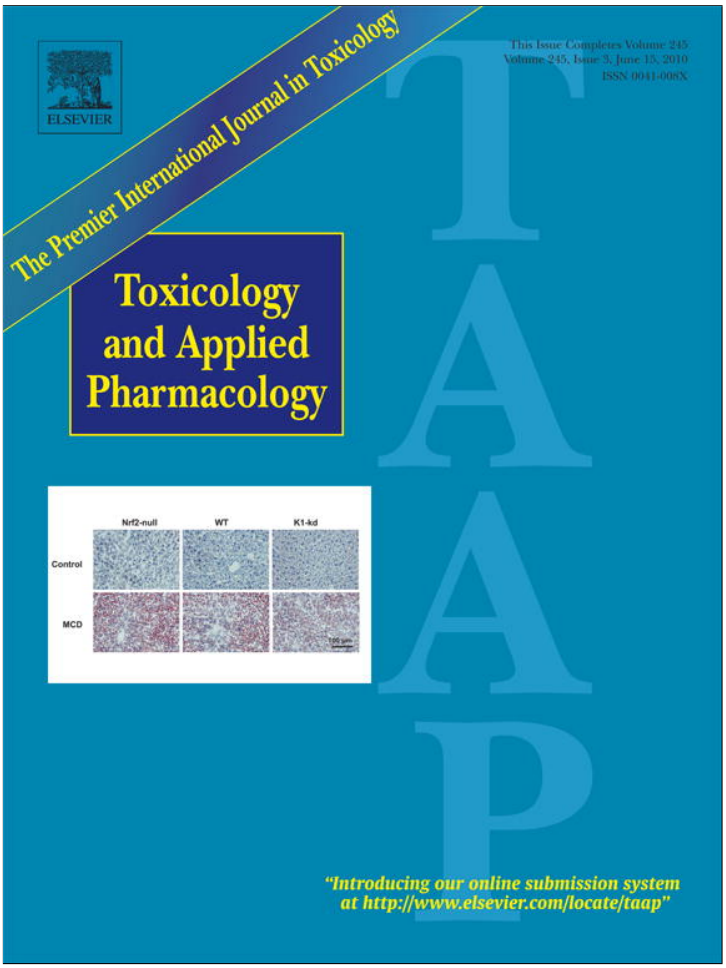

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Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers *in vitro*

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ABSTRACT

In vitro tests are needed to replace animal tests to screen for the skin sensitization potential of chemicals. Skin sensitizers are electrophilic molecules and the Nrf2-electrophile-sensing pathway comprising the repressor protein Keap1, the transcription factor Nrf2 and the antioxidant response element (ARE) is emerging as a toxicity pathway induced by skin sensitizers. Previously, we screened a large set of chemicals in the reporter cell line AREc32, which contains an eight-fold repeat of the rat GSTA2 ARE-sequence upstream of a luciferase reporter gene in the human breast cancer cell line MCF7. This approach was now further developed to bring it closer to the conditions in the human skin and to propose a fully standardized assay. To this end, a luciferase reporter gene under control of a single copy of the ARE-element of the human *AKR1C2* gene was stably inserted into HaCaT keratinocytes. A standard operating procedure was developed whereby chemicals are routinely tested at 12 concentrations in triplicate for significant induction of gene activity. We report results from this novel assay on (i) a list of reference chemicals published by ECVAM, (ii) the ICCVAM list of chemicals for validation of alternative endpoints in the LLNA and (iii) on a more general list of 67 chemicals derived from the ICCVAM database. For comparison, peptide reactivity data are presented for the same chemicals. The results indicate a good predictive value of this approach for hazard identification. Its technical simplicity, the high-throughput format and the good predictivity may make this assay a candidate for rapid validation to meet the tight deadline to replace animal tests for skin sensitization by 2013 set by the European authorities.

Introduction

Skin sensitization and skin irritation are critical toxicological endpoints in the development of novel ingredients for cosmetic products. Whereas alternative tests have been validated to screen for the skin irritation potential, skin sensitization testing still relies on animal tests. The current model of choice is the local lymph node assay in mice (LLNA) measuring cellular proliferation in the draining lymph nodes after repeated topical application of the test compound onto the ears of mice (Basketter et al., 2002; Gerberick et al., 2004a, 2000). Results are expressed as EC3 values indicating the extrapolated % concentration inducing a threefold increase in cellular proliferation. With the forthcoming ban on animal testing for cosmetic ingredients in the EU by 2013, finding alternative ways of determining the skin sensitization potential has gained a high priority.

Skin sensitization is an immune reaction to small exogenous molecules. In general, skin sensitizing molecules are reactive chemicals (or chemicals metabolically transformed into reactive intermediates) which have the potential to covalently modify skin proteins. The chemically modified proteins are then recognized by the immune system as foreign, which triggers a specific T-cell mediated immune response leading to the disease status known as contact allergy. Thus, a key step in the skin sensitization process is the formation of a covalent adduct between the skin sensitizer and endogenous proteins and/or peptides in the skin. The most straightforward approach to predict the sensitization potential of chemicals is thus to measure their reactivity toward peptides and proteins (reviewed in (Gerberick et al., 2008)). Gerberick et al. (2004b) developed a peptide depletion assay, in which a peptide is incubated with an excess of a test chemical, and peptide depletion by the test chemical is recorded as endpoint. We have further developed this approach by integrating LC-MS detection in order to simultaneously record peptide depletion and peptide-adduct formation (Natsch and Gfeller, 2008; Natsch et al., 2007).

Abbreviations: LLNA, local lymph node assay; Nrf2, nuclear factor-erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; ARE, antioxidant response element; SOP, standard operating procedure; ITS, integrated testing strategy; ECVAM, European Centre for the Validation of Alternative Methods to animal testing; ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; RT-PCR, reverse transcriptase polymerase chain reaction; DMSO, dimethylsulfoxide; HRIPT, human repeat insult patch test; DNCB, 2,4-dinitrochlorobenzene; SDS, sodium dodecyl sulphate.

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Another key approach to develop new *in vitro* assays is based on the innate cellular responses to sensitizers. Specifically, gene expression changes measured with gene chip analysis (Ryan et al., 2004) or RT-PCR (Gildea et al., 2006), altered expression of surface markers detected with flow cytometric analysis (Hulette et al., 2005; Sakaguchi et al., 2006), or changes in cytokine levels (Coquette et al., 2003) have been evaluated.

Whereas the elicitation phase of skin sensitization is a specific immune reaction, with hapten-specific T-cells as effector cells, this specificity does not yet exist during the induction phase of skin sensitization (i.e. the time when the immune system encounters the foreign modified proteins for the first time). Yet it is the induction phase which is attempted to be simulated with most *in vitro* tests. For quite some time it was not clear whether, in the induction phase, there are innate reactions of cells which are specific to sensitizers, and little was known about the potential regulatory pathways mediating such early innate reactions. It has recently been established, that in the induction phase, innate pathways are activated, which to some extend resemble the innate responses to pathogens (Freudenberg et al., 2009). One emerging innate toxicity pathway, which appears to be induced by most sensitizers, is the Keap1-Nrf2-ARE regulatory pathway and this evidence has been reviewed recently (Natsch, 2010). The sensor protein Keap1 (Kelch-like ECH-associated protein 1) contains highly reactive Cys residues. Covalent modification of crucial Cys residues by electrophiles leads to the dissociation of Keap1 from the transcriptional regulator Nrf2 (nuclear factor-erythroid 2-related factor 2). Nrf2 then accumulates in the nucleus where it activates genes having an antioxidant response element (ARE) in their promoter sequence (Dinkova-Kostova et al., 2005; Wakabayashi et al., 2004). Based on the importance of this pathway in the cellular reaction to electrophiles we had tested chemicals of known sensitization potential for luciferase induction in an engineered MCF7 breast cancer cell line (Natsch and Emter, 2008; Natsch et al., 2009) and could show that indeed most skin sensitizers induce this pathway. Evidence from gene chip and RT-PCR studies also indicates that ARE-regulated genes are induced in different cell types after sensitizer challenge. Thus for example *AKR1C2* (coding for an aldo-keto reductase) was one of the three most robust genetic markers up-regulated in a detailed RT-PCR study (Gildea et al., 2006). This gene contains a distant, but functional, ARE sequence in its promoter (Lou et al., 2006). Interleukin-8 mRNA and/or protein were increased by sensitizers in many studies and in different cell types (Bergström et al., 2007; Coquette et al., 2003; Gildea et al., 2006; Python et al., 2007; Ryan et al., 2004). IL-8 formation is also under the control of Nrf2 (Zhang et al., 2005). Most recently, a gene chip study in primary and immortalized dendritic cells (Python et al., 2009) found four robust markers induced by sensitizers, among them the two Nrf2-regulated genes *CES1* and *NQO1*. The importance of this pathway for the *in vivo* reaction to sensitizers was recently confirmed in a study with Nrf2 knockout mice by Kim et al. (Kim et al., 2008).

