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journal homepage: www.elsevier.com/locate/toxinvitIntralaboratory validation of four *in vitro* assays for the prediction of the skin sensitizing potential of chemicalsCaroline Bauch^{a,b}, Susanne N. Kolle^a, Eric Fabian^a, Christina Pachel^{a,c}, Tzutzy Ramirez^a, Benjamin Wiench^{a,d}, Christoph J. Wruck^b, Bennard van Ravenzwaay^a, Robert Landsiedel^{a,*}^a BASF SE, Experimental Toxicology and Ecology, Germany^b RWTH Aachen University, Department of Anatomy and Cell Biology, Germany^c Technical University Kaiserslautern, Germany^d Johannes Gutenberg University Mainz, Faculty of Chemistry, Pharmaceuticals and Geosciences, Germany

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ABSTRACT

Allergic contact dermatitis is induced by repeated skin contact with an allergen. Assessment of the skin sensitizing potential of chemicals, agrochemicals, and especially cosmetic ingredients is currently performed with the use of animals. Animal welfare and EU legislation demand animal-free alternatives reflected in a testing and marketing ban for cosmetic ingredients beginning in 2013. The underlying mechanisms of induction and elicitation of skin sensitization are complex and a chemical needs to comply several properties being skin sensitizing. To account for the multitude of events in the induction of skin sensitization an *in vitro* test system will consist of a battery of various tests.

Currently, we performed intralaboratory validations of four assays addressing three different events during induction of skin sensitization. (1) The peptide reactivity assay according to Gerberick and co-workers (Gerberick et al., 2004) using synthetic peptides and HPLC analysis. (2) Two dendritic cell activation assays based on the dendritic cell like cell lines U-937 and THP-1 and flow cytometric detection of the maturation markers CD54 and/or CD86 (Ashikaga et al., 2006; Python et al., 2007; Sakaguchi et al., 2006). (3) Antioxidant response element (ARE)-dependent gene activity in a HaCaT reporter gene cell line (Emter et al., 2010). We present the results of our intralaboratory validation of these assays with 23 substances of known sensitizing potential. The sensitivity, specificity, and accuracy of the individual tests were obtained by comparison to human epidemiological data as well as to data from animal tests such as the local lymph node assay.

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1. Introduction

Allergic contact dermatitis (ACD) is a common skin disease with a significant social and economic impact. ACD is induced by repeated skin contact with an allergen (hapten, pro-hapten or complete allergen). The assessment of the skin sensitizing potential of haptens like industrial chemicals, agrochemicals, and cosmetic ingredients is crucial to define their safe handling and use. Up to now, animal based methods using guinea pigs or mice are used to

identify skin sensitizing potential (ICCVAM, 2008, 2009; OECD, 2010). Public concern regarding animal welfare, which is also reflected in current legislation such as the 7th Amendment to the Cosmetics Directive (Council Directive 76/768/EEC, 1976) as well as REACH (Council Directive 1907/2006/EEC, 2006), demands animal free alternative methods. Especially for cosmetics and cosmetic ingredients testing with animals is restricted since 2009. The marketing ban for cosmetic with ingredients tested for skin sensitization in animals starts in March 2013. Currently there is no validated and accepted animal free alternative available for skin sensitization testing. The mechanisms of induction and elicitation of skin sensitization are complex. Briefly, the contact allergen needs to penetrate the skin and to be either reactive towards endogenous proteins itself or – as pro-hapten – which has to be metabolically activated in the skin. Subsequently, the elaboration of inflammatory responses by keratinocytes occurs via the secretion of inflammatory cytokines like IL-1 α or IL-18. Following, the activation of dendritic cells (DC), the up-regulation of co-stimulatory cell surface markers, the secretion of cytokines, and migration of DC into lymph nodes

Abbreviations: ACD, allergic contact dermatitis; ARE, antioxidant response element; DC, dendritic cells; DMSO, dimethyl sulfoxide; DNCB, 1-chloro-2,4-dinitrobenzene; DPRA, direct peptide reactivity assay; FBS, fetal bovine serum; h-CLAT, human Cell Line Activation Test; Kathon CG, mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one; LLNA, local lymph node assay; MUSST, myeloid U-937 skin sensitization test; Nrf2, nuclear factor-erythroid 2-related factor 2; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

* Corresponding author. Address: BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany. Tel.: +49 621 60 56203; fax: +49 621 60 58134.

E-mail address: robert.landsiedel@basf.com (R. Landsiedel).

takes place. These mature DC interact in the local lymph node with naive T cells and prime them to proliferate and differentiate into specific T cells (Kimber et al., 2002; Ryan et al., 2007). Due to the complexity of the underlying mechanisms, the interaction of different cell types like dendritic cells, keratinocytes and fibroblast a single *in vitro* test is likely not able to cover the all steps of skin sensitization (dos Santos et al., 2009; Corsini et al., 2009). Hence, an *in vitro* test system will consist of a battery of various tests covering several steps of the described process. The aim of this study was the intralaboratory validation of four *in vitro* assays to detect skin sensitizer and to compare their predictivity using a panel of 23 test substances, including the performance standards of the LLNA.

