizers according to LLNA								
1,2-Dibromo-2,4-dicyanobutane	35691-65-7	0.9	>7000	0.0	71.4	7.79	18.14	25.59
Squaric acid diethyl ester	5231-87-8	0.9	>7000	97.6	98.4	>2000	>2000	>2000
1,2-cyclohexane dicarboxylic anhydride	85-42-7	0.84	1037	84.3	65.4	>2000	>2000	>2000
4-(Methylamino)phenol sulfate	55-55-0	0.8	>7000	0.0	55.3	9.40	2.68	11.70
Formaldehyde	50-00-0	0.61	53.3	39.6	88.8	63.21	72.20	201.63
4-(N-Ethyl-N-2-methan-sulphonamido-ethyl)-2-methyl-1,4-phenylenediamine	25646-71-3	0.6	5.5	9.9	86.4	1.98	11.17	30.80
N,N-dimethyl-4-nitrosoaniline	138-89-6	0.48	1.1	0.0	100.0	0.50	2.20	15.05
2-Aminophenol	95-55-6	0.4	2.9	3.8	81.9	1.06	4.37	138.22
2-Nitro-1,4-phenylenediamine	5307-14-2	0.4	44.4	6.7	100.0	11.46	786.42	>2000
2,5-Diaminotoluene sulphate	615-50-9	0.4	25.0	21.6	85	5.40	30.60	177.00
Propyl gallate	121-79-9	0.32	8.9	40.1	73.4	199.84	290.07	650.33
Lauryl gallate	1166-52-5	0.3	>7000	9.1	91.3	1.11	>2000	8.76
Benzyl bromide	100-39-0	0.2	6.4	0.0	77.9	4.91	14.20	25.79
Phthalic anhydride	85-44-9	0.16	1080	98.1	25.0	>2000	>2000	>2000
Maleic anhydride	108-31-6	0.16	1591	0.0	59.0	1475.9	>2000	>2000
1,4-Phenylenediamine	106-50-3	0.16	28.7	7.0	76.5	5.01	46.74	438.95
Fluorescein-5-isothiocyanate	3326-32-7	0.14		0.0	38.9	645.05	1177.86	>2000
Hydroquinone	123-31-9	0.11	2.7	16.7	48.9	9.80	51.29	130.73
Glutaraldehyde	111-30-8	0.1	2.0	69.8	14.6	24.26	69.43	242.56
Extreme sensitizers according to LLNA								
4-Nitrobenzyl bromide	100-11-8	0.05	>7000	0.0	75.8	1.31	2.09	9.10
1-Chloro-2,4-dinitrobenzene	97-00-7	0.05	1.5	0.0	85.3	2.50	3.89	8.20
Tetrachloro-salicylanilide	1154-59-2	0.04	>7000	63.2	91	1.18	2.06	11.06
3-Methylcatechol	488-17-5	0.02	3.3	12.8	20.2	15.80	18.72	50.91
Bandrowski's Base	20048-27-5	0.02	1.3	12.5	95.8	0.50	1.95	12.08
p-Benzoquinone	106-51-4	0.01	1.3	1.0	9.0	6.48	32.77	104.50
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	0.009		3.7	64.9	2.3	4.79	16.9
7,12-Dimethylbenz[α]anthracene	57-97-6	0.006	>7000	100.0	98.2	1.32	>2000	36.3
Benzoyl peroxide	94-36-0	0.004	15.7	0.0	18.7	>2000	>2000	567.63
Oxazolone	15646-46-5	0.003	10.6	24.5	50.4	175.52	490.42	1370.9
Diphenylcyclopropanone	886-38-4	0.003	1.9	1.2	100.0	0.50	1.84	5.99

sensitizers only. Interestingly, however, the best correlation was observed for the combination 'average of cysteine and lysine depletion'. As had already been noted (Gerberick *et al.*, 2007),

the average of the two values from the Cys-peptide and Lys-peptide assay may, on its own, give the best discrimination of potency classes in the DPRA. In addition, this simple arithmetic

Table 2. Yes/No (1/0) rating of individual chemicals according to the specific prediction models and WoE prediction