The first cells which come into contact with compounds applied topically to the skin are the keratinocytes. Whereas the primary immune cells in the skin are the Langerhans cells, the keratinocytes are also involved in the immune reaction and they are particularly known for their ability to produce a number of cytokines. Thus, keratinocyte-derived tumor necrosis factor α is important in the initiation of emigration of Langerhans cells (Cumberbatch et al., 2003) and keratinocytes are also an important source of interleukin-18 (Van Och et al., 2005), a key mediator involved in the induction of emigration of Langerhans cells (Antonopoulos et al., 2008). Most importantly, keratinocytes have some important metabolic functions (Bergström et al., 2007). Since certain skin sensitizers are not protein-reactive but act as prohaptens (i.e. they need metabolic activation to become protein-reactive (Bergström et al., 2006)), this intrinsic metabolic potential of keratinocytes appears to be an important parameter.

Since skin sensitization is a complex process involving many steps, it is assumed that no single test can model this toxicity endpoint. It had thus been proposed to apply an integrated testing strategy (ITS) accumulating different lines of evidence for a final assessment of the sensitization potential of a molecule (Basketter and Kimber, 2009). Such an ITS would combine evidence from peptide reactivity assays, cell based assays and information derived from the molecular structure of a molecule.

In our previous studies we had adapted the reporter cell line AREc32 for skin sensitization testing. This cell line had originally been developed for drug discovery (Xiu et al., 2006). It contains an eight-fold repeat of the rat glutathione-S-transferase-A2 ARE-sequence upstream of a luciferase gene in the human breast cancer cell line MCF7. Here, we report on the further development of this approach to bring it closer to the conditions in the human skin. We report a novel cell line based on the human HaCaT keratinocyte cell line (Boukamp et al., 1988) containing a reporter construct with a single copy of the ARE-element of the human *AKR1C2* gene. We report the different steps in the optimization of the genetic constructs and in the development of a fully standardized standard operating procedure, and then present detailed results on the performance of this novel test vs. different lists of reference chemicals.

Materials and methods

Chemicals. All fragrance chemicals are commercial qualities obtained from Givaudan Schweiz AG, Geneva, Switzerland. All other test chemicals were purchased from Sigma-Aldrich, Buchs, Switzerland. The chemical names, the structures, along with CAS-numbers, LLNA data and if available human and guinea pig evidence of the sensitization potential for the test chemicals are summarized in Supplementary Table S1. This information is derived from the ICCVAM database (http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-pot/appx/LLNApotencyAppB18Jan08FD.xls) and from the sources cited in our previous publication (Natsch et al., 2009). Many of the chemicals used in this study are moderate to extreme skin sensitizers, and therefore skin contact with the neat chemicals should be avoided.

Plasmids and genetic constructs. The reporter plasmids pGL3-Promoter and pGL4.17 were obtained from Promega (Duebendorf, Switzerland). A synthetic double-stranded DNA fragment consisting of two complementary oligonucleotides with the sequences 5'-CACTAGTGTGACAAAGCAGCTAGTGTGACAAAGCAGCT-AGTGTGACAAAGCAGCTAGTGTGACAAAGCAG-3' and 5'-GATCTGCTAGCTGCTTTGTCACACTAGCTGCTTTGTCACACTAGCTGCT-TTGTCACACTAGCTGCTTTGTCACACTAGTGGTAC-3' containing four copies of the rat GST2 ARE sequence (underlined) (Xiu et al., 2006) was synthesized by Microsynth (Balgach, Switzerland). One to four tandem copies of this sequence were cloned into the *Kpn*I/*Bgl*II site of the vector pGL3-Promoter upstream of the SV40 promoter resulting in a luciferase gene under the control of 4, 8, 12 , or 16 tandem copies of the ARE element and the SV40 promoter. These constructs are similar to the one used by Xiu et al. (Xiu et al., 2006) for the creation of the AREc32 cell line (8 copies of the rat GST2 ARE element in front of the SV40 promoter). In addition, a synthetic double-stranded DNA element consisting of the oligonucleotides 5'-CTGGTCGCAAGGTGTGCAAGCTGCTGAGTCAC-CCTGACTGCATCAACCCCAGGAGCTA-3' and GATCTAGCTCCTGGGG-TTGATGCAGTCAGGGTGACTCAGCAGCTTGCACACCTTGCGACCAGGTAC containing the region around the functional ARE element (underlined according to Lou et al., 2006) in the promoter of the human *AKR1C2* gene was inserted between the *Kpn*I and *Bgl*II sites of the vector pGL3-Promoter.

In order to obtain the vector for selecting the stable cell line, the fragment between the restriction sites *Kpn*I and *Hin*dIII containing the AKR1C2-ARE insert and the SV40 promoter was excised from the

pGL3-based vector and inserted into the *Kpn*I and *Hind*III sites in the vector pGL4.17. The resulting vector pGL4.17-*AKR1C2*-ARE-SV40 contains the regulatory construct upstream of the new synthetic version of the luciferase gene *luc2*, which is codon-optimized for improved expression and which contains a reduced number of consensus transcription factor binding sites as compared to the luciferase gene in pGL3. This new vector also contains a synthetic neomycin phosphotransferase gene for selection of stable clones.

Cell culture, transfection and selection of stable cell lines. Wild-type HaCaT cells were maintained in Dulbecco's modified Eagle's medium containing glutamax (Gibco/Invitrogen) supplemented with 9% fetal calf serum at 37 °C in the presence of 5% CO_2. The medium for the stable engineered cell line KeratinoSens was supplemented with 500 µg/ml G418.

For transient transfections and for the generation of the stable cell line KeratinoSens, HaCaT cells were transfected using the Nucleofector® System (Lonza, Switzerland) with the program U-020.

For the generation of the stable cell line KeratinoSens, HaCaT cells were transfected with 1 µg of a mix of circular and linearized (*Bam*HI or *Not*I) plasmid pGL4.17-AKR1C2-ARE-SV40. Stable clones were selected by supplementing the growth medium with 500 µg/ml of G418. Single colonies were isolated, expanded, and frozen.

Testing of chemicals in transiently transfected cells. For transient transfection experiments, 10^6 HaCaT cells were transfected with 2 µg of the respective plasmid and seeded in 96-well plates at 10^4 cells per well. Twenty-four hours later, fresh medium containing the test chemicals was added to the cells. After 24 h incubation with the test chemicals, the cells were washed once with PBS, lysed using Passive Lysis buffer (Promega, Duebendorf, Switzerland), and the Luciferase activity was determined as described below.