2. Materials and methods

Test substances. The test substances for this intralaboratory validation included the 22 performance standards of the local lymph node assay according the OECD guideline 429 and 2,4,6-trinitrobenzene sulfonic acid (OECD, 2010). For these compounds results from murine LLNA as well as human patch test data are available (Kimber et al., 2003; Basketter et al., 1999b). Substances with skin sensitizing potential are 1-chloro-2,4-dinitrobenzene (98%; DNCB); 2,4,6-trinitrobenzene sulfonic acid (5% (w/v); TNBS); 2-mercaptobenzothiazole (>99%); cinnamic alcohol (98%); citral cis/trans mixture (95%); cobalt(II)-chloride (97%); ethylene glycole dimethacrylate (98%); eugenol (99%); α -hexyl cinnamic aldehyde (85%); imidazolidinylurea (95%); isoeugenol cis/trans mixture (98%); Kathon CG (1.2% 5-chloro-2-methyl-4-isothiazolin-3-one and 0.3% 2-methyl-4-isothiazolin-3-one); methylmethacrylate (99%); methyle salicylate (>99%); nickel(II)-chloride (98%); paraphenyldiamine (>99%); phenylbenzoate (99%). Test Substances without sensitizing potential are chlorobenzene (99%); isopropanol (>99%); D/L-lactic acid (90%); salicylic acid (>99%); sodium dodecylsulfate (98%); xylene (98.5%). All chemicals were purchased from Sigma–Aldrich Chemie GmbH, Germany.

3. Dendritic cell activation assay – U-937

The dendritic cell activation assay using U-937 cells, also known as Myeloid U-937 Skin Sensitization Test (MUSST), was performed as described by Python and co-workers (Python et al., 2007) with minor modifications (Fig. 1a). Briefly, U-937 cells, human monocytic leukemia cell line, were obtained from the German Resource Center for Biological Material (DMSZ; ACC 5). Cells were cultured in complete RPMI 1640 medium with 25 mM HEPES buffer and 2 mM L-glutamine (Invitrogen, Germany) supplemented with 10% fetal bovine serum (FBS; Biochrom AG, Germany) and 1% penicillin (100 U/mL)–streptomycin (100 μ g/mL) (Biochrom AG, Germany) in T75 culture flasks (TPP, Switzerland). The cells were kept in a humidified atmosphere at 37 °C and 5% CO₂. They were subcultured every 3–4 days with an initial cell density of 1×10^5 c/mL. Cells were passaged for 5 weeks to a maximum of 15 weeks. Pre-cultures were performed by diluting the cells 1:2 for experiments at the following day and 1:4 for experiments after 2 days. For substance incubation cells were seeded in 96 well microtiter plates by pipetting 100 μ L of 0.5×10^6 c/mL cell suspension. Substances were dissolved in medium (2 $\times$) or DMSO (400 $\times$). DMSO solved substances were further diluted in medium to obtain 2 $\times$ stock solution. Final DMSO concentration on the cells did not exceed 0.25%. For each substance five concentrations were tested in duplicates. Concentrations were chosen according to preliminary propidium iodide (PI) cytotoxicity assays. Therefore, cells were exposed to 12 concentrations of 1:2 serially dilution starting at 2000 μ g/mL for solid test compound or 1000 μ g/mL for liquid test

compound, respectively. Assessment of viable cells was performed according to the main experiment procedures but cells were stained using propidium iodide only. The highest tested concentration in the main experiment was 2 $\times$ concentrations causing a cytotoxicity of 25% (CV75). The additional concentrations were obtained by a 1:2 dilution serie of the 2 $\times$ CV75. Each concentration was run in duplicates and each experiment was performed minimum in two independent times. Cells were exposed to the test substance for 48 h under general cell culture conditions. After treatment, cells were pelleted by centrifugation at 400 g for 5 min at RT and washed once with PBS containing 5% FBS. Cells were resuspended in 100 μ L PBS w/ 5% FBS and labeled for 30 min at 4 °C in the darkness with 5 μ L IgG-FITC (Cat. No. 555748, BD Pharmingen, Germany) or 5 μ L anti-CD86-FITC antibody (Cat. No. 555657, BD Pharmingen, Germany). Follow incubation, cells were washed twice with PBS w/ 5% FBS and resuspended in PBS. Cells nuclei were stained with propidium iodide (PI, 50 μ g/mL in PBS) for 5 min at RT in the darkness. Cells were analyzed by flow cytometry using a BeckmanCoulter FC500MPL equipped with MXP software. The analysis was performed in 10,000 living cells.

4. Dendritic cell activation assay – THP-1

The dendritic cell activation assay using THP-1 cells, also known as human Cell Line Activation Test (h-CLAT), was performed as described by Ashikaga and co-workers (Ashikaga et al., 2006) and Sakaguchi and co-workers (Sakaguchi et al., 2006) with minor modifications (Fig. 1a). Briefly, THP-1 cells, human monocytic leukemia cell line, were obtained from the German Resource Center for Biological Material (DMSZ; ACC 16). THP-1 cells were cultured in complete RPMI 1640 with 25 mM HEPES buffer and 2 mM L-glutamine (Invitrogen, Germany) supplemented with 10% FBS (Biochrom AG, Germany), 1% penicillin (100 U/mL)–streptomycin (100 μ g/mL) (Biochrom AG, Germany) and 0.05 mM 2-mercaptoethanol (Invitrogen, Germany) in T150 culture flasks (TPP, Switzerland). The cells were kept in a humidified atmosphere at 37 °C and 5% CO₂. They were subcultured every 3–4 days with an initial cell density of 2×10^5 c/mL. Cells were passaged for 8 weeks to a maximum of 24 passages. For experiments, cells were seeded in 24 well plates (TPP, Switzerland) by adding 1×10^6 cells in 500 μ L per well. Substances were dissolved in medium (2 $\times$) or DMSO (500 $\times$). DMSO solved substances were further diluted in medium to obtain 2 $\times$ stock solution. Final DMSO concentration on the cells did not exceed 0.2%. For each substance eight concentrations were tested in duplicates. Concentrations were chosen according to preliminary propidium iodide (PI) cytotoxicity assays. Therefore, cells were exposed to nine concentrations of 1:2 serially dilution starting at 2000 μ g/mL for solid test compound or 1000 μ g/mL for liquid test compound, respectively. Assessment of viable cells was performed according to the main experiment procedures but cells were stained using propidium iodide only. The highest tested concentration in the main experiment was 1.2 $\times$ CV75. The additional concentrations were obtained by a 1:1.2 dilution serie of the 1.2 $\times$ CV75. Each concentration was run in duplicates and each experiment was performed minimum in two independent times. Treatment was performed by applying 500 μ L of the test substance dilution to each well for 24 h. For analysis cells were transferred into 1.5 mL tubes (Eppendorf, Germany). Cells were spun down by centrifugation at 200 $\times$ g and 4 °C for 5 min and washed twice by centrifugation at 200 $\times$ g and 4 °C for 5 min with 1 mL staining buffer, PBS w/ Ca²⁺–Mg²⁺ (Biochrom AG, Germany) and 0.1% BSA (Sigma, Germany). Cells were resuspended in 600 μ L staining buffer containing 0.01% Cohn fraction (Sigma, Germany) and incubated for 15 min at 4 °C in the