Chemical	Case no.	LLNA EC3 values %	U937-CD86 Test	DPRA	KeratinoSens™ Assay	Weight of evidence (2 or 3 tests)
Non-sensitizers according to LLNA						
1-Bromobutane	109-65-9	NC	0	1	0	0
1-Butanol	71-36-3	NC	0	0	0	0
4-Hydroxybenzoic acid	99-96-7	NC	0	0	0	0
Diethyl phthalate	84-66-2	NC	0	0	0	0
Glycerol	56-81-5	NC	0	0	0	0
Hexane	110-54-3	NC	0	0	0	0
Methylparaben	99-76-3	NC	1	0	1	1
Octanoic acid	124-07-2	NC	1	0	0	0
Propylene glycol	57-55-6	NC	0	0	0	0
Sulphanilic acid	121-57-3	NC	0	0	0	0
Benzaldehyde	100-52-7	NC	1	0	1	1
Coumarin	91-64-5	NC	0	0	1	0
Propyl paraben	94-13-3	NC	1	0	1	1
Streptomycin sulfate	3810-74-0	NC	0	0	0	0
Sulphanilamide	63-74-1	NC	0	0	0	0
4-Methoxyacetophenone	100-06-1	NC	1	0	1	1
6-Methylcoumarin	92-48-8	NC	0	0	1	0
Benzalkonium chloride	8001-54-5	NC	1	0	0	0
Vanillin	121-33-5	NC	1	0	0	0
Ethyl vanillin	121-32-4	NC	0	0	1	0
Nonanoic acid	112-05-0	NC	1	0	0	0
Tartaric acid	87-69-4	NC	0	0	0	0
4-Carboxyphenylacetate	2345-34-8	NC	0	1	0	0
Benzoic acid	65-85-0	NC	0	1	0	0
3-Chloro-4-methoxybenzaldehyde	4903-09-7	NC	1	1	0	1
Octanenitrile	124-12-9	NC	0	0	0	0
3-Phenoxypropionitrile	3055-86-5	NC	1	1	0	1
Benzocaine	94-09-7	NC	1	1	1	1
2-Acetyl-cyclohexanone	874-23-7	NC	1	1	1	1
Clofibrate	637-07-0	NC	0	0	0	0
Ethyl benzoylacetate	94-02-0	NC	0	0	1	0
Saccharin	81-07-2	NC	0	0	0	0
Vinylidene dichloride	75-35-4	NC	0	0	0	0
Isopropyl myristate ^a	110-27-0	44.0	0	0	0	0
Ethanol-2-butoxy-, acetate	112-07-2	NC	0	1	0	0
Sodium lauryl sulphate	151-21-3	14.0	0	0	0	0
Chlorobenzene	108-90-7	NC	0	0	0	0
Isopropanol	67-63-0	NC	0	0	0	0
Lactic acid	50-21-5	NC	0	0	0	0
Methyl salicylate	119-36-8	NC	0	0	0	0
Salicylic acid	69-72-7	NC	0	1	0	0
1-Iodohexane	638-45-9	NC	0	1	1	1
2-Hydroxypropyl methacrylate	923-26-2	NC	1	1	1	1
Weak sensitizers according to LLNA						
Aniline	62-53-3	89.0	1	0	0	0
Diethyl acetaldehyde	97-96-1	76.0	0	1	1	1
Hydroxycitronellal	107-75-5	33.0	1	1	1	1
Butyl glycidyl ether	2426-08-6	31.0	1	1	1	1
Penicillin G	61-33-6	30.0	1	1	0	1
Linalool	78-70-6	30.0	1	0	0	0
Ethyl acrylate	140-88-5	28.0	1	1	1	1
Ethylene glycol dimethacrylate	97-90-5	28.0	1	1	1	1
2,2,6,6-Tetramethyl-3,5-heptanedione	1118-71-4	27.0	0	0	0	0

(Continues)

Table 2. (Continued)