Standard testing of chemicals in the stable cell line. Test chemicals were dissolved in DMSO at a concentration of 200 mM. They were serially diluted in DMSO to obtain 12 final concentrations ranging from 0.1 mM to 200 mM. These DMSO solutions were diluted 25-fold in culture medium containing 1% FCS. The few chemicals not soluble in DMSO were dissolved in H_2O, and the DMSO level was adjusted to the same level in the dilutions in cell culture medium. The KeratinoSens cells were seeded in 96-well plates at a density of 10'000 cells per well in 125 µl growth medium without G418. Medium was replaced after 24 h with 150 µl fresh medium containing only 1% of FCS. Then, 50 µl of the 1% FCS medium containing the different dilutions of the DMSO solutions was added to the different wells. Final solvent concentration was thus 1% and test concentrations for each chemical ranged from 1 µM to 2000 µM. In each experiment each chemical was tested in triplicate at all the 12 concentrations. As a control *tert*-butyl-hydroquinone was always included in each test plate, and each plate contained six control wells with cells and solvent. In parallel all chemicals were tested for cytotoxicity with the MTT reduction test in a parallel plate in each repetition. All the plates were covered with a foil (Sealing tape SI, Nunc). After 48 h incubation with the test chemicals, the medium was removed and cells were washed once with PBS. To each well, 20 µl of passive lysis buffer (Promega, Duebendorf, Switzerland) was added and the cells were incubated for 20 min at RT. Plates were then read in a Promega Glomax luminometer with automatic injection of 50 µl of the luciferase substrate to each well and integration of the luciferase activity for 2 s. For the cell viability assay, 27 µl of a MTT solution (5 mg/ml in DPBS) was added to each well. After 4 h incubation, the medium was removed and 200 µl of a 10% SDS solution was added to each well. After the cells have dissolved completely, the absorption at 600 nm was determined for each well.

All tests were repeated at least twice with triplicate analysis at 12 concentrations in both repetitions. Based on these experiments, for each test chemical (i) the average maximal induction of gene activity (I_{max}) and (ii) the average concentration inducing significantly enhanced gene activity >50% above control values (EC1.5) were determined. The latter calculations were performed with linear extrapolation from the values above and below the induction threshold (as for the EC3 value determination in the LLNA). Each independent repetition was statistically evaluated. A chemical was rated positive, if it statistically significantly induced the luciferase activity more than 50% above control values at any of the tested concentrations in both independent repetitions. For chemicals with significant induction in only one repetition, two further repetitions were made. These chemicals were rated positive if the luciferase induction was statistically significant in at least 3 out of the total 4 independent repetitions. In addition, also the EC2 and EC3 for 100% and 200% enhanced luciferase expression were calculated with linear extrapolation and added as additional information.

Results

Transient transfections with different reporter plasmids

In a first step, ARE-sequences were evaluated for their transcriptional activity in the keratinocyte background. A relatively weak reporter gene induction was found in HaCaT cells with constructs containing multiple copies of the minimal ARE sequence, which had also been used to create the AREc32 cell line (Xiu et al., 2006). Results for the chemicals DNCB (S_NAr mechanistic domain (Roberts et al., 2007) and β-damascone (Michael acceptor mechanistic domain) are shown in Fig. 1. Testing the same chemicals in the HaCaT cells transiently transfected with a construct containing a single copy of a 56-bp sequence containing the ARE element of the *AKR1C2* gene gave a much broader dynamic range (Fig. 1). Thus, this element obtained

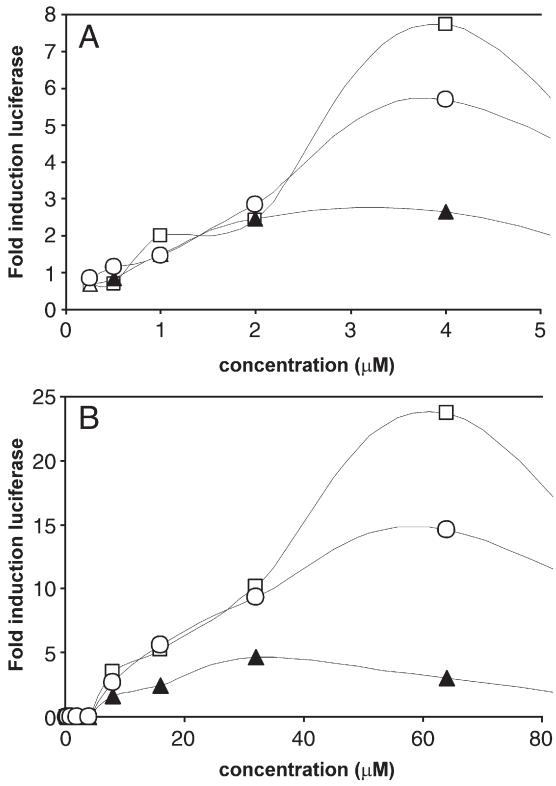

Fig. 1. Induction of luciferase activity by DNCB (A) and β-Damascone (B) in HaCaT cells transiently transfected with the vector pGL3-AKR1C2-ARE (open squares) and with pGL3-12 x ratGST2A-RE (filled triangles). Induction of luciferase activity by the same chemicals in mouse keratinocytes transfected with the vector pGL3-AKR1C2-ARE is shown in parallel (open circles).

from a promoter known to be induced by skin sensitizers appears to be a better choice in a keratinocyte background.

It had been shown that some ARE-dependent genes such as *NQO1* are much more inducible in a mouse keratinocyte cell line as compared to human HaCaT keratinocytes (Dinkova-Kostova et al., 2006). We thus tested the *AKR1C2*-ARE construct in a stable mouse keratinocyte cell line (Durchdewald et al., 2007). Luciferase activity in these transiently transfected mouse cells was clearly inducible (Fig. 1) but the dynamic range was not further enhanced as compared to human HaCaT cells, and thus, the mouse keratinocyte model appears to yield no advantage when working with this particular ARE-element. Based on these results with transient transfections, the HaCaT cell line with the *AKR1C2*-ARE construct appears to give an optimal dynamic range to detect skin sensitizers, and this combination was thus chosen to generate stably transformed cell lines.

Selection of a stable reporter cell line and development of the standard operating procedure

Nine stable, recombinant HaCaT clones based on the plasmid pGL4.17-*AKR1C2*-ARE-SV40 were tested in detail each with 6 reference chemicals (weak to strong sensitizers and methyl salicylate as non-sensitizer, data not shown). For each clone the absolute light output and the dynamic range of luciferase induction by sensitizers were evaluated. Clone 8 was selected based on the following criteria: (i) best signal to noise ratio and (ii) highest dynamic range if treated with the weak sensitizers Lyral and benzyl salicylate. We have called this clone "KeratinoSens" and it was used for the further development of a standard operating procedure (SOP) for testing chemicals. Various parameters were optimized in order to obtain a test set-up with a maximal dynamic range. Cell number at seeding was varied between 5000 and 20,000 cells per well in 96-well plates, incubation time with the chemicals was varied between 7 h and 48 h, solvent concentration was varied between 0.06% and 1% DMSO and the serum level was varied between 0 and 10%. In all these experiments 5 sensitizers were tested including the weak sensitizers Lyral and benzyl salicylate. Form this large number of experiments it could be concluded that an incubation time of 48 h with an inoculum of 10,000 cells per well is optimal: Especially for the weaker sensitizers, such as Lyral and benzyl salicylate, only a prolonged incubation yielded significant luciferase induction. Interestingly the dynamic range was much higher in the presence of 1% DMSO as compared to lower DMSO concentrations, whereas this solvent level did not inhibit proliferation of the cells. Finally, a serum level of 1% gave a higher dynamic range as compared to 10%, probably due to a reduced binding of reactive chemicals to serum proteins (as many skin sensitizers are highly protein-reactive). On the other hand, this serum level was still sufficient to maintain the cellular viability during the course of the experiment.

Selection of test chemicals for assessing the performance of the assay

Currently there exists no accepted 'gold-standard-list' of test chemicals for the development of alternative tests. Casati et al. (2009) have recently proposed a short list of 16 chemicals, further referred to as the 'ECVAM list'. ICCVAM has published a list of chemicals to validate alternative endpoints in the LLNA.[1] Within the large European project Sens-it-iv a different list of chemicals significantly overlapping with the above lists is used. We created an extended list covering the three lists mentioned above as well as further chemicals selected from the ICCVAM database downloaded in December 2008 from: http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-pot/appx/LLNApotencyAppB18Jan08FD.xls.

Chemicals were selected, which have congruent results in the LLNA and guinea pig tests or congruent results in the LLNA and human predictive tests, with an emphasis on chemicals for which evidence from all three test systems is reported in the ICCVAM database. Furthermore, the chemical set was balanced to contain chemicals of all important applicability domains and to cover a broad range of potencies. This test set, the different sub-lists, and the rational for selecting chemicals along with the animal and human *in vivo* results are shown in detail in the Supplementary Table S1. Since no 'gold-list' of test chemicals exists we refer to this list as the 'Silver-list'.

