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# Putting the parts together: Combining *in vitro* methods to test for skin sensitizing potentials

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ABSTRACT

Allergic contact dermatitis is a common skin disease and is elicited by repeated skin contact with an allergen. In the regulatory context, currently only data from animal experiments are acceptable to assess the skin sensitizing potential of substances. Animal welfare and EU Cosmetic Directive/Regulation call for the implementation of animal-free alternatives for safety assessments. The mechanisms that trigger skin sensitization are complex and various steps are involved. Therefore, a single *in vitro* method may not be able to accurately assess this endpoint. Non-animal methods are being developed and validated and can be used for testing strategies that ensure a reliable prediction of skin sensitization potentials. In this study, the predictivities of four *in vitro* assays, one *in chemico* and one *in silico* method addressing three different steps in the development of skin sensitization were assessed using 54 test substances of known sensitizing potential. The predictivity of single tests and combinations of these assays were compared. These data were used to develop an *in vitro* testing scheme and prediction model for the detection of skin sensitizers based on protein reactivity, activation of the Keap-1/Nrf2 signaling pathway and dendritic cell activation.



## 1. Introduction

As the interface between the environment and the body, the skin is continuously exposed to environmental insults, pathogens and xenobiotics. In particular, consumers and workers are often exposed to chemicals via cosmetic and household products or in industrial settings on a daily basis and to a significant degree. One of the adverse effects that can occur as a result of skin exposure to xenobiotics is contact sensitization, the clinical manifestation of which is allergic contact dermatitis (ACD). The principle objective of toxicological testing is to provide a basis for the assessment of hazards and to identify potential risks from use and handling of products, such as chemicals or cosmetic formulations, thus ensuring that adverse effects to human health do not occur. The evaluation of the sensitization potential of a substance has therefore been of central importance for hazard and risk assessments for decades. Currently, most toxicological endpoints in the regulatory context are assessed via animal testing. This is also the case for the sensitization potentials for which generally only the animal studies described in OECD 406 (guinea pig tests according to Buehler or Magnusson & Kligman) or OECD 429 and OECD 442 (murine local lymph node assays, LLNA) are accepted by the regulatory bodies.

The increasing emphasis on the ethics of animal testing has manifested itself in a regulatory context in the recent chemicals legislation on the registration, evaluation, authorization and restriction of chemicals (REACH (EU, 2006)) and even more so in

*Abbreviations:* ARE, antioxidant response element; AUC, area under the curve; C.C, control cells; CV75, concentration reducing viability to 75%; DC, dendritic cells; DMSO, dimethyl sulfoxide; DNCB, 1-chloro-2,4-dinitrobenzene; DPRA, Direct Peptide Reactivity Assay; ECHA, European Chemicals Agency; ECVAM, European Centre for the Validation of Alternative Methods; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; h-CLAT, human Cell Line Activation Test; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Keap1, Kelch-like ECH-associated protein 1; ITS, integrated testing strategy; LLNA, local lymph node assay; MCI/MI, mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one; MFI, mean fluorescence intensity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; mMUSST, modified myeloid U937 dendritic cell activation-based skin sensitization test); NADPH, nicotinamide adenine dinucleotide phosphate; Nrf2, nuclear factor (erythroid-derived 2)-like factor 2; OECD, Organisation for Economic Cooperation and Development; PBS, phosphate buffered saline; PI, propidium iodide; QSAR, quantitative structure-activity relationship; REACH, Registration Evaluation Authorisation and Restriction of Chemicals; RLU, relative luminescent unit; RP HPLC, reverse phase high performance liquid chromatography; RT, room temperature; SDS, sodium dodecyl sulfate; T.C, treated cells; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

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the amendments of the European Cosmetics Directive (Council Directive 76/768/EEC, 1976; EU, 2003). With the advent of REACH, an enormous number of animal tests were estimated to be necessary to address the various toxicological endpoints (Hartung and Rovida, 2009). Hazard assessment of the sensitization potential is one of the endpoints in the base set needed for all chemicals registered under REACH. REACH mandates that every effort must be made to avoid animal testing and that animal testing should only be conducted as a last resort. Usage of validated and adequately documented *in vitro* methods for the adaptation of the standard testing regime is authorized in Annex XI of the REACH legislation. Recently, the European Chemicals Agency (ECHA) published a guideline on the use of alternative to animal testing under REACH to address this issue (European Chemicals Agency, 2011). The 7th Amendment of the Cosmetics Directive (Council Directive 76/768/EEC, 1976; EU, 2003) stipulates a progressive phasing out of animal tests for the purpose of safety assessments of cosmetics and includes a concomitant marketing ban. An animal testing ban on finished cosmetic products has already been in place since 2004. A testing ban on chemicals for the purposes of the Cosmetics Directive for acute toxicity testing has been in place since 2009 with the final deadline for animal testing for the more complex toxicological endpoints including skin sensitization foreseen for 2013. A full marketing ban for cosmetics containing ingredients tested for the purpose of the Cosmetics Directive/Regulation regardless of whether non-animal alternative methods exist is foreseen for 2013. For the safety of consumers and workers, it is therefore important to develop prediction models that do not involve animal testing and yet still reliably assess toxicological endpoints.

The ethical and legislative demands have triggered intense research in the field of alternative methods (e.g. reviewed in Adler et al., 2011; Goebel et al., 2012; Mehling et al., in press). With the current innovation in this field, a number of scientific advancements have been made to assess skin sensitization. Various promising methods have been developed and four methods are currently in the process of undergoing formal validation at the European Centre for Validation of Alternative Methods (ECVAM). These test methods have already been extensively studied and address a number of key steps in the skin sensitization process: Protein-binding/haptenization (e.g. the Direct Peptide Reactivity Assay, DPRA) (Gerberick et al., 2004), induction of the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor (erythroid-derived 2)-like factor 2 (Nrf2) pathways in keratinocytes (e.g. KeratinoSens™ assay) (Emter et al., 2010), and the activation of antigen presenting cells such as dendritic cell-like cell lines (modified myeloid U937 dendritic cell activation test, (mMUSST) or the human Cell Line Activation Test, h-CLAT) (Ashikaga et al., 2006; Bauch et al., 2011; Nukada et al., 2011; Sakaguchi et al., 2006, 2007). However, due to the complexity of the sensitization process, it is unlikely that a single assay will be sufficient to adequately assess the sensitization potential (Corsini et al., 2009; dos Santos et al., 2009). Therefore, results from test batteries will have to be incorporated into integrated testing strategies (ITS) in order to obtain the best possible predictivity for skin sensitization without the use of animals (Jaworska et al., 2011).

A number of substances, e.g. eight surfactants: (Ball et al., 2011) and 23 substances (Bauch et al., 2011) have been tested in parallel in the above mentioned assays. The purpose of the present study was to expand the data base of comparative testing available to the scientific community and to describe the path to a prediction model for the assessment of the skin sensitizing potential which could be used in a regulatory setting. For this purpose, the DPRA, KeratinoSens™, h-CLAT and mMUSST assays, which address three different key events of the skin sensitization process, namely peptide binding, activation of Keap-1/Nrf2 signaling pathway and dendritic cell activation, were performed with 54 test substances. Taking into account that certain chemicals require metabolic activation to acquire their sensitizing potential, a selection of pro- and prehaptens of different chemical classes were included. An additional *in-house* model which gives readout on the Keap-1/Nrf2 pathway, the LuSens assay, was also evaluated. In addition, the OECD quantitative structure–activity relationship (QSAR) Toolbox Version 2.0 was used to assess protein reactivity and to gain insights into the possible mechanisms involved. The predictivity of individual assays and combinations thereof were compared with the aim of identifying a prediction model which would allow a partial or complete replacement of animal testing for sensitization testing for a wide range of substances.

## 2. Materials and methods

### 2.1. Test substances

Originally 59 test substances were selected for this study of which 5 (*Chlorobenzene, 1-bromobutane, geraniol, Tween 80 and Triton-X 100)* were excluded from further evaluation and testing due to technical reasons (see discussion). Table 1 summarizes the test substances and their properties. It includes molecular weight, purities, supplier, CAS number, chemical classes, proposed reaction mechanisms, information about known pro- or prehapten properties, human literature data, EC3 (%) value of LLNA data and the respective literature reference. The mMUSST, h-CLAT, KeratinoSens™ and DPRA data for 23 substances has been previously published (Bauch et al., 2011) and was included for further assessment of the test battery. Of the 54 substances, 34 are considered to be skin sensitizers and 19 nonsensitizers according to LLNA data (no LLNA data was available for one substance); 21 of the substances used are performance standards recommended to assess the reliability of modifications made to the local lymph node assay test protocol (ICCVAM, 2009; OECD, 2010).

### 2.2. LLNA data and human data

For all 54 substances, results from animal tests, primarily from the murine LLNA, and/or human data were available and described in the literature. (Andersen and Frankild, 1997; Aptula and Roberts, 2006; Basketter et al., 2008, 1999b; Casati et al., 2009; Gerberick et al., 2001, 2005; Hansson and Thoerneby-Andersson, 2003; Kimber et al., 2003; Kligman, 1966; Kreiling et al., 2008; Natsch et al., 2008; OECD, 2010; Patlewicz et al., 2008; Robinson, 1989; Ryan et al., 2000; Schneider and Akkan, 2004; SSCP/0863/05 2005; Uter et al., 2007). No LLNA data were available for hexadecyltrimethylammonium bromide and glucose, whereas no suitable human patch test data were available for 2,4,6-trinitrobenzensulfonic acid, 2,3-butandione and glucose. For glucose it was assumed to be negative in humans and mice as it is part of cellular metabolism and exposure is high. Where available, human data were used to compare with the LLNA and assessment of sensitization potential was based solely on the data reported for the available clinical studies and the assessments made by other authors (Basketter et al., 1999a,b; De Groot et al., 1994; Rietschel and Fowler, 1995). Results and the literature references are listed in Table 1.