Chemical	Case no.	LLNA EC3 values %	U937-CD86 Test	DPRA	KeratinSens™ Assay	Weight of evidence (2 or 3 tests)
5-Methyl-2,3-hexanedione	13706-86-0	26.0	1	1	1	1
Geraniol	106-24-1	26.0	1	0	1	1
Imidazolidinyl urea	39236-46-9	24.0	1	0	1	1
cis-6-nonenal	2277-19-2	23.0	1	0	1	1
Cyclamen aldehyde	103-95-7	22.0	1	1	1	1
Cinnamyl Alcohol	104-54-1	21.0	1	1	1	1
Methyl acrylate	96-33-3	20.0	1	1	1	1
Pentachlorophenol	87-86-5	20.0	0	1	0	0
Lilial	80-54-6	19.0	1	1	0	1
1-Iodoheptadecane	544-77-4	19.0	1	1	1	1
4-Allylanisole	140-67-0	18.0	0	1	0	0
Lylal	31906-04-4	17.0	1	1	1	1
Benzyl benzoate	120-51-4	17.0	0	0	1	0
Abietic acid	514-10-3	15.0	1	1	1	1
Oxalic acid anhydrous	144-62-7	15.0	0	0	1	0
Eugenol	97-53-0	13.0	1	1	0	1
Farnesal	502-67-0	12.0	0	1	1	1
2,3-Butanedione	431-03-8	11.0	1	1	1	1
Butyl acrylate	141-32-2	11.0	1	1	1	1
α -Amyl cinnamic aldehyde	122-40-7	11.0	1	0	0	0
Hexyl cinnamic aldehyde	101-86-0	11.0	1	0	1	1
2-Ethylhexyl acrylate	103-11-7	10.0	0	1	1	1
2-Methylundecanal	110-41-8	10.0	1	1	1	1
Moderate sensitizers according to LLNA						
1-(p-Methoxyphenyl)-1-penten-3-one	104-27-8	9.3	1	1	1	1
Citral	5392-40-5	9.2	1	1	1	1
Trimellitic anhydride	552-30-7	9.2	0	1	0	0
Perillaldehyde	2111-75-3	8.1	1	1	1	1
Methyl methanesulphonate	66-27-3	8.1	1	1	1	1
1,1,3-Trimethyl-2-formylcyclohexa-2,4-diene	116-26-7	7.5	1	1	1	1
Dihydroeugenol	2785-87-7	6.8		0	1	1
2-Phenylpropionaldehyde	93-53-8	6.3	1	1	1	1
Diethyl maleate	141-05-9	5.8	1	1	1	1
2-methoxy-4-methylphenol	93-51-6	5.8	1	0	0	0
3,4-Dihydrocoumarin	119-84-6	5.6	0	1	0	0
trans-2-Hexenal	6728-26-3	5.5	0	1	1	1
Resorcinol	108-46-3	5.5	1	0	0	0
Tetramethylthiuram disulfide	137-26-8	5.2	1	1	1	1
α -methyl-trans-cinnamaldehyde	101-39-3	4.5	1	1	1	1
Squaric acid	2892-51-5	4.3	0	1	0	0
2,4-Heptadienal	5910-85-0	4.0		1	1	1
Benzylidene acetone	122-57-6	3.7	1	1	1	1
3-Propylidenephthalide	17369-59-4	3.7	0	1	0	0
5-Amino-2-methylphenol	2835-95-2	3.4	1	1	1	1
3-Aminophenol	591-27-5	3.2	1	0	0	0
Cinnamic aldehyde	104-55-2	3.0	1	1	1	1
Phenylacetaldehyde	122-78-1	3.0	0	1	1	1
Methyl 2-nonynoate	111-80-8	2.5	0	1	1	1
trans-2-decenal	3913-71-1	2.5	1	1	1	1
1,2-Benzisothiazolin-3-one	2634-33-5	2.3	1	1	1	1
1-Bromohexane	111-25-1	2.3	0	1	1	1
3-Dimethylamino propylamine	109-55-7	2.2	0	0	1	0
Ethylenediamine free base	107-15-3	2.2	1	0	1	1

(Continues)

Table 2. (Continued)