Screening for luciferase induction by chemicals of the 'Silver-list' and Cooper statistics

For each of the chemicals in Supplementary Table S1, the full dose response curves were measured for both luciferase induction and cytotoxicity. Fig. 2 shows the results (average from two repetitions with three replicates in each) for the chemicals cinnamic aldehyde and *p*-phenylendiamine. A strong induction of luciferase activity over a wide concentration range can be observed, and most notably, the induction of luciferase starts more than an order of magnitude below the cytotoxic concentrations. The dose–response curves were statistically evaluated: The I_{max} (average maximal fold-gene induction), the

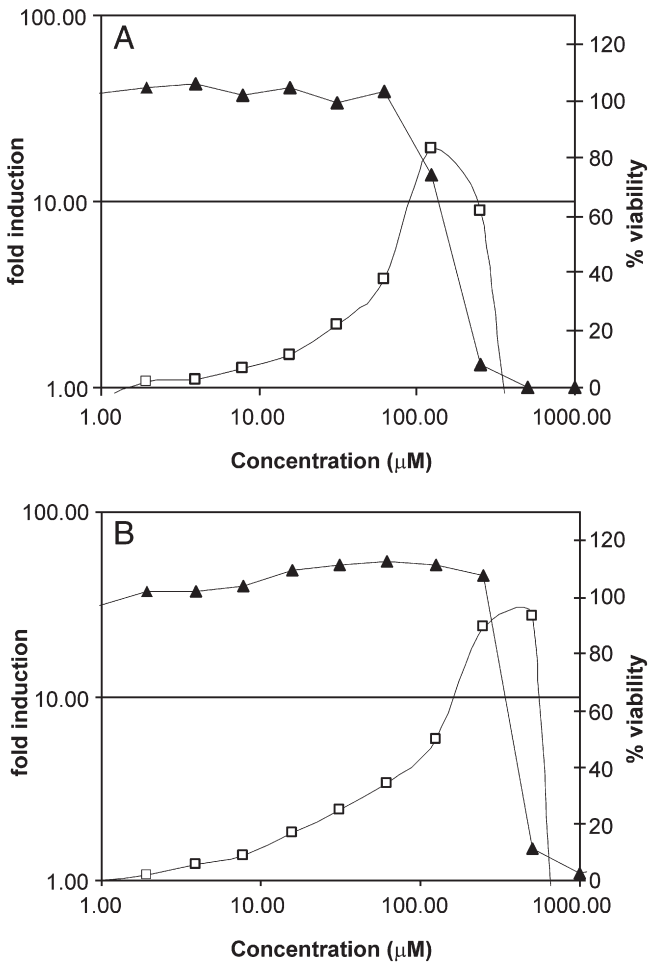

Fig. 2. Induction of luciferase activity (open squares) and cellular viability (closed triangles) in the KeratinoSens assay. Cells were treated with (A) cinnamic aldehyde and (B) with p-phenylenediamine in full dose–response analysis according to the SOP.

[1] The list referred to here is the revised draft for recommended performance standards published in January 2008. A later version published after the initiation of this study has changed two of the reference chemicals.

EC1.5 value (concentration for a statistically significant induction of 50% above background) and the IC50 (concentration leading to 50% cytotoxicity) were calculated. All these results are listed in Table 1. In addition the EC2 and EC3 values (concentration for a two- and threefold induction above background) were also calculated and included in the same table for those compounds for which the I_{max} was above this threshold. The average variability in the six control wells within an experiment was found to be 11.3% (average from 36 experiments each with triplicate plates), thus a 1.5-fold induction was in almost all cases statistically significant. Among the 43 sensitizers, 38 significantly induced luciferase-activity above this threshold, with five false-negatives, whereas among the 24 non-sensitizers 19 compounds were negative and five false-positive; these are discussed below. This resulted in an overall test accuracy of 85.1%. Calculating the Cooper statistics for the three sub-lists, an accuracy of 87.5% for the Sens-it-iv list, 93.8% for the ECCVAM-list and 90.9% for the ICCVAM-list was obtained (Table 2). Table 1 also contains data on the peptide reactivity of all the compounds in the silver list, in order to allow for comparison of the cell based *in vitro* data with this straightforward alternative assay for skin sensitization testing.

Relationship between luciferase induction and cytotoxicity

In a number of cell-based assays to screen for skin sensitizers, compounds are tested at partly cytotoxic concentrations only, since some endpoints are dependent on cytotoxicity (Coquette et al., 2003; Sakaguchi et al., 2009). We thus evaluated the dose–response curves for cytotoxicity and induction of luciferase for all test compounds and evaluated whether the EC1.5-determining concentration (i.e. lowest concentration with a significant induction higher then 1.5 fold) falls within the cytotoxic range. For most chemicals the IC50 (concentration for 50% reduction of the viability) is at least a factor of four above the EC1.5 value (compare EC1.5 values and IC 50 in Table 1; see Fig. 2) and the difference may be almost two orders of magnitude in the case for *p*-phenylenediamine. Not too surprisingly, this difference is much lower for some strongly cytotoxic biocides such as formaldehyde, imidazolidinyl urea and methyldibromo glutaronitrile. Only for formaldehyde and for α-hexyl cinnamic aldehyde and only in one repetition for each of these chemicals, the EC1.5-determining concentration was partly cytotoxic. SDS as a cytotoxic skin irritant and typical false-positive in the LLNA was studied in more detail. In the general screening according to the SOP it was negative (see Table 1). With the two-fold dilution steps, viability was reduced from 85% to 0% within one dilution step without luciferase induction at any test concentration (Fig. 3A). When SDS was tested in dilution series with higher resolution (Fig. 3B), a significant luciferase induction was seen, but only at one level and at exactly 60% cellular viability. Thus, whereas for all typical sensitizers in Table 1 luciferase induction occurs at non-toxic concentrations, a false-positive result may be generated in this case at partly cytotoxic concentrations.

Relationship between luciferase induction and potency

From inspection of Table 1, no direct correlation between EC1.5 and potency of chemicals is evident at first sight. However, particularly low EC1.5 values below 10 µM were recorded for many strong sensitizers, and for the weaker sensitizers with an LLNA EC3 of >20% the EC1.5 was in general above 50 µM. The picture becomes clearer when considering individual chemical classes: Thus Fig. 4A compares data for three halogenated compounds of the S_nAr reactivity domain whereas Fig. 4B compares some fragrance aldehydes, clearly indicating that the weaker sensitizers within these structural domains are separated from the moderate or strong sensitizers based on the luciferase-inducing concentrations.

Discussion

Test set-up

In vitro tests for toxicological endpoints should reflect as close as possible the biological system they are trying to model. Keratinocytes are the first cells skin sensitizers come into contact with, and this cell type is considered to play a role in the formation of danger signals upon contact with sensitizers. Thus, the goal was to develop a test system that measures early, sensitizer-induced cellular events in keratinocytes, although the dendritic cells are certainly the key players in more downstream steps of the sensitization reaction. The evaluation of different functional ARE-elements in different cellular backgrounds allowed us to create a genetic reporter construct with a high dynamic range in a keratinocyte background. The ARE-element in this reporter construct stems from the regulatory region of the gene AKR1C2, which had been identified as a reliable marker to identify skin sensitizers in gene chip and RT-PCR studies in primary dendritic cells (Gildea et al., 2006; Ryan et al., 2004) and this sensitizer-induced activity appears not to be cell-type specific. Although the ARE-element is located 5.5 kb upstream in the native sensitizer-induced *AKR1C2* gene, placing this isolated element directly in front of the SV40 promotor was sufficient to create a highly sensitive sensor system. The stable cell line generated with this construct has a particularly high dynamic range with a very low variation in the background signal against which significant gene induction by test compounds can be compared. Based on this cell line, a simple SOP could be developed, which allows a high-throughput analysis with full dose–response measurements and a parallel assessment of cytotoxicity. This testing approach is compatible with the paradigm proposed by the National Academy of Science of the US for toxicity testing into the 21st century (Andersen and Krewski, 2009): It targets an established toxicity pathway (Natsch, 2010), brings in a routine dose–response measure, evaluates perturbations in the toxicity pathway at subtoxic concentrations and is amenable to high-throughput screening to generate a large database.