### 2.3. OECD QSAR Toolbox Version 2.0

The protein binding module contained in the OECD QSAR Toolbox (Version 2.0, 2010, http://www.oecd.org/document/54/0,3746,en_2649_34379_42923638_1_1_1_1,00.html) was used to make an initial *in silico* assessment of the sensitization potential of the test substances, to identify whether they contained any

**Table 1**
Overview of test substances.

| Substance | molecular weight | Purity (%) | Supplier | CAS # | Chemical class | Mechanism | Literatur | Pro/ Prehapten | Human | Literature | EC3 (%) | potency | LLNA | Literature |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Oxazolone | 217.22 | 98 | Sigma | 15646-46-5 | oxazole | Acyl-transfer | Patlewicz et al. (2008) | | + | Basketter et al. (1999a,b) | 0.003 | extreme | + | Kimber et al. (2003) |
| MCI/MI | 60.1 | 99 | Sigma-Aldrich | 26742-55-4/ 2682-20-4 | aryl halide | Michael acceptor | Patlewicz et al. (2008) | | + | Basketter et al. (1999a,b) | 0.009 | extreme | + | Kimber et al. (2003) |
| p-Benzoquinone | 108.1 | 99 | Sigma-Aldrich | 106-514 | quinone | Michael acceptor | Patlewicz et al. (2008) | | + | Basketter et al. (1999a,b) | 0.0099 | extreme | + | Gerberick et al. (2005) |
| l-Chloro-2,4-dinitrobenzene | 202.6 | 95 | Aldrich | 97-00-7 | nitromatic/aryl halide | SNAr | Patlewicz et al. (2008) | | + | Basketter et al. (1999a,b) | 0.049 | extreme | + | Kimber et al. (2003) |
| 4-Phenylenediamine | 108.14 | 97 | Sigma | 106-50-3 | arylamine | Pro-Michael acceptor | Patlewicz et al. (2008) | pre | + | Basketter et al. (1999a,b) | 0.16 | strong | + | Gerberick et al. (2005) |
| Propyl gallate | 2l2.2 | 99 | Fluka | 121-79-9 | benzoate (ester)/ phenolic | Acyl-transfer | author's data | | + | Basketter et al. (1999a,b) | 0.32 | strong | + | Natsch and Emter (2008) |
| 2,4,6-Trinitrobenzenesulfonic acid | 293.17 | 98 | Sigma | 2508-19-2 | nitromatic | SNAr | author's data | | | no suitable data available | 0.36 | strong | + | Robinson et al. 1989 |
| Phthalic anhydride | 148.1 | 5 | Fluka | 8544-9 | aromatic carboxylic acid anhydride | Acyl-transfer | Aptula et al. 2006 | | + | Basketter et al. 2001 | 0.36 | strong | + | Kimber et al. (2003) |
| Formaldehyde >36% (1% in DMSO) | 220.35 | 99 | Sigma | 50-00-0 | | Schiff base | Patlewicz et al. (2008) | | + | Basketter et al. (1999a,b) | 0.61 | strong | + | Gerberick et al. (2005) |
| Methyldibromo glutaronitrile | 256.94 | 98 | Aldrich | 35691-65-7 | nitrile | Pro-Michael acceptor | Casati et al. (2009) | | + | SCCP 2005 | 0.9 | strong | + | Basketter et al. (2008) |
| Isoeugenol | 388.29 | 99 | Sigma-Aldrich | 97-54-1 | phenylpropanoid | Pro-Michael acceptor | Aptula et al. 2006 | pro/pre | + | Basketter et al. (1999a,b) | 1.5 | moderate | + | Kimber et al. (2003) |
| Diethyl maleate | 129.84 | 99 | Sigma | 141-05-9 | carboxylic acid ester | Michael acceptor | Patlewicz et al. (2008) | | + | Ryan et al. (2000) | 2.1 | moderate | + | Kimber et al. (2003) |
| Ethylene diamine | 90.08 | 99 | Sigma | 107-15-3 | alkyl amine/ alkylenediamine | pro-Schiff base | Patlewicz et al. (2008) | pro | + | Basketter et al. (1999a,b) | 2.2 | moderate | + | Gerberick et al. (2005) |
| Benzylidene acetone | 93.13 | 99 | Aldrich | 122-57-6 | keton/aromat | Michael acceptor | Aptula et al. 2006 | | + | Schneider and Akkan (2004) | 3.7 | moderate | + | Gerberick et al. (2005) |
| Cobalt chloride | 152.2 | 98 | Aldrich | 7791-13-1 | heavy metal salt | coordination bonds | author's data | | + | Basketter et al. (1999a,b) | 4.8 | moderate | + | OECD (2010) |
| 2-Phenylpropionaldehyde | 134.18 | 99 | Sigma-Aldrich | 93-53-8 | aldehyde | Schiff base | author's data | | + | Schneider and Akkan (2004) | 5.911 | moderate | + | Natsch and Emter (2008) |
| a-Hexyl-cinnamic aldehyde | 216.3 | 98 | Aldrich | 101-86-0 | aldehyde | Michael acceptor | Patlewicz et al. (2008) | | + | Basketter et al. (1999a,b) | 8 | moderate | + | Kimber et al. (2003) |
| Tartaric acid | 150.09 | 98 | Aldrich | 133-37-9 | aliphatic carboxylic acid/glycol | non-binding | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | 8.7 | moderate | + | Gerberick et al. (2005) |
| 2-Mercaptobenzothiazole | 167.24 | 99.50 | Sigma | 149-304 | thiazole/heterocyclic | Acyl-transfer | Patlewicz et al. (2008) | | + | Basketter et al. (1999a,b) | 9.7 | moderate | + | Kimber et al. (2003) |
| 2,3-Butanedione | 86.09 | 10.15 | Sigma | 431-03-8 | epoxide | Schiff base | Patlewicz et al. (2008) | | | no suitable data available | 11 | weak | + | Gerberick et al. (2005) |
| Citral | 134.18 | 98.10 | BASF | 5392-40-5 | aldehyde/terpenoids | Schiff base | Patlewicz et al. (2008) | | + | Basketter et al. (1999a,b) | 13 | weak | + | Kimber et al. (2003) |
| Eugenol | 198.2 | 90 | Fluka | 97-53-0 | phenylpropanoid | Pro-Michael acceptor | Patlewicz et al. (2008) | pro | + | Basketter et al. (1999a,b) | 13 | weak | + | Gerberick et al. (2005) |
| Farnesal | 164.2 | >99.5 | Sigma | 19317-11-4 | sesquiterpenalkohol | Schiff base | Patlewicz et al. (2008) | | | no suitable literature | 13 | weak | + | Gerberick et al. (2005) |
| Sodium lauryl sulfate | 288.38 | 99 | Sigma-Aldrich | 151-21-3 | alkyl sulfate | non-binding | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | 14 | weak | + | Gerberick et al. (2005) |
| 4-Allylanisole | 148.2 | 95 | Aldrich | 140-67-0 | aryl alkyl ether // phenylpropanoid | Pro-Michael acceptor | Patlewicz et al. (2008) | pro | | no suitable data available | 18 | weak | + | Gerberick et al. (2005) |
| Hydroxycitronellal | 364.45 | 99 | Sigma | 107-75-5 | terpene aldehyde | Schiff base | Patlewicz et al. (2008) | | + | Basketter et al. (1999a,b) | 20 | weak | + | Kimber et al. (2003) |
| Phenyl benzoate | 198.22 | 99.4 | Fluka | 93-99-2 | benzoate (ester) | Acyl-transfer | Patlewicz et al. (2008) | | + | OECD (2010) | 20 | weak | + | Gerberick et al. (2005) |

(*continued on next page*)

**Table 1** (*continued*)

| Substance | molecular weight | Purity (%) | Supplier | CAS # | Chemical class | Mechanism | Literatur | Pro/ Prehapten | Human | Literature | EC3 (%) | potency | LLNA | Literature |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cinnamic alcohol | 112.6 | 97 | Alfa Aesar | 104-54-1 | aromat | Pro-Michael acceptor | Patlewicz et al. (2008) | pro | + | OECD (2010) | 21 | weak | + | Gerberick et al. (2005) |
| Imidazolidinyl urea | 172.27 | 99 | Sigma | 39236-46-9 | imidazoline | Acyl-transfer | Patlewicz et al. (2008) | | + | Basketter et al. (1999a,b) | 24 | weak | + | Gerberick et al. (2005) |
| Undecylenic acid | 148.28 | >95 | Fluka | 112-38-9 | carboxylic acid | non-binding | author's data | | + | Kreiling et al. (2008) | 25 | weak | + | Kreiling et al. (2008) |
| Ethylene glycol dimethacrylate | 60.1 | 99 | Aldrich | 97-90-5 | acrylate | Michael acceptor | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | 35 | weak | + | Kimber et al. (2003) |
| Pyridin | 92.1 | 99 | Sigma | 110-86-1 | aromatic heterocyclic | non-binding | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | 71.2 | weak | + | Gerberick et al. (2005) |
| Aniline | 160.172 | 99 | Sigma | 62-53-3 | arylamine | SNAr | Patlewicz et al. (2008) | pro | + | Basketter et al. (1999a,b) | 89 | weak | + | Gerberick et al. (2005) |
| Methyl methacrylate | 100.12 | 99 | SAFC | 80-62-6 | acrylate | Michael acceptor | author's data | | + | OECD (2010) | 90 | weak | + | OECD (2010) |
| Xylene | 106.16 | 95 | Aldrich | 1330-20-7 | aromatic hydrocarbon | non-binding | author's data | | | Basketter et al. (1999a,b) | 95.8 | weak | | OECD (2010) |
| Glycerol | 180.16 | 90 | Fluka | 56-81-5 | polyol/alcohol | non-binding | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | 100.02 | non | | Natsch and Emter (2008) |
| 1,2-Propanediol | 76.09 | 99.5 | Supelco | 57-55-6 | glycol/dihydric alcohol | non-binding | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | 100.11 | non | | Natsch and Emter (2008 |
| 4-Hydroxybenzoic acid | 138.12 | 99 | Aldrich | 99-96-7 | phenolic/benzoic acid derivative | non-binding | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | NC | non | | Gerberick et al. (2005) |
| 4-Methoxyacetophenone | 150.18 | 99 | Sigma-Aldrich | 100-06-1 | methylketon | non-binding | Patlewicz et al. (2008) | | | Gerberick et al. (2001) | NC | non | | Gerberick et al. (2005) |
| 6-Methylcoumarin | 149.59 | 99 | Sigma | 9248-8 | lactone | Michael acceptor | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | NC | non | | Gerberick et al. (2005) |
| DL-lactic acid | 172.18 | 99.80 | Sigma | 50-21-5 | alpha-hydroxy carboxylic acid/ alkanoic acid | non-binding | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | NC | non | | Gerberick et al. (2005) |
| Fumaric acid | 30.3 | 99.70 | Sigma | 110-17-8 | dicarbonic acid | non-binding | author's data | | | Hansson and Thoerneby-Andersson, 2003 | NC | non | | Kreiling et al. (2008) |
| Glucose | 154.25 | 98 | Aldrich | 55-99-7 | carbohydrate | non-binding | author's data | | | human metabolism (see methods) | | | | human metabolism (see methods) |
| Isopropanol | 164.2 | 99 | Fluka | 67-63-0 | akanol/aliphatic secondary alcohol | non-binding | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | NC | non | | Gerberick et al. (2005) |
| Methyl salicylate | 152.1 | 99 | Aldrich | 119-36-8 | benzoate (ester)/ phenolic | non-binding | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | NC | non | | Gerberick et al. (2005) |
| n-Butanol | 74.12 | 99 | Sigma-Aldrich | 71-36-3 | primary alkyl halide | non-binding | Patlewicz et al. (2008) | | | Ryan et al. (2000) | NC | non | | Gerberick et al. (2005) |
| n-Hexane | 86.18 | 99 | Sigma | 110-54-3 | alkane | non-binding | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | NC | non | | Gerberick et al. (2005) |
| Nickel chloride | 129.60 | 98.5 | Fluka | 7718-54-9 | heavy metal salt | coordination bonds | author's data | | + | Basketter et al. (1999a,b) | NC | non | | OECD (2010) |
| p-Aminobenzoic acid | 137.14 | 98 | Fluka | 150-13-0 | arylamine/benzoic aid derivative | non-binding | Aptula et al. 2006 | | | Basketter et al. (1999a,b) | NC | non | | Basketter et al. (2008) |
| Propyl 4-hydroxybenzoate | 180.2 | 99.80 | Sigma | 94-13-3 | benzoate (ester) // phenolic | non-binding | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | NC | non | | Gerberick et al. (2005) |
| Salicylic acid | 138.12 | 98 | Sigma-Aldrich | 69-72-7 | benzoic acid derovative/phenolic | non-binding | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | NC | non | | Gerberick et al. (2005) |



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**Table 1** (*continued*)

| Substance | molecular weight | Purity (%) | Supplier | CAS # | Chemical class | Mechanism | Literatur | Pro/ Prehapten | Human | Literature | EC3 (%) | potency | LLNA | Literature |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sulfanilamide | 172.2 | 99 | Sigma | 63-74-1 | benzenesulfoneamide/ arylamine | non-binding | Patlewicz et al. (2008) | | | Basketter et al. (1999a,b) | NC | non | | Gerberick et al. (2005) |
| Vanillin | 152.15 | 98.5 | T.J.Baker | 121-33-5 | phenolic aldehyde | non-binding | Patlewicz et al. (2008) | | | Uter et al. (2010) | NC | non | | Gerberick et al. (2005) |
| Hexadecyltrimethyl-ammonium bromide | 79.1 | 99 | Sigma | 57-09-0 | alkyl ammonium | non-binding | author's data | | | Andersen et al. (1997) | | | | no suitable data available |

chemical structure able to react with proteins and, if yes, to identify possible mechanisms involved.