Chemical	Case no.	LLNA EC3 values %	U937-CD86 Test	DPRA	KeratinoSens™ Assay	Weight of evidence (2 or 3 tests)
2,4-Dinitrobenzenesulfonic acid, sodium salt	885-62-1	2.0	1	1	1	1
2-Methyl-2H-Isothiazol-3-one	2682-20-4	1.9	1	1	1	1
2-Mercaptobenzothiazole	149-30-4	1.7	1	1	1	1
4-Vinyl pyridine	100-43-6	1.6	1	1	1	1
Bisphenol A-diglycidyl ether	1675-54-3	1.5	1	1	1	1
4-Amino-m-cresol	2835-99-6	1.5	1	1	1	1
2-Hydroxyethyl acrylate	818-61-1	1.4	1	1	1	1
Glyoxal	107-22-2	1.4	1	1	1	1
1-Phenyl-1,2-propanedione	579-07-7	1.3	1	1	1	1
1-Naphthol	90-15-3	1.3	1	1	1	1
Isoeugenol	97-54-1	1.2	1	1	1	1
Strong sensitizers according to LLNA						
1,2-Dibromo-2,4-dicyanobutane	35691-65-7	0.90	0	1	1	1
Squaric acid diethyl ester	5231-87-8	0.90	0	0	0	0
1,2-cyclohexane dicarboxylic anhydride	85-42-7	0.84	1	1	0	1
4-(Methylamino)phenol sulfate	55-55-0	0.80	0	1	1	1
Formaldehyde	50-00-0	0.61	1	1	1	1
4-(N-Ethyl-N-2-methan-sulphonamido-ethyl)-2-methyl-1,4-phenylenediamine	25646-71-3	0.60	1	1	1	1
N,N-dimethyl-4-nitrosoaniline	138-89-6	0.48	1	1	1	1
2-Aminophenol	95-55-6	0.40	1	1	1	1
2-Nitro-1,4-phenylenediamine	5307-14-2	0.40	1	1	1	1
2,5-Diaminotoluene sulphate	615-50-9	0.40	1	1	1	1
Propyl gallate	121-79-9	0.32	1	1	1	1
Lauryl gallate	1166-52-5	0.30	0	1	1	1
Benzyl bromide	100-39-0	0.20	1	1	1	1
Phthalic anhydride	85-44-9	0.16	1	1	0	1
Maleic anhydride	108-31-6	0.16	1	1	0	1
1,4-Phenylenediamine	106-50-3	0.16	1	1	1	1
Fluorescein-5-isothiocyanate	3326-32-7	0.14		1	1	1
Hydroquinone	123-31-9	0.11	1	1	1	1
Glutaraldehyde	111-30-8	0.10	1	1	1	1
Extreme sensitizers according to LLNA						
4-Nitrobenzyl bromide	100-11-8	0.05	0	1	1	1
1-Chloro-2,4-dinitrobenzene	97-00-7	0.05	1	1	1	1
Tetrachloro-salicylanilide	1154-59-2	0.04	0	1	1	1
3-Methylcatechol	488-17-5	0.02	1	1	1	1
Bandrowski's Base	20048-27-5	0.02	1	1	1	1
p-Benzoquinone	106-51-4	0.01	1	1	1	1
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	0.01		1	1	1
7,12-Dimethylbenz[α]anthracene	57-97-6	0.01	0	0	1	0
Benzoyl peroxide	94-36-0	0.004	1	1	0	1
Oxazolone	15646-46-5	0.003	1	1	1	1
Diphenylcyclopropenone	886-38-4	0.003	1	1	1	1

combination may be the only one which integrates the two potentially separate MIE 'Lys-residue binding' and 'Cys-residue binding'.

Discussion

Analysis of the data for hazard assessment

Most *in vitro* tests for skin sensitization were developed and first validated vs. a set of chemicals, for which strong evidence

regarding their sensitization potential is available, i.e. congruent data from at least two sources, namely LLNA, guinea pig tests and/or human evidence (Casati *et al.*, 2009; Emter *et al.*, 2010). Often, for these thoroughly investigated chemicals, the experimental data from animals were even based on multiple measurements, especially repeated tests in the LLNA (ICCVAM, 2008). As the analysis of the performance of *in vitro* tests is expanded to larger datasets as presented here, there are less *in vivo* data to compare to. Thus many of the chemicals investigated here had either *in vivo* evidence from a single LLNA test

Table 3. Cooper statistics against the LLNA result for individual tests and for WoE 'positive if 2 of 3 tests positive'

	U937-CD86 Test	DPRA	KeratinoSens™ Assay	WoE (2 or 3 tests)
Sensitivity	71	82	79	82
Specificity	70	74	72	77
Accuracy	71	80	77	81
n	141	145	145	145

or, in some cases, contradicting predictions from animal and human tests. Not surprisingly therefore, the accuracy of all three tests on this enlarged database is slightly lower as compared with the predictivity reported for the more limited chemical collections investigated before (Emter *et al.*, 2010; Gerberick *et al.*, 2007). This lowered predictivity when moving to larger datasets can theoretically be attributed to (i) limitations in the applicability domains of the *in vitro* test, (ii) experimental inaccuracies in the LLNA dataset coming from multiple sources and in many cases from individual measurements only and (iii) limitations in the LLNA-only predictions. Based on the analysis presented in Tables 4 and 5, we can detect in this dataset both limitations in the applicability domain of the *in vitro* tests and also possible limitations in the LLNA-dataset.

It should be kept in mind, that the WoE for hazard assessment may be constructed giving different weight to different observations including the quantitative dose–response readouts. To note a simple example: a strong positive result in one assay, mimicking an essential step, may be sufficient for a positive overall rating, whereas a weakly positive response in one of three tests would not be sufficient. Thus we strongly advocate for a quantitative approach to data integration taking into account dose–response information such as the one presented in the accompanying paper (Jaworska *et al.*, 2013). Nevertheless, we also think it is warranted to perform the '2 of 3 WoE' analysis based on the individual prediction models followed by a careful analysis of the cases with two or even three wrong *in vitro* predictions for three reasons: (i) it allows for a comparison of the results obtained on a smaller dataset published recently (Bauch *et al.*, 2012), (ii) it may indicate general weaknesses of the current testing battery and (iii) it can indicate limitations of evaluating alternative test information when comparing against LLNA results only.