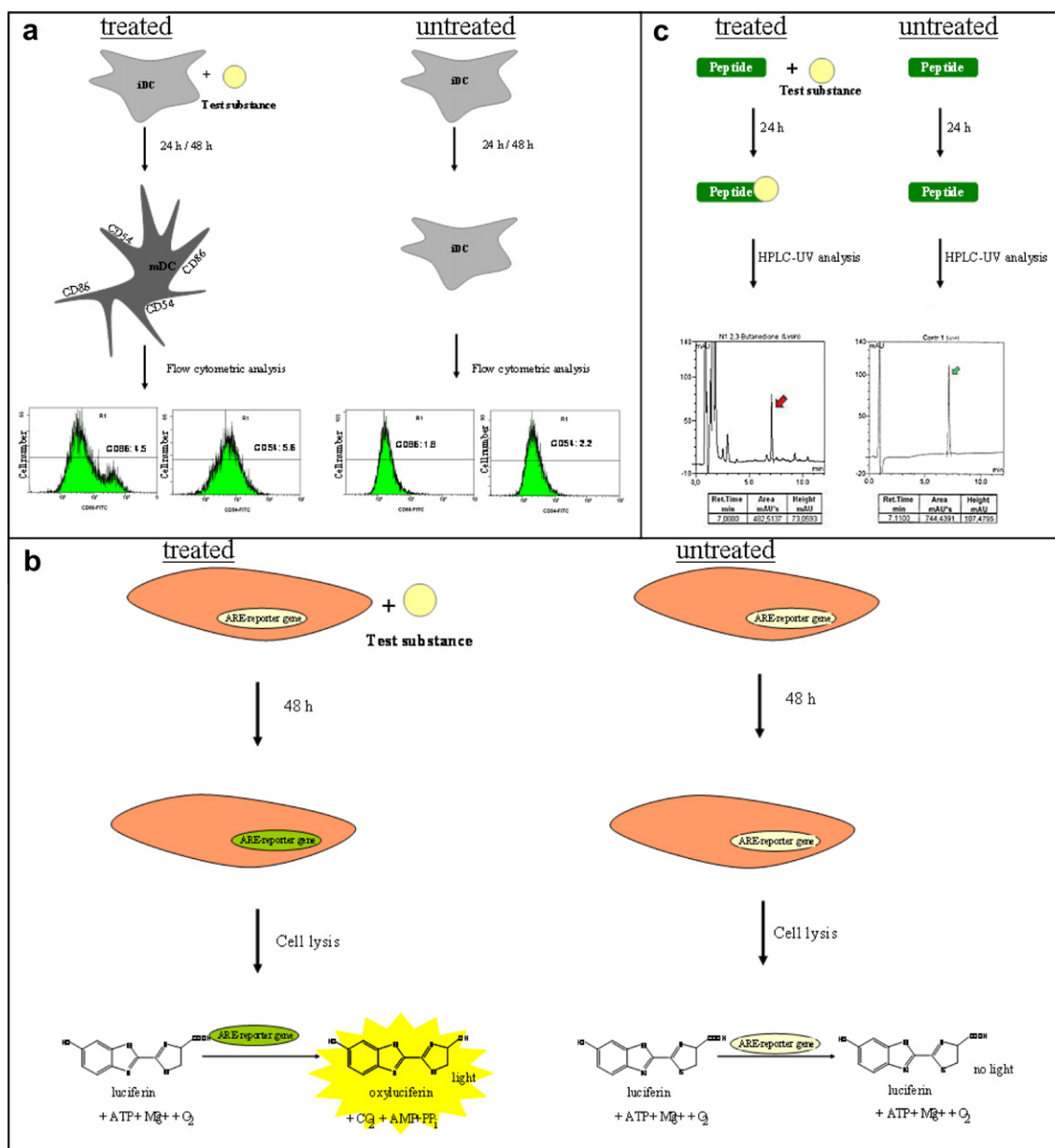


Fig. 1. Schematic representation of the *in vitro* models used in our test battery. (a) Dendritic cell activation assays (MUSST/h-CLAT): immature cells (iDC) are exposed to test substance for 48 h. Sensitizers activate iDC and lead to the maturation of DC (mDC). Consequently, specific markers are overexpressed on the cell surface (CD86 for MUSST and CD86/CD54 for h-CLAT). Cell surface markers are analyzed by flow cytometry. (c) Reporter gene analysis with KeratinoSens[®]: cells expressing luciferase are exposed to test substance for 48 h and analyzed according to the luciferase activity after cell lysis using SteadyGlo[™] (Promega). (d) Peptide Binding Assay: cysteine and lysine peptides are exposed to test substance for 24 h and analyzed according to the peptide peak depletion using HPLC-UV technique.

dark. Then cells were distributed in V-shaped 96 well microtiter plate (TPP, Switzerland). Each concentration replicate was split into 3 wells by pipetting 180 μ L cell suspension per well. Cells were pelleted by centrifugation at $200 \times g$ and $4^\circ C$ for 5 min and resuspended in 50 μ L 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) anti-CD86-FITC (1:7 diluted in PBS; Cat. No. 555657, BD Pharmingen, Germany). Incubation was performed at $4^\circ C$ in the dark for 30 min. Then, cells were washed twice with 200 μ L staining buffer by centrifugation at $200 \times g$ and $4^\circ C$ for 5 min and resuspended in 200 μ L staining buffer. PI (50 μ g/mL in PBS) was added 5 min before flow cytometric analysis and incubated at RT in the dark. Flow cytometric analysis was performed with a BeckmanCoulter FC500MPL and the MXP software.