### 2.4. Direct Peptide Reactivity Assay (DPRA)

The DPRA was conducted using the synthetic lysine or cysteine model peptides and methods described by Gerberick and co-workers (Gerberick et al., 2004, 2007). In brief, the test substances were preferably dissolved in acetonitrile (Sigma–Aldrich, Germany) to prepare a 100 mM solution. If the test substances were not soluble in acetonitrile, solutions were prepared in water, methanol, propanol, isopropanol, acetone or mixture of these solvents which is in accordance with the DPRA protocol used in the interlaboratory ring trials. Substances were freshly prepared prior to use. The cysteine model peptide (Ac-RFAACAA-COOH) was prepared as 0.667 mM stock solutions in 100 mM phosphate buffer (100 mM $NaH_2PO_4$, 100 mM $Na_2HPO_4$, both Sigma–Aldrich, Germany, pH 7.5) and lysine model peptide (Ac-RFAAKAA-COOH) was prepared 100 mM ammonium acetate buffer (1.542 g ammonium acetate in 200 mL $H_2O$; pH 10.2; both peptides were obtained from Synbiosci, Livermore CA, USA). Peptide/test sample solutions were prepared in a ratio of 1:4:15 (v/v/v) substance:acetonitrile:cysteine peptide or in a ratio of 1:3 (v/v) substance:lysine peptide and then incubated in the dark for 24 h at room temperature and analyzed in triplicates for each peptide. Analysis of free peptides was performed by RP-HPLC (Agilent 1200 Series) with UV detection at 220 nm using a C18 Phenomex Luna® column (2.0 × 100 mm × 3 μm particle; Phenomex). Separation of peptides was performed by isocratic flow with a flow rate of 0.35 mL/min and a linear gradient from 10% to 25% acetonitrile over 10 min, followed by an increase to 90% acetonitrile.

### 2.5. LuSens assay

The reporter gene cell line LuSens was prepared in collaboration with Dr. Christoph J. Wruck, RWTH Aachen University. This keratinocyte cell line derived from HaCaT cells carries a reporter gene for luciferase under the control of an antioxidant-response-element (ARE) and hence monitors Nrf-2 transcription factor activity. The ARE promoter belongs to the NADPH:quinone oxidoreductase 1 gene from rats. The LuSens assay was performed similarly to that of the KeratinoSens™ assay and as follows: LuSens cells were cultured in complete DMEM culture medium with high glucose (PAA, Germany) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG, Germany), 100/mL penicillin – 100 μg/mL streptomycin (Biochrom AG, Germany), and 0.5 μg/mL puromycin (Sigma–Aldrich, Germany) in T75 culture flasks (TPP; Switzerland). Cells were grown for 24 h in 96-well plates prior to the substance treatment ($1 \times 10^4$ cells in 120 μL medium). Stock solutions of the substances were prepared by dissolving in distilled water or in DMSO (final concentration of 200 mM) and diluted in 2-fold dilutions. Test substance solutions were further diluted in medium containing 1% FBS w/o puromycin to obtain a DMSO concentration of 1%. Treatment was performed by replacing regular cell culture medium with medium containing the test substance. For substances dissolved in water, the final DMSO concentration was adjusted to 1%. Each substance was tested in triplicates and in three independent experiments in dilutions ranging from 1 to 2000 μM. Cinnamic aldehyde (Sigma, Germany) was used as positive control in the concentration range of 2–32 μM. Substance incubation was performed under standard cell culture conditions for 48 h and luciferase activity then determined using SteadyGlo™ (Promega, Germany) according to manufacturer's instructions and the PerkinElmer Victor3 photometer (PerkinElmer, Germany). Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay

(Mosmann, 1983). Briefly, a 5 mg/mL MTT stock solution was prepared in phosphate buffered saline (PBS), and then diluted (1:10) in medium containing 1% FBS prior to use. This working solution (200 μL) was added to each well of the 96-well microtiter plate and incubated for further 2 h under standard cell culture conditions. For analysis medium was aspirated and cells were lysed by adding 100 μL of lysis solution (99.6 mL DMSO, Sigma–Aldrich, Germany; 10 g SDS, Sigma–Aldrich, Germany; and 0.4 mL glacial acetic acid, Merck, Germany). Absorbance was measured at 570 nm with reference wavelength at 690 nm using the PerkinElmer Victor$^3$ photometer.

### 2.6. KeratinoSens™ Assay

The KeratinoSens™ cell line was kindly provided by Andreas Natsch, Givaudan. This cell-line and the optimization of the protocol has been previously described in detail (Emter et al., 2010). The cell-line contains a stable insertion of a luciferase gene under the control of the ARE-element of the gene AKR1C2. The KeratinoSens™ assay was performed according to Givaudan's protocol (Emter et al., 2010). Briefly, KeratinoSens™ cells were cultured in complete DMEM culture medium with GlutaMAX™-1 (Invitrogen, Germany) supplemented with 10% FBS (Biochrom AG, Germany) and 0.55 mg/mL Geneticin (G-418; Invitrogen, Germany) in T75 culture flasks (TPP; Switzerland). For chemical exposure, cells were grown for 24 h in 96-well plates ($1 \times 10^4$ cells in 120 μL medium). Prior to treatment, medium was aspirated and renewed by adding 150 μL medium containing 1% FBS w/o G-418. Stock solutions of the substances were prepared by diluting in distilled water or in DMSO (final concentration of 200 mM) and diluted in a common ratio of 1:2. Substances were further diluted in a 25-fold manner by adding 10 μL stock solution to 240 μL medium containing 1% FBS w/o G-418 resulting in 4× stock solution. Treatment was performed by adding 50 μL of each 4× stock solution. For chemicals dissolved in water the DMSO level was adjusted to 1%. Each compound was tested at dilutions ranging from 1 and 2000 μM as triplicates and repeated in three independent experiments. Cinnamic aldehyde was used as positive control in the concentration range of 4–64 μM. Substance incubation was performed under standard cell culture conditions for 48 h and luciferase activity then determined using SteadyGlo™ (Promega, Germany) according to manufacturer's instructions and the PerkinElmer Victor$^3$ photometer (PerkinElmer, Germany). Luciferase activity was then compared to the vehicle controls. Cytotoxicity was assessed using the MTT cytotoxicity assay. Briefly, 5 mg/mL stock solution was prepared in phosphate buffered saline (PBS), and then diluted (1:10) in medium containing 1% FBS prior to use. 200 μL of this working solution were added to each well of the 96-well microtiter plate and incubated for further 2 h. For analysis medium was aspirated and cells were lysed by adding 100 μL of lysis solution (99.6 mL DMSO, Sigma–Aldrich, Germany; 10 g SDS, Sigma–Aldrich, Germany; and 0.4 mL glacial acetic acid, Merck, Germany). Absorbance was measured at 570 nm with reference wavelength at 690 nm using the PerkinElmer Victor$^3$ photometer.

### 2.7. Modified myeloid U937 skin sensitization test (mMUSST)

The mMUSST assay uses the human histiocytic lymphoma cell line U937 (Ashikaga et al., 2006). The assay was performed as described previously (Bauch et al., 2011). Briefly, U937 cells were obtained from the German Resource Center for Biological Material (*DMSZ*; ACC 5). Cells were cultured in suspension as described using complete RPMI 1640 medium with 25 mM HEPES buffer and 2 mM L-glutamine (Invitrogen, Germany) supplemented with 10% FBS (Biochrom AG, Germany) and 100 U/mL penicillin – 100 μg/mL streptomycin (Biochrom AG, Germany). For substance incubation, cells were seeded in 96-well microtiter plates (100 μL of $0.5 \times 10^6$ cells/mL per well). Substances were dissolved either in medium (2× stock solution) or DMSO (400× stock solution). DMSO-solved substances were further diluted (1:200) in medium to obtain 2× stock solution. Final DMSO concentration in the test medium did not exceed 0.25%. Treatment was performed by addition of 100 μL of test substance to the cells. For each substance five concentrations were tested in duplicate. Concentrations were chosen based on preliminary propidium iodide (PI) cytotoxicity assays. To this accord, cells were exposed for 48 h to 12 concentrations obtained in a 2-fold dilution series starting at 2000 μg/mL for solid test substances or 1000 μg/mL for liquid test substances. Assessment of viable cells was performed as described previously (Python et al., 2007). The highest tested concentration in the main experiment was the two times the concentration causing a cytotoxicity of 25% (CV75). The additional concentrations were obtained in a 2-fold dilution series of the CV75. Each concentration was run in duplicate and each experiment was performed minimum in two independent experiments. Cells were exposed to the test substance for 48 h under standard cell culture conditions. After treatment, cells were centrifuged at 400*g* for 5 min at room temperature (RT) and washed once with PBS containing 5% FBS. Cells were resuspended in 100 μL PBS w/ 5% FBS and labeled for 30 min at 4 °C in the dark with 5 μL IgG-FITC (Cat. No. 555748, BD Pharmingen, Germany) or 5 μL anti-CD86-FITC antibody (Cat. No. 555657, BD Pharmingen, Germany). Following incubation, cells were washed twice with PBS w/5% FBS and resuspended in PBS. For cell viability analysis cell nuclei were stained with propidium iodide (PI; 50 μg/mL in PBS) for 5 min at RT in the dark. Analysis of the plasma membrane markers was performed in 10,000 living cells by flow cytometry using a BeckmanCoulter FC500MPL equipped with MXP software.