On the dataset of 54 chemicals, Bauch *et al.* (2012) reported for the DPRA an accuracy of 79% vs. LLNA, for KeratinoSens™ 81% and for the U937 assay 74%. The respective values for our extended dataset on 145 chemicals are: 80% (DPRA), 77% (KeratinoSens) and 71% (U937). With the 'at least 2 of 3 WoE' approach, Bauch *et al.* (2012) reported an accuracy vs. LLNA of 83% whereas we find 81% for our extended dataset. Thus, when

Table 4. Discussion of false positives occurring in parallel in several tests

Chemical	Case no.	Discussion
Group1: false-positive with no conflicting human data		
3-Chloro-4-methoxybenzaldehyde	4903-09-7	Positive U937 and DPRA, no human or other data
3-Phenoxypropionitrile	3055-86-5	Positive U937 and DPRA, no human or other data
4-Methoxyacetophenone	100-06-1	Positive U937 and KeratinoSens, no human or other data
Group2: Very weak human sensitizers based on patch tests		
Methylparaben	99-76-3	Positive U937 and KeratinoSens, rare positive patch test reactions (Uter <i>et al.</i> , 2010)
Propyl paraben	94-13-3	Positive U937 and KeratinoSens, rare positive patch test reactions (Uter <i>et al.</i> , 2010)
Group3: Human sensitizers according to predictive tests		
Benzaldehyde	100-52-7	Positive U937 and KeratinoSens, positive HRIPT at 2760 µg/cm ² (Natsch <i>et al.</i> , 2012)
Benzocaine	94-09-7	All test positive, ICCVAM database lists positive HMT and HRIPT, with mean at 1228 µg/cm ² , variable in LLNA and GPMT (Basketter <i>et al.</i> , 1995)
Group4: Clearly peptide reactive molecules with no human predictive tests to compare against		
2-Acetyl-cyclohexanone	874-23-7	Positive in all three tests, no human data, peptide adduct forming in LC-MS test
1-Iodoheptane	638-45-9	Positive DPRA and KeratinoSens, no human data, peptide adduct forming in LC-MS test
2-Hydroxypropyl methacrylate	923-26-2	Positive in all three tests, peptide adduct forming in LC-MS test; frequent positive human patch tests (Kanerva <i>et al.</i> , 1997; Lazarov, 2007)

Table 5. Discussion of false negatives occurring in parallel in several tests

Chemical	Case no.	LLNA EC3	Discussion
Group 1			
Oxalic acid anhydrous	144-62-7	15	No human data, weakly positive only in KeratinoSens, not peptide adduct forming in LC-MS test
2,2,6,6-Tetramethyl-3,5-heptanedione	1118-71-4	27	No human data, all tests negative, not peptide adduct forming in LC-MS test
Benzyl benzoate	120-51-4	17	Very weak in humans with no induction in HRIPT ^a at 59'000 µg/cm ² , positive only in KeratinoSens, not peptide adduct forming in LC-MS test
α-Amyl cinnamic aldehyde	122-40-7	11	Very weak in humans with no induction in HRIPT ^a at 23'600 µg/cm ² , positive only in U937, not peptide adduct forming in LC-MS test
Linalool	78-70-6	30	Very weak in humans with no induction in HRIPT ^a at 15'000 µg/cm ² , positive only in U937, not peptide adduct forming in LC-MS test
Pentachlorophenol	87-86-5	20	Weakly positive in humans (Kligman, 1966), positive only in DPRA, not peptide adduct forming in LC-MS test
Group 2			
Trimellitic anhydride	552-30-7	9	Only DPRA positive, higher reactivity with lysine-peptide
3-Propylidenephthalide	17369-59-4	4	Only DPRA positive, higher reactivity with lysine-peptide
3,4-Dihydrocoumarin	119-84-6	6	Only DPRA positive, higher reactivity with lysine-peptide
Squaric acid diethyl ester	5231-87-8	1	Negative in all assays, but known to be reactive with Lysine in proteins (Kamath <i>et al.</i> , 1996; Wurm <i>et al.</i> , 2012)
Squaric acid	2892-51-5	4	Only DPRA positive
Group 3			
4-Allylanisole	140-67-0	18	putative prohaptens, positive only in DPRA
Resorcinol	108-46-3	6	putative prohaptens, positive only in U937
3-Aminophenol	591-27-5	3	putative prohaptens, positive only in U937
2-methoxy-4-methylphenol	93-51-6	6	putative prohaptens, positive only in U937
7,12-Dimethylbenz[α]anthracene	57-97-6	0	putative prohaptens, positive only in KeratinoSens
3-Dimethylamino propylamine	109-55-7	2	putative prohaptens, positive only in KeratinoSens
Aniline	62-53-3	89	putative prohaptens, positive only in U937, positive in humans and guinea pigs