Cooper statistics and false-positives

Cooper statistics yielded accuracies of over 85% for all lists and thus indicates a good predictivity of the assay for the chosen set of reference chemicals. The cell based assay that is currently most advanced in development is the h-CLAT (human cell line activation test), for which a large set of data has been published and which is currently undergoing prevalidation by ECVAM. For a set of 29 chemicals an overall accuracy of 93% was reported (Sakaguchi et al., 2009), whereas the accuracy was 82% for a more extended set of 100 chemicals (Ashikaga et al., 2008) and 85% on a set of 60 test chemicals (Sakaguchi et al., 2007). Thus, the overall accuracy of the two tests is very similar, and a careful evaluation of larger data sets will be needed to judge whether the two assays have largely overlapping or complementary applicability domains.

We have carefully selected the test-set, referred to as the 'Silver-list', which covers several accepted lists of reference chemicals and wide range of chemical classes and sensitization potencies. Yet, even this silver list includes some chemicals, for which question marks regarding their true sensitization potential may remain: Propyl-paraben is rated as a non-sensitizer by both guinea pig and LLNA tests but is repeatedly found positive in human patch tests. Benzaldehyde was generally assumed to be non-sensitizing based on animal tests for a long time and is included as a negative control in the Sens-it-iv list.

A recent standardized HRIPT conducted by the Research Institute for Fragrance materials (RIFM) confirmed benzaldehyde to be a clear human sensitizer and it is now considered positive by the industry and risk management measures have been put in place. These two examples of negative controls should thus rather be considered positive, which would raise specificity of the assay to 86% and

Table 1
Full data set for the chemicals in the silver list tested in the KeratinoSens assay along with peptide reactivity data.

		KeratinoSens results						Peptide reactivity results LC-MS Cor1 assay[a]	
Name	LLNA EC 3	ARE I_{max}	ARE EC1.5	Reps. positive[b]	ARE IC50	ARE EC2	ARE EC3	% depletion	Characterization of reactivity
Sensitizers									
Oxazolone	0.003	**2.4**	175.5	4/4	1370.9	335.3	490.4	>98	Adduct forming
Benzoquinone	0.01	**15.2**	6.5	4/4	104.5	25.0	32.8	98.4	Adduct forming
(5-chloro)-Methylisothiazolinone	0.01	**7.2**	8.7	2/2	7.1	0.9	1.7	>98	Oxidizing+adduct forming
2,4-Dinitrochlorobenzene	0.05	**14.8**	2.5	2/2	8.2	3.3	3.9	>98	Adduct forming
4-nitrobenzylbromide	0.05	**6.9**	1.3	2/2	9.1	1.7	2.1	100	Adduct forming
4-Phenylenediamine	0.11	**26.8**	5.0	2/2	438.9	13.3	46.7	>98	Oxidizing+adduct forming
Glutaraldehyde	0.12	**80.7**	24.3	2/2	242.6	57.2	69.4	>98	Crosslinking?
Benzoyl peroxide	0.22	1.4	n.i.	0/2	567.6	n.i.	n.i.	99.9	Adduct forming*
Glyoxal	0.75	**28.2**	89.1	4/4	677.9	192.4	307.8	>98	Adduct forming
4-Methylaminophenol sulphate	0.80	5.9	9.4	2/2	11.7	2.1	2.7	95.7	Adduct forming
Formaldehyde	0.84	**16.9**	63.2	2/2	201.6	66.4	72.2	91.5	Oxidizing+adduct forming
Methyldibromo glutaronitrile	0.90	**4.0**	7.8	2/2	25.6	12.4	18.1	>98	Oxidizing+adduct forming
Cinnamic aldehyde	1.3	**16.2**	16.1	4/4	194.4	36.6	63.9	47.9	Oxidizing+adduct forming
2-Hydroxyethyl acrylate	1.4	**54.9**	32.3	2/2	207.2	59.5	92.9	98.4	Adduct forming
Isoeugenol	1.5	**6.4**	16.1	4/4	731.4	72.6	259.4	91.1	Oxidizing+adduct forming
Ethylenediamine	2.2	**13.2**	99.9	4/4	>2000	188.2	453.4	10.7	Not reactive*
Benzylidene Acetone	2.2	**503.9**	9.7	2/2	174.5	19.7	31.5	64.9	Adduct forming
Methyl-2-nonynoate	2.5	**33.1**	1.8	2/2	121.9	8.9	26.7	>98	Adduct forming
2-Mercaptobenzothiazole	2.5	**8.8**	48.1	4/4	1003.1	108.0	340.1	97.8	Oxidizing+adduct forming
Benzyl salicylate	2.9	**5.5**	8.4	2/2	111.0	18.7	40.9	−6.8	Not reactive*
Tetramethylthiuramdisulfide	3.1	**6.8**	0.8	2/2	39.1	5.3	10.7	>98	Adduct forming
Diethylenetriamine	3.3	**1.7**	1259.4	2/4[c]	>2000	n.i.	n.i.	7.8	Not reactive*
Thioglycerol	3.5	1.5	n.i.	1/4	>2000	n.i.	n.i.	28.7	Adduct forming*
Phenylacetaldehyde	4.5	**11.3**	28.5	2/2	116.2	50.4	69.0	95.7	Oxidizing
Resorcinol	5.9	1.0	n.i.	0/2	>2000	n.i.	n.i.	7.8	Not reactive*
Dihydroeugenol	6.8	**1.5**	462.0	2/2	759.2	n.i.	n.i.	−4.8	Not reactive
Benzoisothiazolione	7.8	**24.0**	3.2	2/2	50.9	6.3	14.8	95.8	Oxidizing+adduct forming
Citral	9.8	**96.4**	23.2	2/2	182.8	53.9	67.4	3	Adduct forming
Hexyl cinnamic aldehyde	9.9	**2.7**	17.3	2/2	26.3	24.9	n.i.	93.3	Oxidizing
Eugenol	10.1	1.3	n.i.	0/4	1505.7	n.i.	n.i.	52.8	Oxidizing
Abietic acid	11.6	**11.4**	16.6	2/2	104.6	30.8	34.7	90.7	Oxidizing*
Phenyl benzoate	13.6	1.3	n.i.	1/4	191.6	n.i.	n.i.	25.5	Adduct forming*
Lyral HMPCC	17.1	**16.1**	79.6	2/2	355.4	117.3	197.1	27.5	Oxidizing*
Benzocaine	17.1	**3.0**	18.2	2/2	>2000	101.6	n.i.	−6.2	Not reactive*
Benzyl cinnamate	18.4	**8.7**	11.0	2/2	>2000	26.4	38.3	1.5	Adduct forming
2,4-Dichloronitrobenzene	20.0	**2.9**	68.3	4/4	816.0	197.3	456.0	0.7	Adduct forming*
Cinnamyl alcohol	21.0	**1.7**	123.6	4/4	774.6	n.i.	n.i.	3.4	Not reactive*
Hydroxycitronellal	23.0	**137.1**	79.4	2/2	>2000	110.1	142.9	34.2	Oxidizing
Imidazolidinyl urea	24.0	**2.9**	45.4	3/4	90.4	44.4	41.9	97.9	Oxidizing+adduct forming
Butyl glycidyl ether	30.9	**340.7**	218.5	2/2	>2000	289.8	381.6	32.5	Adduct forming
Ethylene glycol dimethacrylate	32.9	**188.4**	57.4	2/2	1655.8	135.9	253.4	62.9	Adduct forming
Cobalt chloride	Pos.	**23.3**	298.6	2/2	1330.2	450.0	661.4		n.a.
Nickel sulfate	var.	**4.2**	329.0	4/8[d]	998.7	235.2	291.5		n.a.
Non-sensitizers									
Sodium lauryl sulfate	var.[e]	1.2	n.i.	0/2	44.7	n.i.	n.i.	65.8	Not reactive
Salicylic acid	var.[e]	1.1	n.i.	0/2	>2000	n.i.	n.i.	−9.2	Not reactive
Methyl salicylate	var.[e]	1.2	n.i.	0/2	>2000	n.i.	n.i.	−1.2	Not reactive
Sulfanilamide	NC[f]	1.4	n.i.	0/2	>2000	n.i.	n.i.	6.6	Not reactive*
Diethyl phthalate	>100%	1.1	n.i.	0/2	>2000	n.i.	n.i.	−2	Not reactive
Glycerol	>100%	1.2	n.i.	0/4	>2000	n.i.	n.i.	−5.8	Not reactive*
Propylene glycol	>100%	1.2	n.i.	0/2	>2000	n.i.	n.i.	−9.9	Not reactive*
Benzoic acid	>20%	1.1	n.i.	0/2	>2000	n.i.	n.i.	−4.3	Not reactive*
1-Butanol	>20%	1.1	n.i.	0/2	>2000	n.i.	n.i.	4.6	Not reactive
4-Hydroxybenzoic acid	>25%	1.1	n.i.	0/2	>2000	n.i.	n.i.	4.1	Not reactive
Sulfanilic acid	>25%	1.3	n.i.	0/2	>1000	n.i.	n.i.	1.4	Not reactive
Tartaric acid	>25%	1.2	n.i.	0/2	>2000	n.i.	n.i.	−6	Not reactive*
Propylparaben	>25%	**9.7**	14.5	2/2[f]	813.1	41.9	n.i.	−4.4	Not reactive*
Ethyl vanillin	>50%	**5.4**	161.7	2/2[g]	>2000	534.5	700.6	3.8	Not reactive*
Isopropanol	>50%	1.2	n.i.	0/2	>2000	n.i.	n.i.	7.2	Not reactive*
Benzyl alcohol	>50%	1.2	n.i.	0/2	>2000	n.i.	n.i.	1.9	Not reactive
Dimethylisophtalate	NC[h]	**2.1**	694.9	3/4	>2000	1253.5	n.i.	−8	Not reactive*
Dextran	NC[h]	1.5	n.i.	0/2	>2000	n.i.	n.i.	−8.5	Not reactive*
Tween 80	NC[h]	**2.7**	19.3	2/2	399.8	53.2	n.i.	2.8	Not reactive*
Chlorobenzene	Neg.[i]	1.2	n.i.	0/2	>2000	n.i.	n.i.	2.8	Not reactive*
Lactic acid	Neg.[i]	1.3	n.i.	1/4	>2000	n.i.	n.i.	−5.3	Not reactive
Phenol	Neg.[i]	1.3	n.i.	0/2	>2000	n.i.	n.i.	−4.6	Not reactive*
Benzaldehyde	>25	**2.3**	443.1	2/2[g]	>2000	n.i.	n.i.	5.3	Not reactive
Octanoic acid	>50	1.1	n.i.	0/2	>2000	n.i.	n.i.	5.3	Not reactive