5. KeratinoSens[®] assay

KeratinoSens[®] cell line was kindly provided by Andreas Natsch, Givaudan. 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 Nrf2 transcription factor activity (Fig. 1b). 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). Cells were kept in a humidified atmosphere at $37^\circ C$ and 5% CO₂. They were subcultured every 3–4 days without exceeding a confluence greater 90%. Cells were passaged for a

maximum of 12 weeks. For chemical exposure KeratinoSens® cells were seeded in three white 96 well microtiter plates (PerkinElmer, Germany) and one transparent 96 well microtiter plate (TPP, Switzerland). Per well 1×10^4 cells in 120 μ L medium containing 10% FBS w/o G-418 were added and allowed to adhere for 24 h under standard cell culture conditions. Prior to treatment, medium was aspirated and renewed by adding 150 μ L medium containing 1% FBS w/o G-418. Substances were diluted in distilled water or in DMSO (final concentration of 200 mM). Stock solutions were prepared by diluting the substances in a common ratio of 1:2 with the appropriate vehicle. Substances were further diluted in a 25-fold manner by adding 10 μ L stock solution to 240 μ L medium containing 1% FBS w/o G-418 resulting in 4 $\times$ stock solution. Treatment was performed by adding 50 μ L of each 4 $\times$ stock solution. For chemicals dissolved in water the DMSO level was adjusted to 1%. Substance incubation was performed under standard cell culture conditions for 48 h. Analysis were performed using SteadyGlo™ (Promega, Germany) according to manufacturer instructions and the PerkinElmer Victor³ photometer (PerkinElmer, Germany). Cytotoxicity was assessed using MTT cytotoxicity assay. Briefly, 5 mg/mL stock solution was prepared in PBS, sterile filtrated and diluted in medium (1:10) containing 1% FBS. 200 μ L of this working solution were added to each well of the transparent 96 well microtiter plate and incubated for further 2 h. For analysis medium was aspirated and cells were lysed by adding 100 μ L 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 read at 570 nm with reference wavelength at 690 nm using the PerkinElmer Victor³ photometer.

6. Direct peptide reactivity assay

The Peptide Binding Assay, also known as DPRA (Direct peptide reactivity assay), is a cell free, *in chemico* assay with the use of artificial model peptides according to Gerberick and co-workers (Gerberick et al., 2004) (Fig. 1c). In brief, test substances were dissolved in acetonitrile (Sigma–Aldrich, Germany) or if not soluble in acetonitrile/water mixture or water to obtain a concentration of 100 mM. Substances were freshly prepared every time before use. Cysteine model peptides (Synbiosci, Livermore CA, USA) were prepared in 0.667 mM stock solution in 100 mM phosphate buffer (100 mM NaH₂PO₄, 100 mM Na₂HPO₄, both Sigma–Aldrich, pH = 7.5) and lysine model peptides (Synbiosci, Livermore CA, USA) were prepared 100 mM ammonium acetate buffer (pH = 10.2). Substance stocks were diluted with peptide solutions in a ratio of 1:4:15 (v/v/v) substance:acetonitrile:cysteine peptide and 1:3 (v/v) substance:lysine peptide. Each substance was analyzed in triplicates for each peptide. Incubation was performed at room temperature in the dark for 24 h. 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 mm $\times$ 100 mm $\times$ 3 micron particle; Phenomex). Separation of peptides was achieved by isocratic flow with a flowrate of 0.35 mL/min and a linear gradient from 10% to 25% acetonitrile over 10 min, followed by a rapid increase to 90% acetonitrile.

7. Results

Each assay described above was performed to assess 23 test substances of the LLNA performance standards list and TNBS. Substances were classified as skin sensitizer according to the following criteria: 1.2 $\times$ or greater the expression of CD86 in the MUSST; 1.5 $\times$ or greater induction of CD54 and/or CD86 in h-CLAT; 1.5 $\times$ or greater luciferase expression in KeratinoSens®; and greater than 6.4% of the mean peptide depletion of cysteine and lysine peptide in the

DPRA. Results were expressed as positive if the test substance was predicted as skin sensitizer and as negative if they were predicted as non-skin sensitizer. For all compounds human patch test data are available from the literature and were described offering significant skin sensitizing potential based on available clinical studies (Basketter et al., 1999b; ICCVAM, 2009). Compared to human data all cell based assays were able to predict 20 of 23 substances correctly; the *in chemico* DPRA was able to predict 21 of 23 substances correctly. The MUSST assay predicted three sensitizing compounds false negative, isoeugenol, cinnamic alcohol and α -hexyl cinnamic aldehyde (Table 1). Evaluation of data with Cooper statistics (Cooper et al., 1979) (Figs. 2 and 3) revealed that the MUSST assay offered a sensitivity of 81%, a specificity of 100%, a positive predictive value (PPV) of 100% and a negative predictive value (NPV) of 70%. The overall accuracy for MUSST assay was 87% (Fig. 2a). The h-CLAT assay predicted two sensitizing compounds false negative: imidazolinidyl urea and nickel chloride, and salicylic acid false positive. Cooper statistics revealed a sensitivity of 81%, a specificity of 86%, a PPV of 93% and a NPV of 67%. The overall accuracy for h-CLAT assay was 83% (Fig. 2b). The KeratinoSens® assay predicted three of the sensitizing compound false negative: methyl methacrylate, nickel chloride and phenyl benzoate. Cooper statistics estimated a sensitivity of 81%, a specificity of 100%, a PPV of 100% and a NPV of 70%. The overall accuracy of the KeratinoSens® assay was 87% (Fig. 2c). The *in chemico* approach DPRA predicted α -hexyl cinnamic aldehyde false negative and sodium dodecyl sulfate false positive. Cooper statistics estimated a sensitivity of 94%, a specificity of 86%, a PPV of 94% and a NPV of 86%. The overall accuracy of the DPRA was 91% (Fig. 2d).