^aThe HRIPT data were kindly provided by RIFM, the Research Institute for Fragrance materials.

compared with LLNA, the two studies provide very similar overall results even if our analysis is based on an almost threefold enlarged dataset. Bauch *et al.* (2012) reported a clearly better predictivity for all three tests and for the WoE prediction when compared against human data. The accuracy was at 86% for the DPRA, at 80% for KeratinoSens™ and at 86% for the U937 test. For the '2 of 3 WoE', the overall accuracy even reached 94% vs. human data. A similar analysis on our extended dataset is not possible, as for a large part of the database the human data is simply not available. However, it is worthwhile to take human evidence into account when discussing the cases with false predictions in two or three of the assays. These cases are individually discussed in Table 4 and Table 5. For this discussion we also mention additional unpublished evidence on covalent adduct formation with peptides obtained with an LC-MS based method (Natsch and Gfeller, 2008).

The 10 chemicals which are false-positive in two of the three assays (Table 4) may be grouped into four small groups. For 3-chloro-4-methoxybenzaldehyde, 4-methoxyacetophenone and 3-phenoxypropiononitrile we do not have human data or

contradicting evidence and would just label them as false-positives. For the parabens the human predictive data indicate no sensitization potential whereas positive patch test reactions are regularly found in the clinic. However, taking the frequent use into account, these are relatively rare (Schnuch *et al.*, 2011) and it had been argued that parabens are too weak to merit classification as sensitizers (Basketter *et al.*, 2006). For benzaldehyde and benzocaine human predictive tests (HRIPT and/or HMT) predict a weak but significant sensitization potential. For these two chemicals we must thus consider the possibility of a false-negative LLNA result and a correct *in vitro* prediction. The three chemicals 2-acetyl-cyclohexanone, 1-iodohexane and 2-hydroxypropyl methacrylate were positive in two or even all three tests, and they were also confirmed in an independent test (our unpublished data) to form covalent adducts with peptides. In the case of 2-hydroxypropyl methacrylate frequent positive human patch tests clearly indicate a sensitization potential. For these three chemicals, the positive *in vitro* evidence and the confirmed potential to modify peptides could in our view overrule the negative LLNA result.