### 2.8. Human cell line activation test (h-CLAT)

The dendritic cell activation assay using the human monocytic leukemia cell line THP-1 was performed according to previous reports (Ashikaga et al., 2006, 2010; Sakaguchi et al., 2006, 2007) with minor modifications (Bauch et al., 2011). Briefly, THP-1 cells were obtained from the German Resource Center for Biological Material (DMSZ; ACC 16) and cultured in suspension as described in complete RPMI 1640 with 25 mM HEPES buffer and 2 mM L-glutamine (Invitrogen, Germany) supplemented with 10% FBS (Biochrom AG, Germany), 100 U/mL penicillin-100 μg/mL streptomycin (Biochrom AG, Germany) and 0.05 mM of 2-mercaptoethanol (Invitrogen, Germany). For experiments, cells were seeded in 24-well plates (TPP, Switzerland; $1 \times 10^6$ cells in 500 μL per well). Substances were dissolved in medium (2× stock solution) or DMSO (500× stock solution). DMSO-solved substances were further diluted (1:250) in medium to obtain 2× stock solution. Final DMSO concentration on the cells did no exceed 0.2%. Treatment was performed by applying 500 μL of the test substance dilution to each well for 24 h. Each substance was tested in eight concentrations in duplicate. Only if the classification in both tests differed, was a third test conducted. Concentrations were chosen according to preliminary PI cytotoxicity assays. To this accord, cells were exposed to 12 concentrations obtained in a 2-fold dilution series starting at 2000 μg/mL for solid test substances or 1000 μg/mL for liquid test substances. Assessment of viable cells was performed as described previously (Ashikaga, 2010) (Bauch et al., 2011). The highest tested concentration in the main experiment was 1.2 × CV75. The additional concentrations were obtained by a 1:1.2 dilution series of the 1.2 × CV75. Each concentration was run in duplicate and each experiment was performed minimum in two independent runs. For analysis cells were transferred into 1.5 mL tubes (Eppendorf, Germany). Cells were

centrifuged at 200g and 4 °C for 5 min and washed twice with 1 mL staining buffer, PBS w/$Ca^{2+}$ $Mg^{2+}$ (Biochrom AG, Germany) and 0.1% BSA (Sigma, Germany. Cells were resuspended in 600 µL staining buffer containing 0.01% Cohn fraction (Sigma, Germany) and incubated for 15 min at 4 °C in the dark (original protocol: 10 min incubation). After incubation, cells were distributed in V-shaped 96-well microtiter plate (TPP, Switzerland). Each concentration replicate was split into 3 wells by pipetting 180 µL cell suspension per well. Cells were centrifuged at 200g and 4 °C for 5 min and resuspended in 50 µL antibody working solution. Cells were stained for IgG1-FITC (1:16 diluted in PBS; DAKO/DAK X092701) and anti-CD54-FITC (1:16 diluted in PBS; DAKO /DAKO F7143) or anti-CD86-FITC (1:7 diluted in PBS; Cat. No.: 555657, BD Pharmingen, Germany). Staining was performed at 4 °C in the dark for 30 min. After incubation, cells were washed twice in 200 µL staining buffer; centrifugation was performed at 200g and 4 °C for 5 min. Cells were resuspended in 200 µL staining buffer. For DNA staining, 5 µL PI solution (50 µg/mL in PBS) was added for 5 min in the dark before flow cytometric analysis (original protocol: 1.2 µg/mL). Flow cytometry was performed with a Beckman Coulter FC500MPL and the MXP software.

### 2.9. Data evaluation and statistics

Data analysis was performed in MS Excel. The peptide depletion in the DPRA was determined by dividing the areas under the curves (AUC) of peptide (treated and untreated) by the AUC of peptide incubations with the vehicle alone. Mean peptide depletion was calculated using following equation: mean peptide depletion = [(AUC T.C. (Cys-peptide)/(AUC V.C. (Cys-peptide) + (AUC T.C. (Lys-peptide)/(AUC V.C. (Lys-peptide)/2∗100. For the LuSens and KeratinoSens™ assays, those concentrations inducing less than 70% of cell viability were excluded from further analysis. The fold induction of the luminescent signal was calculated by dividing the relative luminescence units (RLU) of the treated cells by the RLU of control cells using following equation: FI = ($RLU_{T.C.}$)/($RLU_{C.C.}$). For the mMUSST assays, concentrations inducing viability less than 70% were not considered for further assessment of the sensitization potential, whereas for the h-CLAT a cell viability threshold of 50% was used. The mMUSST assay was evaluated by setting the isotype control of medium or vehicle treated cells to 0.6% and fold induction was calculated by dividing the value of substance treated cells by the value of control cells using the following formula: FI = (%$CD86_{T.C.}$ − %$isotype_{T.C.}$)/(%$CD86_{C.C.}$ − %$isotype_{C.C.}$). The h-CLAT assay was evaluated using the mean fluorescence intensity (MFI) and fold induction was calculated by dividing the MFI of substance treated cells by the MFI of non-treated cells using following equation for CD86, FI (CD86) = (MFI $CD86_{T.C.}$ − MFI $isotype_{T.C.}$)/(MFI CD86 $_{C.C.}$ − MFI $isotype_{C.C.}$), or CD54, FI (CD54) = (MFI $CD54_{T.C.}$ − MFI $isotype_{T.C.}$)/(MFI $CD54_{C.C.}$ − MFI $isotype_{C.C.}$), calculations.

Substances that induced mean peptide depletion of cysteine- and lysine-containing peptide above 6.4% were rated as being peptide reactive, while in the LuSens and KeratinoSens™ assay, those substances inducing greater luciferase expression than 1.5-fold induction were considered to have an ARE activating potential. In the DC-like activation assays, those substances that induced the expression of CD86 higher than 1.2-fold compared to the controls in mMUSST assay were predicted to have a DC activating potential, while in the h-CLAT assay substances inducing a fold induction greater than 2.0-fold for CD54 and/or 1.5-fold increase for CD86 were predicted to have a DC activating potential in at least 2 of 3 experiments (new prediction model as described in Sakaguchi et al. (2010); original protocol: CD54 considered to be positive if >1.5) . For the purpose of predicting the sensitization potential of a substance, if the above mentioned thresholds are exceeded, the substance would be categorized as being a skin sensitizer. Statistical significance was assessed using a t-test. The predictivity of single assays or combinations was calculated according to Cooper statistics (Cooper et al., 1979) using the following to assess sensitivity, specificity, positive and negative predictive value and accuracy: Sensitivity: (RP/[RP + FN] × 100); Specificity: (FP/[RN + FP] × 100); Positive predictivity: (RP/[RP + FP] × 100); Negative predictivity: ((RN/[RN + FN] × 100); Accuracy: (RP + RN/[RN + RP + FP + FN] × 100).

## 3. Results

In this study, comparative testing using the DPRA, LuSens, KeratinoSens™, mMUSST and h-CLAT assays was performed to establish (LuSens) or expand (DPRA, KeratinoSens™, mMUSST and h-CLAT) the available database for the test methods. The results obtained from these tests along with the predictions obtained from the OECD QSAR Toolbox Version 2.0 are depicted in Tables 2 and 3. The predictivity of the individual methods and their combinations were compared to human and LLNA data using Cooper statistics (Table 4). The results were then used to propose first approaches for developing testing strategies to assess skin sensitization using non-animal alternatives. Of the 59 test substances initially selected, 54 could be assessed using the chosen test systems whereas five substances where excluded due to technical limitations (see discussion). An overview of the test results is given in Table 5.

### 3.1. Local lymph node assay (LLNA)

In the murine LLNA (Basketter et al., 1999b; Kimber et al., 2003) one substance was assessed as being a false negative, i.e. nickel chloride; and four substances were assessed as being false positive: pyridine, sodium dodecyl sulfate (SDS), tartaric acid and xylene if compared to human data. Based on these results, the LLNA offered a sensitivity of 96% and a specificity of 81% whereas the overall accuracy was 90%.

### 3.2. Reaction mechanism based analysis – OECD QSAR Toolbox and DPRA

The protein reactivity of test substances can be estimated *in silico* via the correlation of their chemical structures with structural alerts. The predictive capacity of the OECD QSAR Toolbox Version 2.0 was assessed and used to discern the potential chemical reactivity involved. The substances were processed both with and without the module of skin metabolism simulation. The results obtained without skin metabolism by this analysis are shown in Table 2. The OECD QSAR Toolbox Version 2.0 was able to correctly predict 40 of 50 or 37 of 53 of the substances when compared to the human data or LLNA data, respectively, whereby in particular the predictions of pro- and prehaptens proved to be difficult. Inclusion of the skin metabolism module identified several protein-binding and non-binding metabolites both for the known pro- or prehaptens and but also for the known nonsensitizing substances (data not shown). For example, when analyzing the skin sensitizer propyl gallate, nine metabolites with four alerts for protein binding were predicted and for the irritant pyridine nine metabolites and two alerts for protein binding were predicted. Only predictions obtained without the metabolism module were evaluated. Evaluation of data with Cooper statistics (Cooper et al., 1979) revealed that the *in silico* method using the OECD QSAR Toolbox Version 2.0, offered a sensitivity of 64% or 56%, a specificity of 100% or 100% and an overall accuracy was 80% or 70% when comparing the predictions to human data or the LLNA, respectively (Table 4).



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**Table 2**
Results of peptide reactivity assessments: OECD QSAR Toolbox v2.0 (*in silico*), the direct peptide reactivity assay (DPRA; *in chemico*), and the LuSens assay and KeratinoSens® assay (both *in vitro*; ARE-reporter gene based assay).