The murine LLNA assay offered for these 23 tested compounds a sensitivity of 94%, a specificity of 86%, a PPV of 94% and a NPV 86%. The overall accuracy of the LLNA is 91%. The murine LLNA (Basketter et al., 1999b; Kimber et al., 2003) predicted nickel chloride as false negative and sodium dodecyl sulfate false positive if compared to human data. Comparison of our results with LLNA data from literature indicated that the h-CLAT assay, KeratinoSens® assay and DPRA offers the same predictivity as compared to human data (Figs. 2 and 3). The MUSST assay showed a reduced predictivity if results were compared to LLNA data than to human data. The MUSST assay predicted four of the sensitizing compounds false negative: isoeugenol, cinnamic alcohol, α -hexyl cinnamic aldehyde and sodium dodecyl sulfate, and nickel sulfate false positive. Cooper statistics displayed that the MUSST assay offered a sensitivity of 75%, a specificity of 86%, a PPV of 92% and a NPV of 60%. The overall accuracy for MUSST assay was about 78% (Fig. 3a).

8. Discussion

In this study we report the intra-laboratory validation of four *in vitro* assays: the *in chemico* Peptide Binding Assay (DPRA; (Gerberick et al., 2004)), the reporter gene based assay – KeratinoSens® assay from Givaudan with an ARE-dependent luciferase reporter gene for Nrf2 activity and the dendritic cell activation assays using the cell lines U-937 cells (MUSST assay) and THP-1 cells (h-CLAT assay). The performance standards of the LLNA (OECD, 2010) were used as test substances comprising 16 sensitizing and 7 non-sensitizing compounds.

The results of each assay were compared to literature data of human patch tests and murine LLNA and were evaluated with so called Cooper statistics. Results for human data where taken from literature (Basketter et al., 1999b). Basketter and co-workers referred to other publications (Gerberick et al., 2000; Kimber et al., 1994; Kligman, 1966; Basketter et al., 1996; 1994; 1999a; De Groot et al., 1994; Rietschel and Fowler, 1995) where those data were based on available clinical studies only and offered significant

Table 1

Summary of the data obtained from the *in vitro* test systems, the human patch test and murine LLNA data were obtained from the literature. Substances indicated as (+) were rated positive and those indicated as (–) were rated as negative.

Substance	Human	Literature <i>In vivo</i>	Experimental data <i>In vitro</i>			
			LLNA	U-937	THP-1	DPRA
1-Chloro-2,4-dinitrobenzene	+	+	+	+	+	+
2-Mercaptobenzothiazole	+	+	+	+	+	+
2,4,6-Trinitrobenzenesulfonic acid	+	+	+	+	+	+
4-Phenylenediamine	+	+	+	+	+	+
Cinnamic alcohol	+	+	–	+	+	+
Citral	+	+	+	+	+	+
Cobalt chloride	+	+	+	+	+	+
Ethylene glycol dimethacrylate	+	+	+	+	+	+
Eugenol	+	+	+	+	+	+
Hexyl-cinnamic aldehyde	+	+	–	+	+	–
Imidazolidinyl urea	+	+	+	–	+	+
Isoeugenol	+	+	–	+	+	+
Kathon CG	+	+	+	+	+	+
Methyl methacrylate	+	+	+	+	–	+
Nickel chloride	+	–	+	–	–	+
Phenyl benzoate	+	+	+	+	–	+
Chlorobenzene	–	–	–	–	–	–
Isopropanol	–	–	–	–	–	–
DL-lactic acid	–	–	–	–	–	–
Methyl salicylate	–	–	–	–	–	–
Salicylic acid	–	–	–	+	–	–
Sodium lauryl sulfate	–	+	–	–	–	+
Xylene	–	–	–	–	–	–