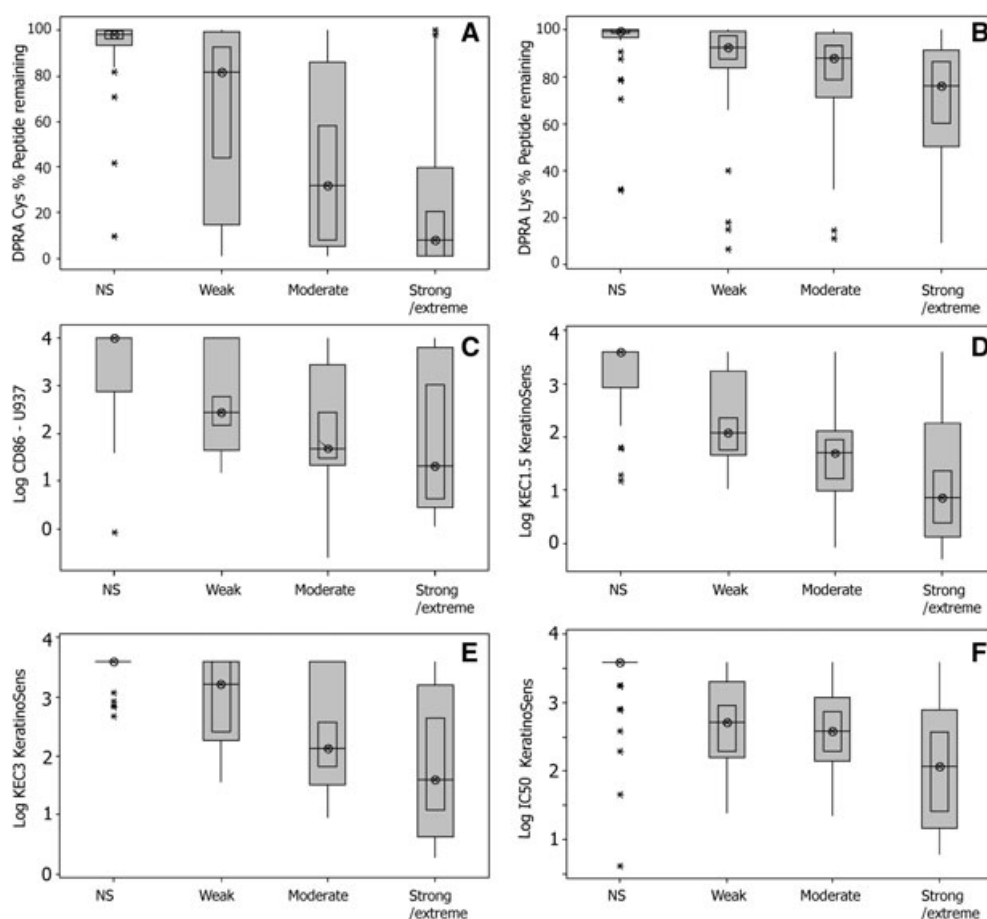


Figure 3. Distribution of the six quantitative read-outs from the alternative assays in the four LLNA classes. Shown are Box plots with a median and interquartile range.

Table 6. Spearman's rank correlations for potency vs. individual endpoints

Test	Endpoint	rho against LLNA classes	P-value against LLNA classes	rho against LLNA values	P-value against LLNA values
U937-CD86	CD86	-0.492	<0.0001	0.502	<0.0001
DPRA	CYS	-0.600	<0.0001	0.608	<0.0001
	LYS	-0.436	<0.0001	0.411	<0.0001
	Cys + Lys	-0.650	<0.0001	0.648	<0.0001
KeratinoSens™	EC1.5	-0.608	<0.0001	0.610	<0.0001
	EC3	-0.594	<0.0001	0.597	<0.0001
	IC50	-0.531	<0.0001	0.579	<0.0001

The 17 'double false-negative' chemicals in Table 5 were attributed to three groups. The chemicals in group 1 are all weak in the LLNA (EC3 11–30%) and we have either no human data, or the predictive human data indicate no sensitization potential even when tested at very high test concentrations. Given the very low frequency of positive patch test reactions in spite of widespread use in topical products for benzyl benzoate, α -amyl cinnamic aldehyde and linalool (Uter *et al.*, 2010), a negative rating and no classification based on *in vitro* data appears acceptable, or even preferable as compared with the classification based on the LLNA. In addition, it should be kept in mind that irritation can be a confounding

factor in the LLNA (Ball *et al.*, 2011), and the published EC3 values alone can give no information on the potential irritation observed in the assay, which could only be judged from the unpublished individual study reports. Group 2 contains chemicals with higher reactivity for lysine as compared with cysteine reactivity. Often this can be observed for acyl-transfer agents which spontaneously transfer acyl groups onto lysine. The *in vitro* battery appears overall more sensitive to cysteine-reactive chemicals, with the DPRA–cysteine readout, the KeratinoSens™ and also CD86-test being more sensitive to Cys-reactive chemicals. Therefore, lysine reactivity in the DPRA is not sufficient for a positive rating of these chemicals in the

simple '2 of 3 WoE' rating. This result indicates that specific reactivity towards lysine in absence of other evidence probably should receive a stronger weighting for a positive rating in an ITS. In the case of squaric acid diethyl ester, the DPRA-Lys was not positive, in spite of the fact that this chemical is widely known for its ability to specifically react with lysine in proteins (Kamath *et al.*, 1996; Wurm *et al.*, 2012). The reason for this may need further investigation.

The third group is comprised of seven chemicals which earlier were predicted as putative prohaptens (Kern *et al.*, 2010). These were all positive in one of the three *in vitro* tests, but are rated negative, if evidence from one positive test is not sufficient. A better ability to recognize prohaptens and discriminate them from non-sensitizers with an occasional false-positive result in a single test will therefore be required in the future. However it should also not be left unmentioned, that a significant number of putative pro-haptens would be correctly predicted by the "2-of 3" WoE, and these include geraniol, cinnamyl alcohol, abietic acid, eugenol, 5-amino-2-methylphenol, ethylenediamine, 4-amino-m-cresol, 1-naphthol and 2-aminophenol.

Correlation of the quantitative data to potency

Potency predictions are important on the one hand for the recently introduced GHS classification, which goes beyond hazard classification by defining two classes of sensitizers (weak and strong). Even more important is potency information for an appropriate risk assessment. It is difficult to manufacture products without any skin sensitizing molecules, but with a careful risk assessment weak sensitizers can be used at a safe dose if potency data are available. This assessment is currently strongly based on the LLNA EC3 values in the majority of cases when human potency data are not available (Api *et al.*, 2008). Without an ability of non-animal testing schemes to support this assessment, the risk assessment on new ingredients will be seriously hampered.