| Substance | DPRA | | | OECD Toolbox v2.0 | | LuSens | KeratinoSens® |
|---|---|---|---|---|---|---|---|
| | Lys-peptide depletion | Cys-peptidedepletion | Mean peptide depletion | Rationale by OECD toolbox | Prediction | EC1.5 in μM | EC1.5 in μM |
| Oxazolone | 44.6 | 71.7 | 58.1 | Nucleophilic acyl substitution | + | 220 | 154.6 |
| MCI/MI | 4.3 | 90.9 | 47.6 | Ring opening at the S–N bond followed by Nucleophilic addition. | + | 2.7 | 3.1 |
| p-Benzoquinone | 95.9 | 99.7 | 97.8 | Michael addition on Quinones/quinone imines | + | 4 | 6.12 |
| 1-Chloro-2,4-dinitrobenzene | 58.3 | 99.9 | 79.1 | Nucleophilic substitution on activated aryl carbon atom. | + | 2 | 2.5 |
| 4-Phenylenediamine | 42.2 | 99.8 | 71.0 | No binding | – | 6 | 4.5 |
| Propyl gallate | 63.6 | 59.2 | 61.4 | No binding | – | 6 | 191.28 |
| 2,4,6-Trinitrobenzenesulfonic acid | 22.6 | 99.7 | 61.1 | No binding | – | 23 | 40.8 |
| Phthalic anhydride | 31.3 | 16.7 | 24.0 | Protein acylation by acid anhydride | + | Below threshold | Below threshold |
| Formaldehyde > 36% (1% DMSO) | 2.4 | 54.8 | 28.6 | Schiff base formation with aldehydes. | + | 101 | 136.69 |
| Methyldibromo glutaronitrile | 28.4 | 75.3 | 51.8 | Nucleophilic substitution on halogenated C $sp^3$ atom | + | 7 | 8.44 |
| Isoeugenol | 21.8 | 99.1 | 60.4 | No binding | – | 3 | 6.2 |
| Diethyl maleate | 78.8 | 99.8 | 89.3 | Michael addition on conjugated systems with electron withdrawing groups | + | 4 | 2.42 |
| Ethylene diamine | 0.7 | 18.6 | 9.7 | No binding | – | Below threshold | 549.1 |
| Benzylidene acetone | −1.4 | 92.4 | 45.5 | Michael addition on conjugated systems with electron withdrawing groups | + | 7 | 7 |
| Cobalt chloride | 35.0 | 30.3 | 32.6 | No binding | – | 75 | 105.9 |
| 2-Phenylpropionaldehyde | 5.1 | 26.6 | 15.8 | Schiff base formation with aldehydes | + | 34 | 26.48 |
| α-Hexyl-cinnamic aldehyde | −0.9 | 12.3 | 5.7 | Michael addition on α,β-aldehydes, Schiff base formation with aldehydes. | + | 13 | 22.9 |
| Tartaric acid | −2.0 | 6.6 | 2.3 | No binding | – | Below threshold | 3.41 |
| 2-Mercaptobenzothiazole | 3.2 | 99.9 | 51.5 | Protein thiol-disulfide interchange | + | 85 | 57.4 |
| 2,3-Butanedione | 34.9 | 92.8 | 63.9 | Nucleophilic cycloaddition to diketones | + | 117 | 73.61 |
| Citral | 8.6 | 78.6 | 43.6 | Michael addition on α,β-aldehydes, Schiff base formation with aldehydes. | + | 6 | 16.8 |
| Eugenol | 4.2 | 38.3 | 21.3 | No binding | – | 79 | 63.7 |
| Farnesal | −4.1 | 37.3 | 16.6 | Michael addition on α,β-aldehydes, Schiff base formation with aldehydes. | + | 6 | 10.79 |
| Sodium lauryl sulfate | 97.1 | −0.1 | 48.5 | No binding | – | Below threshold | Below threshold |
| 4-Allylanisole | −2.2 | 98.5 | 48.1 | No binding | – | 274 | 39.47 |
| Hydroxycitronellal | 32.8 | 61.7 | 47.2 | Schiff base formation with aldehydes | + | 185 | 30.69 |
| Phenyl benzoate | 2.2 | 64.9 | 33.6 | Diarylester aminolysis or thiolysis | + | Below threshold | Below threshold |
| Cinnamic alcohol | 2.7 | 21.3 | 12.0 | No binding | – | 20 | 23.9 |
| Imidazolidinyl urea | 20.6 | 59.0 | 39.8 | Protein acylation by *N*-acylamides | + | 80 | 41.3 |
| Undecylenic acid | −0.1 | 11.4 | 5.7 | No binding | – | 27 | 56.72 |
| Ethylene glycol dimethacrylate | 33.9 | 93.5 | 63.7 | Michael addition on conjugated systems with electron withdrawing groups | + | 18 | 14.1 |
| Pyridin | −2.2 | −0.1 | −1.2 | No binding | – | Below threshold | Below threshold |
| Aniline | 2.4 | −1.0 | 0.7 | No binding | – | 165 | 2.1 |
| Methyl methacrylate | 11.8 | 55.3 | 33.5 | Michael addition on conjugated systems with electron withdrawing groups | + | Below threshold | Below threshold |
| Xylene | 0.7 | −0.7 | 0.0 | No binding | – | Below threshold | Below threshold |
| Glycerol | 1.3 | −0.7 | 0.3 | No binding | – | Below threshold | Below threshold |
| 1.2-Propanediol | 0.0 | −3.0 | −1.5 | No binding | – | Below threshold | Below threshold |
| 4-Hydroxybenzoic acid | 0.7 | 3.0 | 1.9 | No binding | – | Below threshold | Below threshold |
| 4-Methoxyacetophenone | −0.3 | −1.9 | −1.1 | No binding | – | 168 | 56.12 |
| 6-Methylcoumarin | −2.0 | −1.8 | −1.9 | No binding | – | 14 | 5.87 |
| DL-lactic acid | −1.2 | 3.0 | 0.9 | No binding | – | Below threshold | Below threshold |
| Fumaric acid | 4.6 | 10.8 | 7.7 | No binding | – | Below threshold | Below threshold |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Glucose | 15.3 | 0.1 | 7.7 | Schiff base formation with aldehydes | – | Below threshold | Below threshold |
| Isopropanol | −1.7 | −2.0 | −1.9 | No binding | – | Below threshold | Below threshold |
| Methyl salicylate | −0.2 | −2.4 | −1.3 | No binding | – | 383 | Below threshold |
| *n*-Butanol | −1.3 | 0.7 | −0.3 | No binding | – | Below threshold | Below threshold |
| *n*-Hexane | 2.6 | −0.9 | 0.9 | No binding | – | Below threshold | 561.84 |
| Nickel chloride | 0.3 | 43.1 | 21.7 | No binding | – | 355 | Below threshold |
| p-Aminobenzoic acid | 0.5 | 5.9 | 3.2 | No binding | – | Below threshold | Below threshold |
| Propyl 4-hydroxybenzoate | −1.3 | −1.5 | −1.4 | No binding | – | 9 | 5.22 |
| Salicylic acid | 1.0 | 8.7 | 4.8 | No binding | – | Below threshold | Below threshold |
| Sulfanilamide | −10.4 | 3.8 | −3.3 | No binding | – | Below threshold | Below threshold |
| Vanillin | 19.8 | −1.3 | 9.3 | No binding | – | 226 | 83.24 |
| Hexadecyltrimethylammonium bromide | 1.5 | 2.4 | 2.0 | No binding | – | Below threshold | Below threshold |

The DPRA is a method which simulates chemical protein-reactivity in an *in chemico* assay using two synthetic model peptides and specifically assesses the peptide reactivity to cysteine or lysine residues. The DPRA was able to correctly predict 43 of 50 or 42 of 53 of the substances when compared to human data or LLNA data, respectively. Three substances were rated as false negative: aniline, undecylenic acid and α-hexyl cinnamic aldehyde, whereas four substances were considered to be false positive: fumaric acid, glucose, SDS and vanillin (Table 2). The *in chemico* method offered a sensitivity of 89% or 81%, a specificity of 82% or 76% and an overall accuracy of 86% or 79% when compared to human data or the LLNA, respectively (Table 4).

### 3.3. ARE/Nrf-2 based reporter gene assays –LuSens and KeratinoSens™

The LuSens assay and the KeratinoSens™, both based on luciferase reporter cell lines and ARE pathway activation, can also be used to indirectly assess the intracellular cysteine reactivity of a substance and the resulting activation of the keap-1/Nrf2 signaling pathway. The LuSens assay correctly predicted 42 out of 50 or 41 out of 53 substances when compared to human or LLNA data, respectively. Three substances exhibited borderline activation, hexadecyltrimethylammonium bromide, hydroxycitronellal, methylmethacrylate since no dose dependent effect or high variability between experiments was observed. Three substances would be incorrectly rated to be negative: ethylene diamine, phenyl benzoate, phthalic anhydride; and five substances would be incorrectly rated to be positive: 4-methoxyacetophenone, 6-methylcoumarin, methyl salicylate, propyl-4-hydroxybenzoate, and vanillin (Table 2). For the LuSens assay, the Cooper statistics estimated a sensitivity of 89% or 81%; a specificity of 77% or 71% and an overall accuracy of 84% or 77% when compared to the human data or LLNA, respectively (Table 4). The KeratinoSens™ assay correctly predicted 40 out of 50 or 43 out of 53 substances when compared to human or LLNA data, respectively. Four substances would be incorrectly rated to be negative: methyl methacrylate, nickel chloride, phenyl benzoate, phthalic anhydride; and six substances would be incorrectly rated to be positive: 4-methoxyacetophenone, 6-methylcoumarin, *n*-hexane, propyl-4-hydroxybenzoate, tartaric acid and vanillin (Table 2). For the KeratinoSens™ assay, the Cooper statistics estimated a sensitivity of 86% or 83%; a specificity of 73% or 76% and an overall accuracy of 80% or 81% when compared to the human data or LLNA, respectively (Table 4).

### 3.4. Dendritic cell activation assays – mMUSST and h-CLAT

The dendritic cell activation assay mMUSST measures the expression of the cell surface protein CD86 as marker for maturation of dendritic-like cells induced by the test substance. The mMUSST assay was able to correctly predict 43 out of 50 or 39 of 53 substances when compared to human or LLNA data, respectively. The substances citral, imidazolidinyl urea, nickel chloride, diethyl maleate and undecylenic acid exhibited borderline activation. Compared to human data seven substances would be identified as false negative: 2-phenylpropianaldehyde, cinnamic alcohol, isoeugenol, methyldibromo glutaronitrile, oxazolone, phthalic anhydride and α-hexyl cinnamic aldehyde. No false positive result was obtained with the mMUSST whereby if compared to LLNA one substance would be assessed as being false positive (nickel chloride), and 13 substances as false negative. The results are summarized in Table 3. The mMUSST assay offered a sensitivity of 75% or 64%, a specificity of 100% or 94%, and an overall accuracy of 86% or 74% when compared to human data or the LLNA, respectively (Table 4).

The dendritic cell activation assay h-CLAT measures the expression of the cell surface proteins CD86 and CD54 as markers for maturation of dendritic cells induced by the test substance. The h-CLAT assay was able to correctly predict 38 out of 50 or 39 out of 53 substances when compared to human or LLNA data, respectively. The substances 4-phenylenediamine, aniline, farnesal, p-benzoquinone, diethyl maleate, eugenol, salicylic acid, phthalic anhydride, propyl gallate and vanillin gave borderline results. Seven substances would be rated as negative but were actually skin sensitizers according to the human data: 2-mercaptobenzothizole, formaldehyde, nickel chloride, imidazolidinyl urea, oxazolone, phthalic anhydride and undecylenic acid. Whereas five substances would be rated as false positive: 4-hydroxybenzoic acid, 4-methoxyacetophenone, 6-methylcoumarin, *n*-butanol and propyl-4-hydroxybenzoic. The summary of results is given in Table 3. Cooper statistics revealed that the h-CLAT assay has a sensitivity of 75% or 72%, a specificity of 77% or 76%, and an overall accuracy of 76% or 74% when compared to human data or the LLNA, respectively (Table 4).

### 3.5. Incorrect predictions

Despite the generally good predictivities of the single methods, some substances were predicted either false positive or false

**Table 3**
Summary results of DC-like cell activation assays: mMUSST and h-CLAT

| Substance | mMUSST-CD86 | | h-CLAT-CD86 | | h-CLAT -CD54 | |
|---|---|---|---|---|---|---|
| | EC1.2 in μg/mL | EC1.2 in μM | EC1.5 in μg/mL | EC1.5 in μM | EC2 in μg/mL | EC2 in μM |
| Oxazolone | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| MCI/MI | 0 | 2 | 2 | 14 | Below threshold | Below threshold |
| p-Benzoquinone | 1 | 14 | 4 | 36 | Below threshold | Below threshold |
| 1-Chloro-2,4-dinitrobenzene | 0 | 2 | 2 | 12 | 3 | 13 |
| 4-Phenylenediamine | 1 | 5 | 2 | 14 | Below threshold | Below threshold |
| Propyl gallate | 2 | 8 | Below threshold | Below threshold | 10 | 48 |
| 2,4,6-Trinitrobenzenesulfonic acid | 15 | 51 | Below threshold | Below threshold | 21 | 71 |
| Phthalic anhydride | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| Formaldehyde >36% (1% in DMSO) | 1 | 21 | Below threshold | Below threshold | Below threshold | Below threshold |
| Methyldibromo glutaronitrile | Below threshold | Below threshold | 10 | 39 | Below threshold | Below threshold |
| Isoeugenol | Below threshold | Below threshold | 17 | 104 | Below threshold | Below threshold |
| Diethyl maleate | 13 | 75 | 62 | 358 | Below threshold | Below threshold |
| Ethylene diamine | 25 | 412 | 109 | 1818 | Below threshold | Below threshold |
| Benzylidene acetone | 4 | 26 | 17 | 115 | Below threshold | Below threshold |
| Cobalt chloride | 9 | 73 | 29 | 223 | 45 | 346 |
| 2-Phenylpropionaldehyde | Below threshold | Below threshold | 28 | 209 | Below threshold | Below threshold |
| α-Hexyl-cinnamic aldehyde | Below threshold | Below threshold | Below threshold | Below threshold | 21 | 98 |
| Tartaric acid | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| 2-Mercaptobenzothiazole | 7 | 44 | Below threshold | Below threshold | Below threshold | Below threshold |
| 2,3-Butanedione | 13 | 153 | 27 | 319 | Below threshold | Below threshold |
| Citral | Below threshold | Below threshold | 13 | 87 | 13 | 86 |
| Eugenol | 6 | 35 | 77 | 469 | 90 | 548 |
| Farnesal | Below threshold | Below threshold | Below threshold | Below threshold | 9 | 40 |
| Sodium lauryl sulfate | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| 4-Allylanisole | 110 | 744 | 315 | 2125 | 76 | 511 |
| Hydroxycitronellal | 4 | 22 | 22 | 126 | Below threshold | Below threshold |
| Phenyl benzoate | 12 | 63 | 82 | 416 | Below threshold | Below threshold |
| Cinnamic alcohol | Below threshold | Below threshold | 73 | 547 | 97 | 724 |
| Imidazolidinyl urea | 8 | 20 | Below threshold | Below threshold | Below threshold | Below threshold |
| Undecylenic acid | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| Ethylene glycol dimethacrylate | 6 | 31 | 427 | 2156 | 287 | 1447 |
| Pyridine | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| Aniline | 71 | 762 | 280 | 3007 | Below threshold | Below threshold |
| Methyl methacrylate | 1 | 5 | 198 | 1978 | 298 | 2978 |
| Xylene | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| Glycerol | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| 1.2-Propanediol | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| 4-Hydroxybenzoic acid | Below threshold | Below threshold | Below threshold | Below threshold | 1052 | 7617 |
| 4-Methoxyacetophenone | Below threshold | Below threshold | 70 | 469 | not calc | Below threshold |
| 6-Methylcoumarin | Below threshold | Below threshold | 36 | 222 | Below threshold | Below threshold |
| DL-lactic acid | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| Fumaric acid | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| Glucose | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| Isopropanol | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| Methyl salicylate | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| n-Butanol | Below threshold | Below threshold | 921 | 12429 | 1066 | 14382 |
| n-Hexane | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| Nickel chloride | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| p-Aminobenzoic acid | not calc | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| Propyl 4-hydroxybenzoate | Below threshold | Below threshold | Below threshold | Below threshold | 83 | 461 |
| Salicylic acid | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| Sulfanilamide | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| Vanillin | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |
| Hexadecyltrimethylammonium bromide | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold | Below threshold |