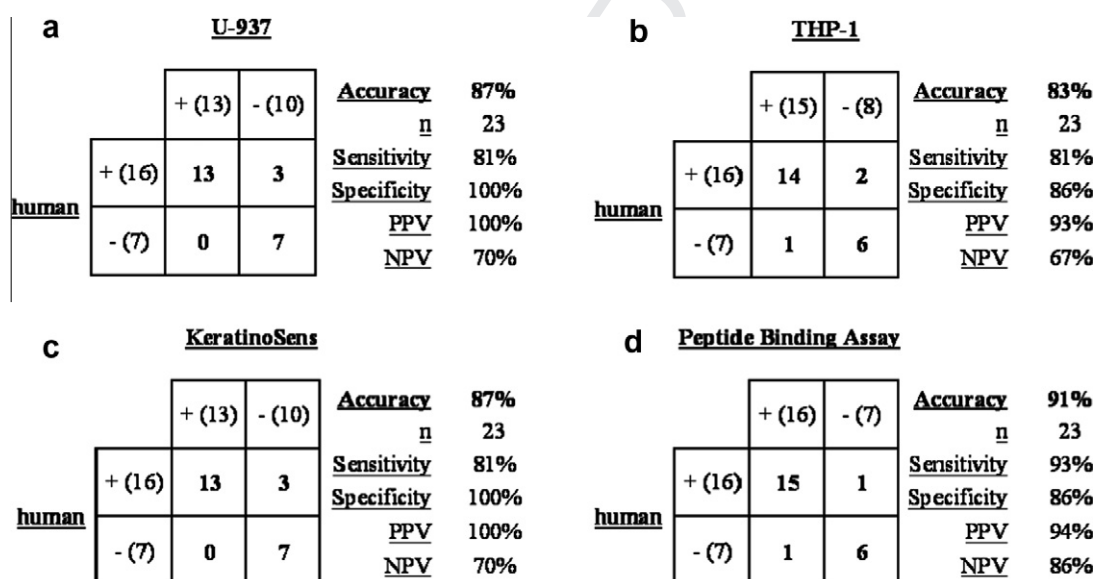


Fig. 2. Predictivity of each assay in comparison to human patch test data. Evaluation was performed using the Cooper statistics (Cooper et al., 1979). Substances predicted as sensitizers are indicated as (+) and substances predicted as non-sensitizer as (–).

evidence for skin sensitization (Basketter et al., 1999b). In summary the assays offered accuracies between 83% and 91% compared to human patch test data and between 78% and 91% compared to LLNA data. Remarkably, the DPRA showed in both comparisons the highest accuracy of 91%, followed by the KeratinoSens[®] assay with 87% and h-CLAT assay with 82%. The MUSST assay had an accuracy of 87% when compared to human data, but only 78% accuracy when compared to the LLNA data. It is important to notice that these predictivities were performed for 23 substances only and that they could be different if a larger (and/or different) set of substances is used to assess the predictivities. Nevertheless, it is remarkable that the parallel testing of the

four different methods with the same set of 23 substances yielded predictivities around 80% for the single assays.

Our data indicate that the combination of different assays, such as dendritic cell activation (MUSST or h-CLAT assay) with DPRA and/or KeratinoSens[®] assay, could create a prediction model with excellent predictivity. According to the hypothesis that a single assay will not be able to replace the animal testing approaches we combined the above mentioned methods in pairs (data not shown). This led to an increased accuracy of each approach with a striking result for the combination of MUSST assay with the KeratinoSens[®] assay: In this simple test battery no false positive or negative prediction was done and the accuracy would be 100% for the 23 test

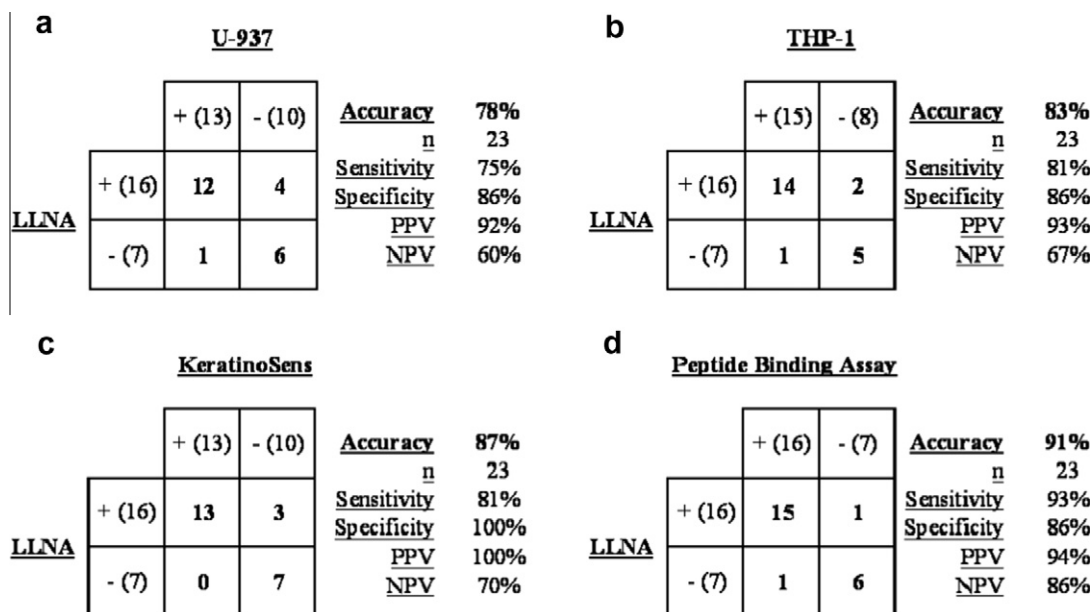


Fig. 3. Predictivity of each assay in comparison to murine local lymph node data (LLNA). Data analysis was performed using the Cooper statistics (Cooper et al., 1979). “+” indicates the number of substances predicted as sensitizer and “-” the number of substances predicted as non-sensitizer of experimental or literature data. This applies for each assay.

compounds tested. Our data for the single assays and the pairing of assays could be further validated with a larger set of test compounds.

As described above the penetration of test substance through the skin is an important factor of the skin sensitization process, since it leads to the bioavailability of haptens in the viable dermis. The stratum corneum is a barrier against the entry of noxious materials, but substances of low molecular weight (<500 Da) with lipophilic properties are able to pass this barrier (Basketter et al., 1999b). Nevertheless, the topically applied dose may not reflect the actual dose in the dermis. Currently, the arising *in vitro* approaches using cell culture do not consider the natural physical barrier that represents the stratum corneum and the reduced bioavailability of test substances *in vivo*. Therefore, there is a need for further development of test strategies that could evaluate dermal penetration on *in vitro* skin models and *in silico* processing, such as QSAR, by which the chemical structure of a compound could be quantitatively correlated to dermal penetration (Basketter et al., 1999b; Pendlington et al., 2008).