Table 2
Cooper statistics for the different test lists.

	SILVER list	Sens-it-iv list	ECVAM list	ICCVAM list	SILVER list with reactivity data[a]
Correct positives	38	12	11	13	41
False negatives	5	2	1	2	2
Correct negatives	19	9	4	7	19
False positives	5	1	0	0	5
n test chemicals	67	24	16	22	67
Sensitivity	88.4	85.7	91.7	86.7	95.3
Specificity	79.2	90.0	100.0	100.0	79.2
Accuracy	85.1	87.5	93.8	90.9	89.6

[a] Chemicals rated positive if either positive in (i) KeratinoSens assay or if (ii) adduct formation is observed in peptide reactivity assay.

sensitivity to 89%. Ethyl vanillin is structurally related to benzaldehyde and, like benzaldehyde, shows no potential for sensitization in the LLNA which is supported by other animal data. However, for ethyl vanillin there is little evidence of human sensitization occurring to this substance. For a number of compounds such as isopropanol, glycerol, propyleneglycol, salicylic acid, methyl salicylate and diethyl phthalate, the ICCVAM database reports some positive human evidence. Nevertheless, these compounds are generally considered true negative controls and some are even included as negative controls in the ICCVAM performance standards. They are widely used at high levels in topical products as excipients or active ingredients without negative health effects. Since they lack typical structural alerts they still appear *bona fide* negatives, although, as stated recently, the correct interpretation of human data to determine positive and negative controls needs a high level of expert knowledge (Basketter and Kimber, 2009).

Prohaptens and false-negatives

The test set contains a number of putative pre- and prohaptens. Prehaptens are compounds known for their potential of spontaneous oxidation leading to reactive metabolites. *P*-phenylendiamine and isoeugenol are the typical prehaptens in the list (Lepoittevin, 2006) and they are strong inducers of luciferase-activity. Prohaptens need metabolic activation, such as enzyme-catalyzed oxidation or oxidative deamination. Typical examples in the test-set are cinnamic alcohol, ethylenediamine, and diethylentriamine (all rated positive by the assay, see Table 1). Ethylenediamine and diethylentriamine were negative in the AREc32 assay, and thus the metabolic capacity of the KeratinoSens to activate amines appears to be better as compared to the breast cancer cell line. On the other hand, outstanding among the false-negatives are the putative prohaptens resorcinol and eugenol. Eugenol is the only chemical which was correctly positive in our previous data set (Natsch and Emter, 2008) but not in the keratinocyte background. One possible explanation is that HaCaT cells lack the necessary enzymes for activation of eugenol. However, we had previously found (our unpublished data) that both HaCaT cells and human skin tissue models can metabolize eugenol to a specific unusual phase II metabolite, and thus, there appears also the possibility that the keratinocytes can efficiently detoxify this

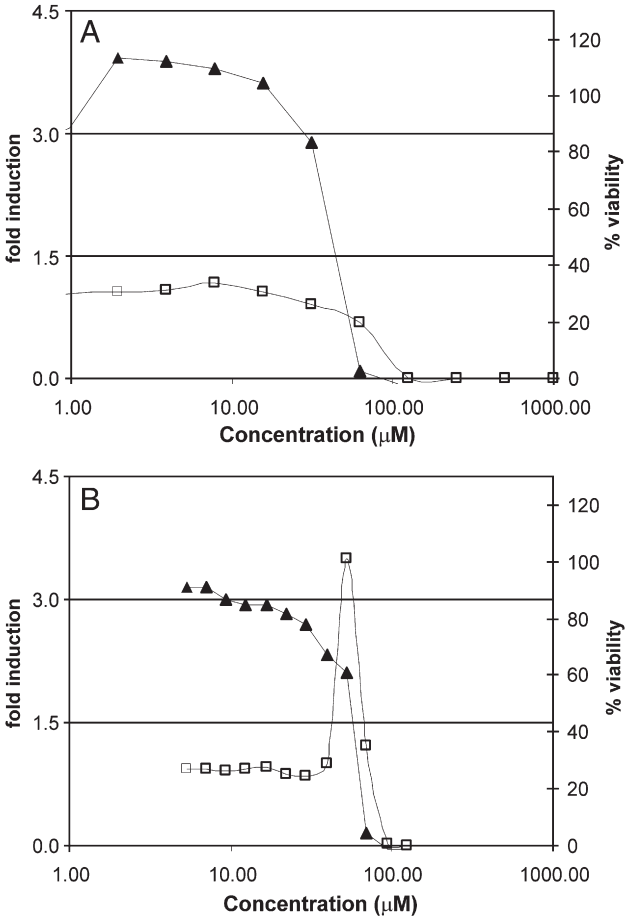

Fig. 3. Luciferase induction (open squares) and cellular viability (closed triangles) for SDS tested with the full dose–response according to the SOP (A) and with a higher resolution dose–response curve (B). Note the single significant luciferase induction at 60% viability in B.

molecule. Thus, the use of a skin cell line as model rather than the breast cancer cell line helps to predict better some of the prohaptens, but some phenolic chemicals may also be rated as false-negatives. Another false-negative is phenyl benzoate: this molecule is a typical acyl-transfer agent reacting with Lysine-residues. It is thus positive in the peptide reactivity assay. The fact that ARE-induction as an endpoint is blind to some Lysine-reactive chemicals has been discussed (Natsch, 2010).