negative. Table 5 summarizes the results of each single assay. Phthalic anhydride, a respiratory allergen, was false negative in three assays, whereas seven substances generated either false negative (oxazolone, α-hexyl cinnamic aldehyde, undecylenic acid) or false positive result (4-methoxyacetophenone, 6 methylcoumarin, propoyl-4-hydroxy benzoic acid and vanillin) in two of the assays. Further 19 substances resulted in a false negative (formaldehyde, methyldibromo glutaronitril, isoeugenol, ethylene diamine, 2-phenylpropianaldehyde, 2-mercaptobenzothiazole, farnesal, phenyl benzoate, cinnamic alcohol, imidazolidinyl urea, aniline, methyl methacrylate and nickel chloride) or false positive (SDS, 4-hydroxybenzoic acid, fumaric acid, glucose, methyl salicylate and *n*-butanol) in at least one of the four assays.

### 3.6. Prediction model

Based on the intended purpose of a specific test, different prediction models can be applied to the results of this study: Predictions can strive for high sensitivity (to minimize false negatives), high specificity (to minimize false positives) or a high overall accuracy.

The DPRA and LuSens assays simulate an essential early step, namely protein reactivity, in the pathway leading to the adverse outcome of skin sensitization. The LuSens assay also yields additional information on the activation of the ARE related genes resulting from the subsequent activation of Keap-1/Nrf2 signaling pathway. The combination of both peptide reactivity assays, DPRA

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**Table 4**
Summary of predictivities of single assays and their combinations based on Cooper statistics.

| Compared to human | | Sensitivity (%) | Specificity (%) | Positive predictivity (%) | Negative predictivity (%) | Accuracy (%) |
|---|---|---|---|---|---|---|
| LLNA | | 96 | 81 | 87 | 94 | 90 |
| single assays | OECD QSAR toolbox | 64 | 100 | 100 | 69 | 80 |
| | DPRA | 89 | 82 | 86 | 86 | 86 |
| | LuSens | 89 | 77 | 83 | 85 | 84 |
| | KeratinoSens® | 86 | 73 | 80 | 80 | 80 |
| | mMUSST | 75 | 100 | 100 | 76 | 86 |
| | h-CLAT | 75 | 77 | 81 | 71 | 76 |
| Combinations | DPRA and LuSens | 100 | 64 | 78 | 100 | 84 |
| | DPRA and KeratinoSens® | 100 | 59 | 76 | 100 | 82 |
| | DPRA and mMUSST | 96 | 82 | 87 | 95 | 90 |
| | DPRA and h-CLAT | 96 | 59 | 75 | 93 | 80 |
| | LuSens and mMUSST | 96 | 77 | 84 | 94 | 88 |
| | LuSens and h-CLAT | 96 | 68 | 79 | 94 | 84 |
| | KeratinoSens® and mMUSST | 97 | 56 | 78 | 91 | 81 |
| | KeratinoSens® and h-CLAT | 93 | 64 | 76 | 88 | 80 |
| Prediction Model (high overall accuracy) | DPRA, LuSens and mMUSST | 93 | 95 | 96 | 91 | 94 |
| | DPRA, KeratinoSens® and mMUSST | 93 | 95 | 96 | 91 | 94 |
| compared to LLNA | Sensitivity | Specificity | PPV | NPV | Accuracy | |
| single assays | OECD QSAR toolbox | 56 | 100 | 100 | 52 | 70 |
| | DPRA | 81 | 76 | 88 | 65 | 79 |
| | LuSens | 81 | 71 | 85 | 63 | 77 |
| | KeratinoSens® | 83 | 76 | 88 | 68 | 81 |
| | mMUSST | 64 | 94 | 96 | 55 | 74 |
| | h-CLAT | 72 | 76 | 87 | 57 | 74 |
| combinations | DPRA and LuSens | 92 | 59 | 83 | 77 | 81 |
| | DPRA and KeratinoSens® | 94 | 59 | 83 | 83 | 83 |
| | DPRA and mMUSST | 86 | 76 | 89 | 72 | 83 |
| | DPRA and h-CLAT | 89 | 53 | 80 | 69 | 77 |
| | LuSens and mMUSST | 86 | 71 | 86 | 71 | 81 |
| | LuSens and h-CLAT | 86 | 59 | 82 | 67 | 77 |
| | KeratinoSens® and mMUSST | 89 | 71 | 86 | 75 | 83 |
| | KeratinoSens® and h-CLAT | 89 | 65 | 84 | 73 | 81 |
| Prediction Model (high overall accuracy) | DPRA, LuSens and mMUSST | 81 | 88 | 94 | 68 | 83 |
| | DPRA, KeratinoSens® and mMUSST | 81 | 88 | 94 | 68 | 83 |

and LuSens (the KeratinoSens™ assay can be used instead of the LuSens assay), is proposed. Based on the results from this study, if the criterion "one assay results in a positive response" is used, 100% of the human skin sensitizers in the substance panel were identified (high sensitivity). Yet, this criterion would also result in eight of 22 nonsensitizers being predicted as positive. Hence it does not allow for reliable identification of skin sensitizers (low specificity; a positive result does not yield information with sufficient predictivity). However, if both assays indicate no protein reactivity, a sensitizing potential can be excluded with high probability. Based on this result, protein reactivity is a necessary, but not a sufficient prerequisite for skin sensitization. Conversely, the mMUSST did not yield a positive response for any human nonsensitizers (high specificity). Yet, this assay predicted seven human skin sensitizers to be negative. Hence it does not allow for reliable identification of nonsensitizers (low sensitivity; a negative result does not yield information with a sufficient predictivity). However, if this assay indicates DC activation, a sensitizing potential can be confirmed with high probability. Based on this result, DC activation is a sufficient, but not a necessary prerequisite for skin sensitization. Yet on occasion, protein reactivity and DC-activation will give inconclusive results (protein reactivity positive and mMUSST negative) or conflicting results (protein reactivity negative and mMUSST positive).

The model proposed is depicted in Fig. 1. It combines the sensitivities and specificities described. If the two peptide-reactivity assays, DPRA and LuSens, yield negative results, then the substance is considered to be nonsensitizing. If substances are predicted to be sensitizing in the mMUSST assay, the substance is considered to be a skin sensitizer. If the predictions are conflicting, or if the h-CLAT is used instead of the mMUSST, a pragmatic 'weight of evidence' approach is used: Any two of the three tests rule the overall result (any two assays must be positive to rate the substance as a skin sensitizer and any two assays must be negative to rate the substance as a nonsensitizer). Using this model, 47 out of 50 or 44 out of 53 test substances were predicted correctly if compared to human or LLNA, respectively. One substance would be rated as false positive (vanillin) and two substances would be rated as false negative (phthalic anhydride and α-hexyl cinnamic aldehyde) if compared to human data; comparison to data from the LLNA would result in two false positives (nickel chloride and vanillin) and seven false negatives (6-methylcoumarin, phthalic anhydride, pyridin, SDS, tartaric acid, xylene and α-hexyl cinnamic aldehyde). Therefore, the predictivities of this prediction model are as follows: sensitivity of 93% or 81%, specificity of 95% or 88% and an overall accuracy of 94% or 83% if compared to human data or LLNA, respectively (Table 4).

To use this prediction model for classification purposes, all three tests need to be performed. If specific questions regarding the sensitization potential are asked (e.g. the reliable exclusion of a sensitization potential for screening purposes) testing of effect, protein reactivity or DC activation, may be sufficient.

## 4. Discussion

The development of animal-free test methods to assess the skin sensitizing potential of substances (e.g. chemicals and cosmetic