In order to understand whether, and to which extend the endpoints from the different alternative tests correlate to potency as measured in the LLNA, the read-outs reported in Table 1 were further analyzed. Both Spearman's rank correlation calculations and the visualization by box plots indicate that the different read-outs have, to a varying degree, a correlation with the LLNA potency classes and/or the actual LLNA EC3 values. These correlations are far from perfect and indicate a significant spread for the quantitative data within individual potency classes. Thus, while the *in vitro* endpoints all contain potency information, this correlation is not sufficient for a 1:1 potency assessment. One approach is to do this potency assessment in a global ITS (integrating all the data in one model for all chemical classes) as shown in the parallel paper by Jaworska *et al.* (2011). The other option (which may be used as a complementary approach) is to use *in vitro*-based read across whenever molecules with related structures and available animal and *in vitro* data are known. The presented database may also find use for this purpose.

However, when assessing the correlations between the *in vitro* parameters and the LLNA result one should also keep in mind that also LLNA and human data only correlate partly with each other, with an R^2 in log-log linear regression between LLNA and human data between 0.45 and 0.75 reported in different studies (ICCVAM, 2011). On the other hand, these relatively low

correlations between LLNA and human data are certainly also due to limited quality of the human data set.

Gaps in the current testing battery and limitations of the applicability domain

After taking into account the discussion of the 'double false-negatives' and the 'double false-positives', the overall predictivity of the current battery is promising. However, this analysis also indicated potential improvements. Thus, the inclusion of an assay to better detect prohaptens, and also to directly recognize these in an assay run in the presence and absence of a metabolic activation system will be helpful, as a number of putative prohaptens are recognized by only one assay. An assay to specifically recognize prohaptens has recently been proposed: in a further development of the DPRA, a H_2O_2 /horse radish peroxidase activation system was included to assess the effect of enzymatic oxidation on peptide binding (Gerberick *et al.*, 2009; Troutman *et al.*, 2011). In addition, this assay also includes a dose-response analysis and LC-MS based analytics.

The other gap might be limitations in potency assessment of the individual tests. These may partly be overcome by combining the data in an ITS and to which extend potency assessment is possible with an ITS based on the current alternative testing battery and with currently available *in silico* models now needs a careful assessment. At the same time we should be aware that we are missing standardized tests for some of the steps of the AOP. Thus, to note a key example, we currently have no routine assay to determine induction of T-cell proliferation. Currently, we do not know how much the T-cell repertoire might be skewed towards specific classes of chemical structures, and whether potency is strongly affected by frequency of matching T-cell receptors in the repertoire. Thus, we cannot yet anticipate how important this additional endpoint will be for potency assessment. Finally, all tests in the current battery are performed in solution, and do not mimic topical application. This will be limiting especially for molecules with a low water solubility. The current dataset contains a limited number of chemicals with a $cLogP > 4$, and in general, these molecules are the most difficult to test in test systems run in aqueous solution.

Finally, having all tests available in one laboratory in order to perform the full assessment may be another hurdle for smaller companies and test laboratories. While the DPRA based on HPLC with UV detection and the KeratinoSens™ assay based on luminescence use broadly available and relatively affordable technologies, the CD86 analysis requires more sophisticated high-throughput flow cytometry. Nevertheless, as shown in the parallel paper (see Jaworska *et al.*, 2011), in an ITS based on a Bayesian approach, partial evidence is often sufficient, and the third test might only be performed if the evidence from the first two tests is not sufficient. The testing in the third test for these specific cases might then need to be performed by more specialized laboratories.

Conclusions

The compiled data on the skin sensitization *in vitro* endpoints indicate that overall the predictivity is not reduced when moving from a dataset on 54 chemicals published before to a dataset on 145 chemicals. The analysis on false-negatives and false-positives indicate that while all these assays have their limitations in predictivity, part of the false-positive and

false-negatives may also come from false predictions in the LLNA or database inaccuracies.

Furthermore, the highly significant correlation between the dose–response data and LLNA EC3 / potency classes show, that the parameters measured in these assays, beyond hazard identification, can also yield partial information on potency.

The extended dataset of this study will find use to construct and improve ITS approaches and to perform *in vitro* based read across for novel chemicals which are tested *in vitro* and which can then be compared to related chemicals in this database. It will also benefit the assessment of new assays, as with the emergence of many new *in vitro* testing proposals for skin sensitization (Mehling *et al.*, 2012) it will be crucial to rapidly find out which test can yield additional information and fill gaps. Furthermore, this database can be analyzed from a chemical perspective to obtain an overview on which types of chemicals have not yet been sufficiently tested to delineate the applicability domain of the existing tests. Thus, to note just one example, the current database does not contain silicone-derived molecules, and the utility of the tests for such previously untested classes of molecules needs further assessment.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article

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