The epidermal inflammatory response induced by skin sensitizers facilitates the response of dendritic cells and T cells via the release of cytokines like IL-18. Corsini and co-workers proposed a test strategy using the keratinocyte cell line NCTC 2544 and the IL-18 release to identify skin sensitizer and to differentiate them from irritants and respiratory allergens (Corsini et al., 2009). Coquette and co-workers described the possibility to distinguish irritants and skin sensitizer by considering the IL-1 α and IL-8 ratio in reconstructed skin models (Coquette et al., 2003). Including inflammatory responses in a tiered test strategy for skin sensitization could further improve the testing strategy and help to discriminate skin sensitizers from irritants.

Isoeugenol and cinnamic alcohol are prohaptens reported to be skin sensitizers without direct reactivity towards proteins (Smith and Hotchkiss, 2001; Barratt and Basketter, 1992; Basketter, 1992; Lepoittevin, 2006). Both chemicals were predicted as true positive in the h-CLAT assay, KeratinoSens and DPRA assay. Whereas the MUSST assay, using the cell line U-937, was not able to predict isoeugenol and cinnamic alcohol as skin sensitizer. If cellular systems like dendritic like cell lines do not offer any metabolic capacity they would not be sufficient to predict prohaptens

(Chipinda et al., 2011). Therefore the metabolic capacity of the cellular systems test systems should be investigated. A lack of metabolic capacity can be compensated. Schreiner and co-workers for instance, established a co-culture of keratinocytes and dendritic cells able to detect pro-haptens like isoeugenol as sensitizers (Schreiner et al., 2008). Chipinda and co-workers featured the THP-1 based testing system with liver S9-mix of aroclor-induced rats (Chipinda et al., 2011). The use of skin models could include the metabolic activation of the various cell types of the skin and dermal penetration in the different layers of the skin (Oesch et al., 2007; Pendlington et al., 2008; Jaechk et al., 2011). Surprisingly, the *in chemico* assay DPRA was able to detect all four prohap-
tens (2-mercaptobenzothiazole, eugenol, isoeugenol and cinnamic alcohol). This may be attributed to a non-enzymatic activation – as discussed for isoeugenol (Gerberick et al., 2009) – or may, indeed, reflect false positives in this assay.

A prediction model for skin sensitization should further be able to classify test substances not only as sensitizers but also as weak, moderate, strong or extreme sensitizer. The LLNA classifies skin sensitizers according to the test concentrations provoking a proliferation of T cells with a fold induction greater than $3\times$ compared to vehicle control and with set thresholds for categorization as weak, moderate, strong and extreme sensitizer (Kimber et al., 2003). Jowsey and co-workers have published a hypothetical prediction model where dose-response and a relative activity in test systems are assigned with a score for a classification of skin sensitizers (Jowsey et al., 2006).

Until now there is no applicability domain for the assays presented. To define the applicability domain it would be useful to increase the substance panel to include chemicals with broader physico-chemical properties. Furthermore, in order to show the robustness and predictivity of these assays, it may be required a (pre)validation with a substance panel including blindly coded test substances. Currently, MUSST, h-CLAT and DPRA are under pre-validation by ECVAM and the KeratinoSens® will be also submitted for its pre-validation.

Our study is the first one in which the four assays were performed in parallel with a defined panel of 23 substances and the direct correlation to human patch test and murine LLNA data,

making it a reliable approach to compare their potential to predict skin sensitizers. Currently, we enhanced our substance panel up to 57 compounds to confirm our findings.

Conflict of Interest Statement

Caroline Bauch, Susanne N. Kolle, Eric Fabian, Tzutzuy Ramirez, Bennard van Ravenzwaay and Robert Landsiedel are employees of BASF SE, a chemical company intending to use the toxicological assays to test their commercial products. These authors are, however, not aware of any conflict of interest.

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References

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

Barratt, M.D., Basketter, D.A., 1992. Possible origin of the skin sensitization potential of isoeugenol and related compounds. *Contact Dermatitis* 27, 98–104.

Basketter, D.A., 1992. Skin sensitization to cinnamic alcohol – the role of skin metabolism. *Acta Dermato-Venereologica* 72, 264–265.

Basketter, D.A., Flyvholm, M.A., Menn[®], T., 1999a. Classification criteria for skin-sensitizing chemicals: a commentary. *Contact Dermatitis* 40, 175–182.

Basketter, D.A., Gerberick, G.F., Kimber, I., Loveless, S.E., 1996. The local lymph node assay: a viable alternative to currently accepted skin sensitization tests. *Food and Chemical Toxicology* 34, 985–997.

Basketter, D.A., Lea, L.J., Cooper, K., Stocks, J., Dickens, A., Pate, I., Dearman, R.J., Kimber, I., 1999b. Threshold for classification as a skin sensitizer in the local lymph node assay: a statistical evaluation. *Food and Chemical Toxicology* 37, 1167–1174.

Basketter, D.A., Scholes, E.W., Kimber, I., 1994. The performance of the local lymph node assay with chemicals identified as contact allergens in the human maximization test. *Food and Chemical Toxicology* 32, 543–547.

Chipinda, I., Ruwona, T.B., Templeton, S.P., Siegel, P.D., 2011. Use of the human monocytic leukemia THP-1 cell line and co-incubation with microsomes to identify and differentiate hapten and prohaptens sensitizers. *Toxicology* 280, 135–143.

Cooper, J.A., Saracci, R., Cole, P., 1979. Describing the validity of carcinogen screening-tests. *British Journal of Cancer* 39, 87–89.

Coquette, A., Berna, N., Vandenbosch, A., Rosdy, M., De Wever, B., Poumay, Y., 2003. Analysis of interleukin-1 alpha (IL-1 alpha) and interleukin-8 (IL-8) expression and release in in vitro reconstructed human epidermis for the prediction of in vivo skin irritation and/or sensitization. *Toxicology in Vitro* 17, 311–321.