Induction threshold and dynamic range

The assay readout has a very broad dynamic range, and with some chemicals inducing luciferase activity by >100 fold, an EC1.5 to rate chemicals positive may be viewed as a low threshold. Indeed for most sensitizers an EC2 or even EC3 can also be calculated (Table 1), yet some putative prohaptens such as cinnamic alcohol, diethylenetriamine and dihydroeugenol would become negative by raising the

Notes to Table 1:
The Imax of chemicals with significant luciferase induction is highlighted in bold characters; n.i. = no significant induction above threshold.
[a] Peptide reactivity data originate from Natsch and Gfeller (2008), data marked with an asterisk are new data generated with the standard method from the original publication.
[b] Indicates number of experiments (each conducted in triplicate) with statistically significant induction >1.5-fold/number of experiments conducted.
[c] Diethylentriamine, 2 negative repetitions with I_{max} 1.46/1.43, but statistically significant induction and clear dose response in all 4 repetitions, considered positive.
[d] Nickel sulfate, only four repetitions positive out of eight, but very strong induction, considered positive.
[e] For details see Supplementary Table S1.
[f] Propylparaben, known to be a weak human sensitizer, nevertheless for Cooper statistics the negative prediction from animal tests was considered.
[g] Benzaldehyde, considered a human sensitizer by the fragrance association due to recent human HRIPT test, still considered false positive for Cooper statistics.
[h] Level not specified.
[i] Negative reference according D. Basketter 1(999), Food Chem. Toxicol. 37, 1167–1174.

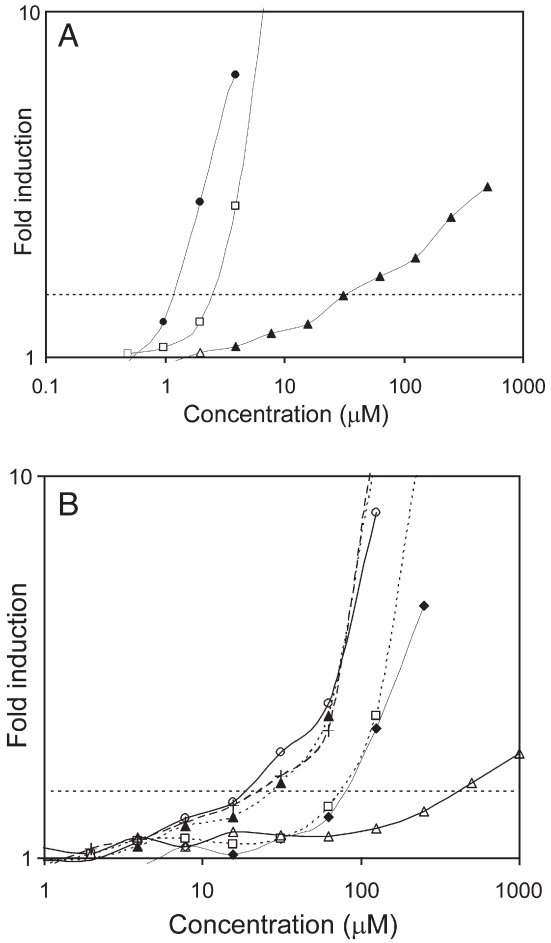

Fig. 4. Luciferase induction by skin sensitizers of differing potency. (A) Halogenated compounds of the S_NAR mechanistic reactivity domain (Roberts et al., 2007). Closed circles: 4-nitrobenzyl-bromide (strong); open squares: DNCB (strong); closed triangles: 2,4-dichloronitrobenzene (weak sensitizer). (B) Different fragrance aldehydes. Open circles: cinnamic aldehyde (moderate); crosses: citral (moderate), closed triangles: phenylacetaldehyde (moderate); open squares: hydroxycitronellal (weak); closed diamonds: Lyral (weak); open triangles: benzaldehyde (not classified, positive in humans). Note: all the aldehydes in panel B are also of lower potency as compared to the strong sensitizers in panel A.

threshold. Nevertheless, if using this assay in conjunction with other assays (in an ITS), it might in the future be desirable to raise the specificity at the cost of sensitivity–thus with a threshold of three, the specificity would be raised to 96% and the sensitivity reduced to 78% while maintaining a similar accuracy. As a high specificity from single assays is needed if, in an integrated testing strategy, positive evidence from different approaches is combined as is currently done in mutagenicity testing, the prediction model might be adapted accordingly in an ITS.

Maximal test concentration

We have tested all chemicals up to the very high level of 2000 μM, as we think that a new test should be tested even beyond its potential limits. Evaluating the data in Table 1, diethylenetriamine is the only sensitizer for which the EC1.5-determining concentration is 2000 μM. For all other chemicals it is at 500 μM or below. On the other hand, the weak false-positive result obtained for dimethylisophtalate was only obtained at 1000 μM and above. Testing chemicals at too high concentrations may in some cases lead to false-positive results either due to (i) unspecific induction of stress response or (ii) contaminants in the test chemical preparation. In the light of significant activation below 500 μM for almost all sensitizers, we might reduce the maximal test concentration in the future. A different approach is taken in the h-CLAT assay: Test concentrations are always adapted in order to cover a partly cytotoxic range for each compound, with maximal concentrations for non-toxic compounds such as DMSO of up to 5000 μg/ ml For a compound with MW 100 this corresponds to 12'500 μM, and thus for some chemicals the h-CLAT protocol leads to the need to test at even higher concentrations (Sakaguchi et al., 2009).

Impact of cytotoxicity

As detailed in the Results section, cytotoxicity of a compound is not a prerequisite for a positive result in the test and the EC1.5 and the IC50 are not closely linked for most chemicals. On the other hand, under very specific test concentrations, a partial cytotoxicity of an irritating chemical may lead to a signal in dying cells. This emphasises the need to rate chemicals as positive only when luciferase induction starts at non-cytotoxic concentrations. This activation under cytotoxic conditions might be due to generation of endogenous Keap1-ligands which certainly would lead to false-positives: Thus, one might consider conditions of oxidative stress, which might lead to the generation of endogenous electrophilic metabolites such as 4-hydroxynonenal, thus causing false-positive responses. Such endogenous metabolites would probably never lead to immunogenic protein adducts, as they are recognized as self by the immune system, yet they still might generate a false-positive induction of the Nrf2-pathway. If these false-positives can be avoided by simply excluding cytotoxic concentrations in the prediction model, much is gained already and based on our current large data set, this seems largely to be the case. Yet a critical question remains, which needs to be addressed as the data set expands: Can non-sensitizing molecules even at non-cytotoxic concentrations induce certain endogenous activators? Thus, the most surprising false-positive result is Tween 80, which also in our previous publication was false-positive at non-cytotoxic concentrations (Natsch and Emter, 2008). Does this non-reactive, non-ionic surfactant induce some subtle changes in cellular membrane structures thereby generating a stress response finally leading to the production of endogenous activation? On the other hand, ethoxylated surfactants such as Tween 80 are also prone to oxidation, and the oxidised ethylene-oxide units may act as true sensitizers. Indeed, Tween 80 had been described as sensitizer in some studies (Bergh et al., 1997; Bergh et al., 1998).

Relationship to potency

As outlined in the Results section, there is a general trend for luciferase-induction at lower concentrations for strong sensitizers whereas higher concentrations are needed in the case of weak sensitizers. This is particularly the case when considering data within specific structural groups (e.g. aldehydes and halogenated compounds). For the Michael acceptors, the picture in some cases is less clear. The weak sensitizer benzyl cinnamate induces luciferase activity already at low concentrations and would be predicted as a too strong sensitizer if the KeratinoSens result is evaluated without further evidence from other tests (i.e. the clearly lower reactivity of benzyl cinnamate as compared to other Michael acceptors had been illustrated by kinetic reactivity data recently; Roberts and Natsch, 2009). There are some other notable exceptions: Glutaraldehyde, formaldehyde, ethylendiamine and glyoxal are (beside oxazolone) the strong to extreme sensitizers in the data set, for which luciferase induction occurs at unusually high concentrations and which would be under-predicted. These are the chemicals within the database with the potential to cross-link proteins. The ability for cross-linking proteins may be a determinant for sensitizers to generate particularly strong antigens and it is a key feature of many respiratory sensitizers (Enoch et al., 2009). Thus, the potency of these chemicals may rather be linked to their cross-linking ability then to their mere

electrophilicity and the induction of an innate pathway such as the Nrf2 pathway. This result highlights the importance of also including chemical information and structural features as parameters in an ITS. Regarding glyoxal and ethylenediamine it is also worth noting that these two chemicals have almost identical dose–response curves (similar EC1.5, EC2 and EC3 in Table 1). Ethylendiamine is considered a prohapten transformed to the reactive glyoxal by oxidative deamination. The identical dose response curve suggests that this process is quite efficient in HaCaT cells, a hypothesis which could be tested by an analytical study.