**Table 5**
Overview of the assays, their results and the results of the proposed prediction model.

| Substance | Reaction mechanism | Pro/ prehapten | Human | EC3 (%) | Potency | LLNA | OECD toolbox | DPRA | LuSens | Keratino Sens® | mMUSST | h-CLAT | DPRA + LuSens* (both negative then nonsensitizer) | mMUSST (if positive then sensitizer) | Initial prediction | WoE (2 of 3) | Prediction |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Oxazolone | Acyl-transfer | | + | 0.003 | extreme | + | + | + | + | + | – | – | | | ? | + | + |
| MCI/MI | Michael | | + | 0.01 | extreme | + | + | + | + | + | + | + | | + | + | | + |
| p-Benzoquinone | Michael | | + | 0.01 | extreme | + | + | + | + | + | + | + | | + | + | | + |
| 1-Chloro-2,4-dinitrobenzene | SNAr | | + | 0.05 | extreme | + | + | + | + | + | + | + | | + | + | | + |
| 4-Phenylenediamine | Pro-Michael | pre | + | 0.16 | strong | + | – | + | + | + | + | + | | + | + | | + |
| propyl gallate | Acyl-transfer | pro | + | 0.32 | strong | + | – | + | + | + | + | + | | + | + | | + |
| 2,4,6-Trinitrobenzenesulfonic acid | SNAr | - | - | 0.36 | strong | + | – | + | + | + | + | + | | + | + | | + |
| Phthalic anhydride | Acyl-transfer | - | + | 0.36 | strong | + | + | + | – | – | – | – | | | ? | – | – |
| Formaldehyde >36% (1% in DMSO) | Schiff base | - | + | 0.61 | strong | + | + | + | + | + | + | – | | + | + | | + |
| Methyldibromo glutaronitrile | Pro-Michael | | + | 0.90 | strong | + | + | + | + | + | – | + | | | ? | + | + |
| Isoeugenol | Pro-Michael | pro/pre | + | 1.50 | moderate | + | – | + | + | + | – | + | | | ? | + | + |
| Diethyl maleate | Michael | | + | 2.10 | moderate | + | + | + | + | + | + | + | | + | + | | + |
| Ethylene diamine | pro-Schiff base | pro | + | 2.20 | moderate | + | – | + | – | + | + | + | | + | + | | + |
| Benzylidene acetone | Michael | | + | 3.70 | moderate | + | + | + | + | + | + | + | | + | + | | – |
| Cobalt chloride | coordination bonds | | + | 4.80 | moderate | + | – | + | + | + | + | + | | + | + | | + |
| 2-Phenylpropionaldehyde | Schiff base | | + | 5.91 | moderate | + | + | + | + | + | – | + | | | ? | + | + |
| a-Hexyl-cinnamic aldehyde | Michael | | + | 8.00 | moderate | + | + | v | + | + | – | + | | | – | | v |
| Tartaric acid | non-binding | | | 8.70 | moderate | + | – | – | – | + | – | – | – | | – | | – |
| 2-Mercaptobenzothiazole | Acyl-transfer | | + | 9.70 | moderate | + | + | + | + | + | + | – | | + | + | | + |
| 2,3-Butanedione | Schiff base | | | 11.00 | weak | + | + | + | + | + | + | + | | + | + | | + |
| Citral | Schiff base | | + | 13.00 | weak | + | + | + | + | + | + | + | | + | + | | + |
| Eugenol | Pro-Michael | pro | + | 13.00 | weak | + | – | + | + | + | + | + | | + | + | | + |
| Farnesal | Schiff base | | | 13.00 | weak | + | + | + | + | + | – | + | | | ? | + | + |
| Sodium lauryl sulfate | non-binding | | | 14.00 | weak | + | – | + | – | – | – | – | | | ? | – | – |
| 4-Allylanisole | Pro-Michael | pro | | 18.00 | weak | + | – | + | + | + | + | + | | + | + | | + |
| Hydroxycitronellal | Schiff base | | + | 20.00 | weak | + | + | + | + | + | + | + | | + | + | | + |
| Phenyl benzoate | Acyl-transfer | | + | 20.00 | weak | + | + | + | – | + | + | | | + | + | | + |
| Cinnamic alcohol | Pro-Michael | pro | + | 21.00 | weak | + | – | + | + | + | – | + | | | ? | + | + |
| Imidazolidinyl urea | Acyl-transfer | | + | 24.00 | weak | + | + | + | + | + | + | – | | + | + | | + |
| Undecylenic acid | non-binding | | + | 25.00 | weak | + | – | + | + | + | – | – | | + | + | | + |
| Ethylene glycol dimethacrylate | Michael | | + | 35.00 | weak | + | + | + | + | + | + | + | | + | + | | + |
| Pyridin | non-binding | | | 71.20 | weak | + | – | – | – | – | – | – | – | | – | | – |
| Aniline | SNAr | pro | + | 89.00 | weak | + | – | – | + | + | + | + | | + | + | | + |
| Methyl methacrylate | Michael | | + | 90.00 | weak | + | + | + | + | – | + | + | | + | + | | + |
| Xylene | non-binding | | – | 95.8 | weak | + | – | – | – | – | – | – | – | | – | | – |
| Glycerol | non-binding | | – | 100.02 | non | – | – | – | – | – | – | – | – | | – | | – |
| 1.2-Propanediol | non-binding | | – | 100.11 | non | – | – | – | – | – | – | – | – | | – | | – |
| 4-Hydroxybenzoic acid | non-binding | | – | NC | non | – | – | – | – | – | – | + | – | | – | | – |
| 4-Methoxyacetophenone | non-binding | | – | NC | non | – | – | – | + | + | – | + | | | – | | – |
| 6-Methylcoumarin | Michael | | – | NC | non | – | – | – | + | + | – | + | | | – | | – |
| DL-lactic acid | non-binding | | – | NC | non | – | – | – | – | – | – | – | – | | – | | – |
| Fumaric acid | non-binding | | – | NC | non | – | – | + | – | – | – | – | | | ? | – | – |
| Glucose | non-binding | | – | NC | non | – | – | + | – | – | – | – | | | ? | – | – |
| Isopropanol | non-binding | | – | NC | non | – | – | – | – | – | – | – | – | | – | | – |
| Methyl salicylate | non-binding | | – | NC | non | – | – | – | + | – | – | – | | | – | | – |
| *n*-Butanol | non-binding | | – | NC | non | – | – | – | – | – | – | + | – | | – | | – |
| *n*-Hexane | non-binding | | – | NC | non | – | – | – | – | + | – | – | – | | – | | – |
| Nickel chloride | coordination | | + | NC | non | – | – | + | + | – | + | – | | + | + | | + |



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**Table 5** (*continued*)

| Substance | Reaction mechanism | Pro/ prehapten | Human | EC3 (%) | Potency | LLNA | OECD toolbox | DPRA | LuSens | KeratinoSens® | mMUSST | h-CLAT | DPRA + LuSens* (both negative then nonsensitizer) | mMUSST (if positive then sensitizer) | Initial prediction | WoE (2 of 3) | Prediction |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | bonds | | | | | | | | | | | | | | | | |
| p-Aminobenzoic acid | non-binding | | – | NC | non | – | – | – | – | – | – | – | – | | – | | – |
| Propyl 4-hydroxybenzoate | non-binding | | – | NC | non | – | – | – | + | + | – | + | | | – | | – |
| Salicylic acid | non-binding | | – | NC | non | – | – | – | – | – | – | – | – | | – | | – |
| Sulfanilamide | non-binding | | – | NC | non | – | – | – | – | – | – | – | – | | – | | – |
| Vanillin | non-binding | | – | NC | non | – | – | + | + | + | – | – | | | ? | + | + |
| Hexadecyltrimethylammonium Bromide | non-binding | | – | | | – | – | – | – | v | – | – | – | | v | | – |

ingredients) has been the goal of many researchers for some time. Much of this is being driven by the European legislation, in particular the Cosmetics Directive (Council Directive 76/768/EEC) and its successor the Cosmetics Regulation (Council Directive 76/768/EEC, 1976; EC, 2009). A number of *in vitro* assays for sensitization testing have been developed and are currently in the validation process at ECVAM, namely the DPRA, h-CLAT, MUSST and KeratinoSens™ assay. Moreover, scientists are also aware of the need to develop test strategies comprising the most reliable methods in order to cover the biological complexity of the skin sensitization process. While the successful intralaboratory method validation of three *in vitro* methods assay reported earlier (Bauch et al., 2011) are presented here, a number of other methods for the prediction of skin sensitization were assessed but not further used due to practical feasibility, e.g. limitations such as dealing with human blood. The assays described in this study have been extensively studied, and most of them are transferable to other laboratories (the interlaboratory validation for LuSens is pending) and thus are considered to be reliable and workable test systems. The LuSens assay is similar to the KeratinoSens™ assay and, according to the data produced for the 54 test substances in this assay, it would be possible to use the KeratinoSens™ assay interchangeably without major differences in predictive capacity. Besides being used for the intralaboratory validation of the individual assays, the test results were also used to develop test strategies by combining different tests which cover key elements of the complex biological pathways leading to sensitization, which lead to a reliable prediction of skin sensitizers.

In the initial phase of this study, six different *in vitro*, *in chemico* and *in silico* assays were assessed for their performance to predict the sensitizing potential of test substances. Test substances were all in a molecular weight range of less than 400 g/mol for which skin penetration can be assumed. Hence dermal penetration was not assessed experimentally in this study. The 54 substances included in the strategy development were sufficiently soluble in vehicle and culture medium and did not interfere with the detection methods. The test methods used in this study address several events in the skin sensitization process: (1) the peptide reactivity of test substances, which was assessed using the *in chemico* DPRA, and ARE reporter gene based assays, namely the LuSens assay and KeratinoSens™ assay, for indirect protein reactivity and concomitant evaluation of keratinocyte responses due to the activation of Keap-1/Nrf2 signaling pathway and induction of genes under the control of ARE; and (2) dendritic cell activation; this was addressed using dendritic cell-like cell lines: the mMUSST assay and h-CLAT assays (Ashikaga et al., 2006; Bauch et al., 2011). The intralaboratory validation study was conducted with a set of 54 substances comprising 32 skin sensitizers with diverse potencies and 22 non-sensitizing industrial substances and cosmetic ingredients that were subjected to all six tests. This study was an extension of the study previously published and which assessed the individual methods (with the exception of OECD QSAR toolbox and LuSens assay) when testing the substances described by the performance standards proposed by ICCVAM and 2,4,6-trinitrobenzene sulfonic acid (Bauch et al., 2011; ICCVAM, 2009; OECD, 2010). These performance standards recommend chemicals to be used to allow a more rapid validation of modifications to the LLNA protocols. The assays and results are summarized as an overview in Table 5. The substances cover fourteen different structural alerts of the OECD QSAR toolbox for protein binding.

Each individual assay offered sensitivities between 64% and 89% or 56% and 83%, and specificities between 77% and 100% or 71% and 100% and the overall accuracies ranged from 76% to 86% or 70% to 81% when compared to human data or LLNA data, respectively. Among single assays, the DPRA showed the highest predictivity (human: 89% sensitivity, 82% specificity and 86% accuracy) and correctly predicted the sensitizing potential of 43 of 50 substances

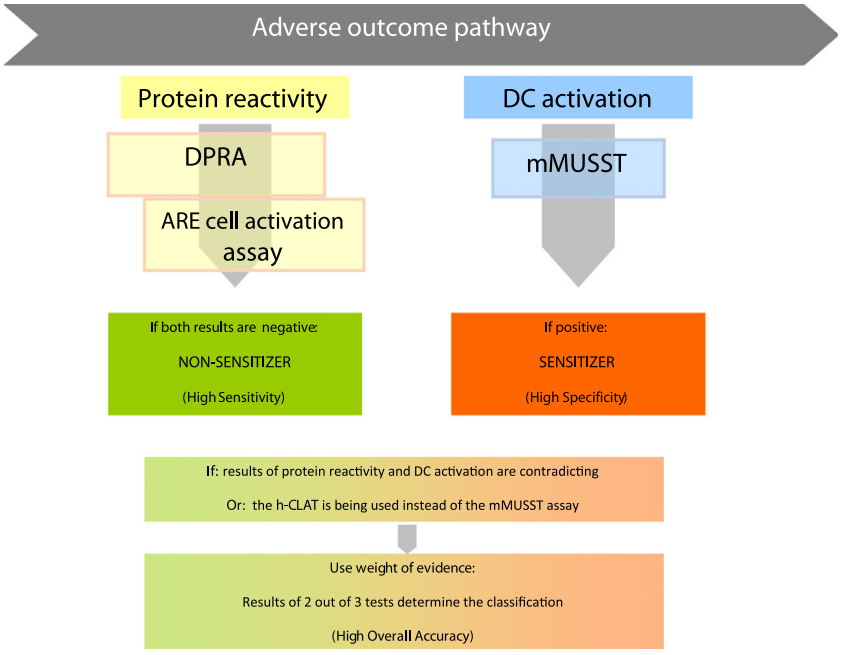


**Fig. 1.** Schematic representation of the proposed prediction model. If the two peptide-reactivity assays, DPRA and ARE cell activation assays (LuSens or KeratinoSens™), yield negative results, then the substance is considered to be nonsensitizing. If substances are predicted to be sensitizing in the mMUSST assay, the substance is then considered to be a sensitizer. If the predictions are conflicting, or if the h-CLAT is used instead of the mMUSST, a pragmatic 'weight of evidence' approach is used: Any two of the three tests rule the overall result (any two assays must be positive to rate the substance as a skin sensitizer and any two assays must be negative to rate the substance as a nonsensitizer).

when compared to human data. This was somewhat higher than the sensitivity of 81%, specificity of 91% and accuracy of 84% reported for cysteine reactivity assay by Gerberick and co-workers in 2007 (Gerberick et al., 2007). In this study the DPRA identified all pro- and prehaptens correctly with the exception of aniline. These correct predictions were unexpected as the assay does not include metabolic activation, i.e. these results should actually be considered to be false positives if the mechanistic accuracy of the assay itself was to be assessed. For particularly sensitive substances, oxygen dissolved in the incubation medium may be sufficient to result in peptide reactivity. It is therefore speculated, that the oxygen in the sample mixture is sufficient to transform prehaptens to prohaptens although the transformation from prohaptens to haptens cannot be explained.