Corsini, E., Mitjans, M., Galbiati, V., Lucchi, L., Galli, C.L., Marinovich, M., 2009. Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens. *Toxicology in Vitro* 23, 789–796.

Council Directive 1907/2006/EEC, 2006. EC: Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. *Off. J. Eur. Union*, L 396/1.

Council Directive 76/768/EEC. Based on the Commission Staff Working Document: Timetables for the Phasing-out of Animal Testing in the Framework of the 7th Amendment to the Cosmetic Directive. 1976. Ref Type: Generic.

De Groot, A.C., Weyland, J.W., Nater, J.P., 1994. Unwanted Effects of Cosmetic and Drugs Used in Dermatology. Elsevier, Amsterdam.

dos Santos, G.G., Reinders, J., Ouweland, K., Rustemeyer, T., Scheper, R.J., Gibbs, S., 2009. Progress on the development of human in vitro dendritic cell based assays for assessment of the sensitizing potential of a compound. *Toxicology and Applied Pharmacology* 236, 372–382.

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

Gerberick, G.F., Troutman, J.A., Foertsch, L.M., Vassallo, J.D., Quijano, M., Dobson, R.L.M., Goebel, C., Lepoittevin, J.P., 2009. Investigation of peptide reactivity of pro-hapten skin sensitizers using a peroxidase-peroxide oxidation system. *Toxicological Sciences* 112, 164–174.

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

Gerberick, G.F., Ryan, C.A., Kimber, I., Dearman, R.J., Lea, L.J., Basketter, D.A., 2000. Local lymph node assay: validation assessment for regulatory purposes. *American Journal of Contact Dermatitis* 11, 3–18.

ICCVAM. Revised Draft ICCVAM Performance Standards Murine Local Lymph Node Assay Performance Standards. 2008. Ref Type: Generic.

ICCVAM. Recommended Performance Standards: Murine Local Lymph Node Assay, NIH Publication Number 09-7357. 2009. Ref Type: Generic.

Jaech, C., Blatz, V., Fabian, E., Guth, K., van Ravenzwaay, B., Reisinger, K., Landsiedel, R., in press 2011. Characterization of enzyme activities of Cytochrome P450 enzymes, Flavin-dependent monooxygenases, N-acetyltransferases and UDP-glucuronyltransferases in human reconstructed epidermis and full-thickness skin models. *Toxicology in Vitro*, Uncorrected proof.

Jowsey, I.R., Basketter, D.A., Westmoreland, C., Kimber, I., 2006. A future approach to measuring relative skin sensitising potency: a proposal. *Journal of Applied Toxicology* 26, 341–350.

Kimber, I., Basketter, D.A., Butler, M., Gamer, A., Garrigue, J.L., Gerberick, G.F., Newsome, C., Steiling, W., Vohr, H.W., 2003. Classification of contact allergens according to potency: proposals. *Food and Chemical Toxicology* 41, 1799–1809.

Kimber, I., Cumberbatch, M., Dearman, R.J., Griffiths, C.E.M., 2002. Danger signals and skin sensitization. *British Journal of Dermatology* 147, 613–614.

Kimber, I., Dearman, R.J., Scholes, E.W., Basketter, D.A., 1994. The local lymph node assay: developments and applications. *Toxicology* 93, 13–31.

Kligman, A.M., 1966. Identification of contact allergens by human assay 3. Maximization test – a procedure for screening and rating contact sensitizers. *Journal of Investigative Dermatology* 47, 393–409.

Lepoittevin, J.P., 2006. Metabolism versus chemical transformation or pro- versus prehapten? *Contact Dermatitis* 54, 73–74.

OECD. Guideline for Testing of Chemicals No. 429, (“Skin Sensitization: Local Lymph Node Assay”). 2010. Ref Type: Generic.

Oesch, F., Fabian, E., Oesch-Bartlomowicz, B., Werner, C., Landsiedel, R., 2007. Drug-metabolizing enzymes in the skin of man, rat, and pig. *Drug Metabolism Reviews* 39, 659–698.

Pendlington, R.U., Minter, H.J., Stupart, L., MacKay, C., Roper, C.S., Sanders, D.J., Pease, C.K., 2008. Development of a modified in vitro skin absorption method to study the epidermal/dermal disposition of a contact allergen in human skin. *Cutaneous and Ocular Toxicology* 27, 283–294.

Python, F., Goebel, C., Aebly, P., 2007. Assessment of the U937 cell line for the detection of contact allergens. *Toxicology and Applied Pharmacology* 220, 113–124.

Rietschel, R.L., Fowler, J.F., 1995. Fisher’s Contact Dermatitis, fourth ed. Williams and Wilkins, Baltimore, MD.

Ryan, C.A., Kimber, I., Basketter, D.A., Pallardy, M., Gildea, L.A., Gerberick, G.F., 2007. Dendritic cells and skin sensitization: biological roles and uses in hazard identification. *Toxicology and Applied Pharmacology* 221, 384–394.

Sakaguchi, H., Ashikaga, T., Miyazawa, M., Yoshida, Y., Ito, Y., Yoneyama, K., Hirota, M., Itagaki, H., Toyoda, H., Suzuki, H., 2006. Development of an in vitro skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT) II. An inter-laboratory study of the h-CLAT. *Toxicology in Vitro* 20, 774–784.

Schreiner, M., Peiser, M., Briehle, D., Stahlmann, R., Zuberbier, T., Wanner, R., 2008. A new dendritic cell type suitable as sentinel of contact allergens. *Toxicology* 249, 146–152.

Smith, C.M., Hotchkiss, S.A., 2001. Allergic Contact Dermatitis: Chemical and Metabolic Mechanisms. Taylor and Francis, London, UK.