The OECD QSAR Toolbox Version 2.0 was used to predict the protein reactivity of the parental molecule structures. The predictions yielded no false positive results but incorrectly classified 10 substances as being non-skin sensitizers. Among the false negatives, eight pro- and prehaptens were not identified as sensitizing substances. If the metabolic pathway and/or correct metabolic activation of the substances is known, the accuracy of this *in silico* method can be increased. As data on metabolites of the pro- or prehaptens tested in this study is available, which is rarely the case, further analyses were performed using the OECD QSAR Toolbox Version 2.0. The skin metabolism module predicts metabolites and these can be again processed for their protein reactivity. Six of eight pro- or prehaptens were then correctly detected as having a skin sensitizing potential since at least one protein binding metabolite was predicted (data not shown). The skin metabolism module was then applied to the 54 substances evaluated in this study and protein binding metabolites were also occasionally predicted for nonsensitizing substances. Therefore, application of the skin metabolism module did not improve overall predictivity. As the metabolites for all substances are not known, the potential metabolites calculated by the program may not sufficiently reflect those found *in vivo*. Application of the protein binding module to assess the protein reactivity of the parent substance did offer sufficiently good predictivities to be used for initial assessments of the potential protein binding, although a better understanding and integration of the skin metabolism could improve the accuracies.

Each method has limitations regardless of whether animal, human or non-animal test and that usually defines its applicability domain. Probably the major limitation of the LuSens, KeratinoSens™, mMUSST and h-CLAT assay, and any submerse cell culture system without an air–liquid interface exposure, is the water solubility of the test substances. In general, the test substance must be soluble in the aqueous culture media to obtain reliable results, albeit limited amounts of a solubilizer such as 1% DMSO can be used. Any precipitation of the test substance can cause inaccurate results. Nukada and co-workers described that water-insoluble substances are the limitation of DC activation assays and decrease their sensitivity (Nukada et al., 2011). For this reason, three test substances were excluded from the original set of 59 test substances: Chlorobenzene, 1-bromobutane and geraniol. No appropriate alternative vehicle was identified that did not have a concomitant negative effect on the cell viability and the basal expression of the CD86 and/or CD54 cell surface marker. A further two substances (Tween 80 and Triton-X 100) were excluded as, in our hands, foam formation interfered with the flow cytometric analyses in spite of the cells being washed prior to analysis. Nickel has been reported to be a skin sensitizer in the h-CLAT but this seems to be dependent from which source the cells were obtained (Kosaka et al., 2008). This may also be one reason why some results generated in this study differed from those reported by Ashikaga (2010). An additional limitation of cell based assays is the metabolic capacity. Cellular systems like dendritic cell-like cell lines are reported to lack some metabolic competence (Chipinda et al., 2011; Roggen et al., 2008). As prehaptens are not reactive towards proteins but need to undergo spontaneous chemical reactions to form a reactive hapten, and prohaptens require a metabolic transformation to form a protein-reactive hapten (Lepoittevin, 2006; Smith and Hotchkiss, 2001) these aspects are not always



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sufficiently covered by the test system used. This is also one of the major disadvantages of the *in chemico* tests, e.g. the DPRA, although in this study most prohaptens were unexpectedly identified. Both prehaptens were predicted correctly probably due to auto-oxidation (in air). In the absence of metabolic activity (either intrinsic or added), prohaptens are typically identified as being non- or weak skin sensitizers. The DPRA as performed here uses peptide depletion as a measure of peptide reactivity but does not identify and measure the newly formed peptide adducts – the representatives of the ultimate antigens. Ball and co-workers reported that some surfactants lead to the dimerization of peptides thereby leading to peptide depletion without adduct formation (Ball et al., 2011). This was also the case for SDS which would explain the false positive result obtained in the DPRA for SDS in this study. These substances would then be incorrectly classified as being skin sensitizers. This has also been addressed by Natsch and co-workers, who then further developed the method by using a cor1 peptide which includes additional cysteine residues, followed by a LC–MS analysis. This modifications enable the identification of the parent peptide, and adducts and oxidization products thereof (Natsch and Gfeller, 2008).

In this study, the newly developed LuSens assay showed an activation of the reporter gene after exposure to all pro- or prehaptens except eugenol. The U937 cells (mMUSST assay) were able to respond to all pre or prohaptenic substances with the exceptions of isoeugenol and cinnamic alcohol, whereas the THP-1 cells (h-CLAT assay) responded to all pro- and prehaptenic substances tested in this study. None of these assays specifically included addition of a metabolizing system, for example S9 mix. Since the prohaptens and prehaptens were correctly identified as skin sensitizers, this may indicate that endogenous metabolic capacity in the cell-based systems is sufficient and/or that the assays allow for adequate non-enzymatic oxidation. For example, the prehapten 4-phenylenediamine is known to be non-enzymatically oxidized to a Bandrowski's base. Several researches have reported initial endeavors to incorporate metabolic activation in both the cell based assays and DPRA. Gerberick et al. (2009) further developed the DPRA to incorporate a certain degree of metabolic activation via the addition of horse radish peroxidase. Chipinda and co-workers detected an activation of THP-1 cells by prohaptens such as benzo[*a*]pyren, carvoxime and cinnamic alcohol in the presence of a rat-liver S9 fraction (Chipinda et al., 2011). Schreiner and co-workers described a co-culture based cell system using primary keratinocytes and dendritic cells. With this system they were able to obtain positive responses with prohaptens (Schreiner et al., 2007,, 2008). Additionally, integration of reconstructed skin models into test strategies could be used to provide metabolic activation (Jaeckh et al., in press; Oesch et al., 2007; Pendlington et al., 2008). Skin models also offer the advantage of having a skin barrier function which would incorporate the factor of dermal penetration and bioavailability into the model and would also facilitate the application of poorly soluble test substances.

Despite the limitations, each individual assay has been shown to generate results with good predictivities for a large number of substances (Gerberick et al., 2007; Natsch and Emter, 2008; Python et al., 2007; Sakaguchi et al., 2007; Ashikaga et al., 2010). The goal of this study was therefore not primarily to assess individual predictivities but to select and combine assays according to rational models. In general, models which answer mechanism specific questions on the sensitization process should ideally be based on steps derived from the biological pathway, as this can be of use when dissecting the individual events leading to an adverse event, e.g. those used in the proposed prediction model. In order to have all options, a test battery of three tests, namely a peptide reactivity assay (e.g. DPRA or the LC–MS based peptide reactivity assay), an assay based on the activation of ARE related genes (e.g. the KeratinoSens™ or LuSens assays), and an assay based of antigen presenting cell activation (e.g. the mMUSST or h-CLAT assays) is proposed. Depending on the selected model, an optimized order of tests (a prediction model) can be designed to make testing more efficient. The model developed uses key steps in the sensitization pathway to assess the sensitization potential. For classification purposes without the use of animal testing, we propose using both the direct and indirect reactivity assays ("if both are negative") to identify nonsensitizers and the DC activation test mMUSST ("if positive") to determine the sensitization potential. If the predictions are contradictory, or if the h-CLAT is used instead of the mMUSST, then a weight of evidence approach ("2 out of 3") should be used. This model strives for a high overall accuracy of the prediction of a skin sensitizing potential of a substance in humans, which has been the aim of most *in vitro* assays developed so far. The accuracy (94%) of the prediction model is equivalent or even surpasses that of the LLNA, and high sensitivities (93%) and specificities (95%) were obtained in this study. This indicates an excellent approach to reducing animal testing for the endpoint sensitization.

The model cannot only be used for classification purposes but also for other applications, e.g. parts of the model can also be used to address concerns that the product developer may encounter, e.g. limited amount of substance available for testing, large number of substances need to be screened. During the screening process it is usually important to primarily identify possible skin sensitizers or to ensure that the probability of the substances being nonsensitizers is given. The model optimizes the predictivity to yield either a high sensitivity or a high specificity – obviously two questions of different nature and profoundly different consequences. A high sensitivity is used to reliably exclude a sensitizing potential, this would be addressed by the reactivity assays and/or activation of the Keap-1/Nrf2 signaling pathway (e.g., DPRA, LuSens). A high specificity reduces the number of false positive results, which can be of importance when making decisions during product development when an otherwise promising candidate is prematurely rejected; this would be addressed by the dendritic cell activation assay (mMUSST).

The models presented here are not yet able to classify substances into weak, moderate, strong or extreme skin sensitizer; i.e. do not allow assessments of potency. Potency is, however, an important parameter used in risk and safety assessments. The LLNA still remains the gold standard for potency assessments. Potency is predicted via estimated concentration leading to a specified fold induction of the proliferative responses of the lymph node cells. Kimber and coworkers described thresholds in the LLNA to categorize skin sensitizer as weak, moderate, strong and extreme skin sensitizer (Kimber et al., 2003). For classification according to the global harmonized system (GHS), it is of interest to distinguish strong and less strong skin sensitizers (Category 1A or 1B). For the *in chemico* method DPRA, Gerberick and coworkers drafted a tree model to classify the reactivity of substances according their mean peptide depletion ability into minimal, low, moderate, or high (Gerberick et al., 2007). Jowsey and co-workers published a hypothetical prediction model where dose–response and a relative activity in test systems are assigned a score for to be used in classification of skin sensitizers. This proposal needs to be further corroborated using experimental data (Jowsey et al., 2006). Natsch and coworkers enhanced predictivities by using a combination of an ARE-reporter assay, peptide reactivity assay, and an *in silico* model (TIMESS). A recently published integrated testing strategy based on experimental data (Jaworska et al., 2011), proposes a way of integrating different *in silico*, *in chemico* and *in vitro* methods that leads to generation of networks. What remains to be seen is whether a suitable predictive model can be developed to use the combination of methods used in this study to allow potency to be assessed.



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## 5. Conclusions and outlook

To the best of our knowledge, this study is the first to test the same 54 substances in all six methods presented in this study and to develop test strategies suitable for the reliable prediction of sensitizing potential for a substantial number of substances. The test battery reflects key steps in the sensitization process namely protein reactivity, Nrf-2/ARE activation as well as dendritic cell activation. The accuracy of the prediction model proposed exceeded that of the LLNA for set of substances tested. These very promising results point to a possible future replacement of animal testing while maintaining the same degree of accuracy and predictivity for human skin sensitizers. Future developments will need to focus on applicability domains and potency. Whether the development of a prediction model to assess the potency of skin sensitizer using the tests described in this study is feasible will be a challenge.

## 6. Conflict of interest statement

Other than employment, the authors are not aware of any conflicts of interest.

## 7. Uncited reference

Q3 Natsch et al. (2009).

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