Applicability domain

Based on the current data set and as discussed above, this assay may predict the hazard for a wide variety of chemical classes of structurally diverse sensitizers and non-sensitizers. Yet some prohaptens may remain undetected and, as recently reviewed in detail (Natsch, 2010), chemicals with a unique reactivity toward amine-groups such as anhydrides do not induce the Nrf2- pathway. Therefore, either alternative cell based assays need to be developed for these amine reactive chemicals or these chemicals need to be detected with reactivity-based assays with amine-nucleophiles in the frame of an ITS.

Cooper statistics in combination with peptide reactivity data

It is widely assumed that such a complex endpoint like skin sensitization cannot be predicted with a single *in vitro* assay, but that rather a battery of tests needs to be combined (Basketter and Kimber, 2009; Jowsey et al., 2006; Natsch et al., 2009) with an integrated testing strategy (ITS). Although the focus of this paper is on the development and performance of the novel KeratinoSens assay, we have included peptide reactivity data from our previous paper (Natsch and Gfeller, 2008) in Table 1, and filled the data gaps with new data obtained with the same SOP to cover the whole silver-list. This allows to better estimate already at this early stage how the two approaches complement each other and especially to determine the effect of a dual screening on the Cooper statistics. As is evident from this comparison, chemical reactivity in the peptide assay and a positive result in the KeratinoSens assay were recorded for many sensitizers. Yet both assays detected a number of compounds, which are negative in the other assay. Particularly, the putative prohaptens cinnamic alcohol, ethylenediamine, diethylentriamine and benzyl salicylate were only positive in the KeratinoSens assay, whereas benzoyl peroxide, phenyl benzoate and thioglycerol were only positive in the LC-MS assay. As we had reported before, adduct formation with peptides is a very specific and mechanistically-based endpoint from the peptide reactivity assay (Natsch and Gfeller, 2008). Thus, rating every chemical, which is either adduct-forming in the LC-MS assay or positive in the KeratinoSens assay as a sensitizer, leads to an improved sensitivity of 95.3% and an overall accuracy of 89.6% for the 'Silver list' (see Tables 1 and 2), while not affecting overall specificity.

Conclusions

With the new standardized assay based on a keratinocyte cell line we present a high- throughput assay to screen large numbers of chemicals. A key advantage of this assay is the measurement of gene induction events at subcytotoxic concentrations and the routine evaluation of chemicals over a wide dose-range to measure full dose–response curves. Another main advantage of this assay is its technical simplicity. The straight-forward luciferase-based readout should make it amenable to lab-to-lab transferability and validation studies. Results from this assay can easily be combined with peptide reactivity data, which should always be part of any *in vitro* assessment of potentially sensitizing chemicals (Basketter and Kimber, 2009) and thus, we presented peptide reactivity data along with the cell-based results. Although a first indication of potency within structural classes may come from the EC1.5 value, the quantitative dose–response data (EC1.5 and optionally EC2 and EC3) should in the future be integrated in an ITS along with more quantitative peptide reactivity data, bioavailability data and some informed rating of structural alerts (such as the ability of chemicals to cross-link proteins). The cytotoxicity determination (IC50) derived from this assay may also be used as a crude measure of irritation potential/danger signal formation in this ITS (Basketter and Kimber, 2009).

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Conflicts of interest

The authors are all employees of Givaudan and receive no other compensation but their salaries for this work and do declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.taap.2010.03.009.

References

Andersen, M.E., Krewski, D., 2009. Toxicity testing in the 21st century: bringing the vision to life. Toxicol. Sci. 107, 324–330.
Antonopoulos, C., Cumberbatch, M., Mee, J.B., Dearman, R.J., Wei, X.Q., Liew, F.Y., Kimber, I., Groves, R.W., 2008. IL-18 is a key proximal mediator of contact hypersensitivity and allergen-induced Langerhans cell migration in murine epidermis. J. Leukoc. Biol. 83, 361–367.
Ashikaga, T., Sakaguchi, H., Nukada, Y., Kosaka, N., Sono, S., Nishiyama, N., Itagaki, H., 2008. Database of h-CLAT (cell-based skin sensitization test) for clarification of applicability domain. Abstracts of the 45th Congress of the European Societies of Toxicology. Toxicol. Lett. 180 (supplement 1), S95.
Basketter, D.A., Kimber, I., 2009. Updating the skin sensitization in vitro data assessment paradigm in 2009. J. Appl. Toxicol. 29, 545–550.
Basketter, D.A., Evans, P., Fielder, R.J., Gerberick, G.F., Dearman, R.J., Kimber, I., 2002. Local lymph node assay—validation, conduct and use in practice. Food Chem. Toxicol. 40, 593–598.
Bergh, M., Magnusson, K., Nilsson, J.L., Karlberg, A.T., 1997. Contact allergenic activity of Tween 80 before and after air exposure. Contact Dermat. 37, 9–18.
Bergh, M., Shao, L.P., Hagelthorn, G., Gafvert, E., Nilsson, J.L., Karlberg, A.T., 1998. Contact allergens from surfactants. Atmospheric oxidation of polyoxyethylene alcohols, formation of ethoxylated aldehydes, and their allergenic activity. J. Pharm. Sci. 87, 276–282.
Bergström, M.A., Luthman, K., Nilsson, J.L.G., Karlberg, A.T., 2006. Conjugated dienes as prohaptens in contact allergy: in vivo and in vitro studies of structure-activity relationships, sensitizing capacity, and metabolic activation. Chem. Res. Toxicol. 19, 760–769.
Bergström, M.A., Ott, H., Carlsson, A., Neis, M., Zwadlo-Klarwasser, G., Jonsson, C.A.M., Merk, H.F., Karlberg, A.T., Baron, J.M., 2007. A skin-like cytochrome P450 cocktail activates prohaptens to contact allergenic metabolites. J. Investig. Dermatol. 127, 1145–1153.
Boukamp, P., Petrussevska, R.T., Breitkreutz, D., Hornung, J., Markham, A., Fusenig, N.E., 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J. Cell Biol. 106, 761–771.
Casati, S., Aeby, P., Kimber, I., Maxwell, G., Ovigne, J.M., Roggen, E., Rovida, C., Tosti, L., Basketter, D., 2009. Selection of chemicals for the development and evaluation of in vitro methods for skin sensitisation testing. Altern. Lab. Anim. 37, 305–312.
Coquette, A., Berna, N., Vandenbosch, A., Rosdy, M., De Wever, B., Poumay, Y., 2003. Analysis of interleukin-1α (IL-1α) 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. Toxicol. in Vitro 17, 311–321.
Cumberbatch, M., Dearman, R.J., Griffiths, C.E.M., Kimber, I., 2003. Epidermal Langerhans cell migration and sensitisation to chemical allergens. APMIS 111, 797–804.
Dinkova-Kostova, A.T., Holtzclaw, W.D., Kensler, T.W., 2005. The role of Keap1 in cellular protective responses. Chem. Res. Toxicol. 18, 1779–1791.
Dinkova-Kostova, A.T., Jenkins, S.N., Fahey, J.W., Ye, L., Wehage, S.L., Liby, K.T., Stephenson, K.K., Wade, K.L., Talalay, P., 2006. Protection against UV-light-induced skin carcinogenesis in SKH-1 high-risk mice by sulforaphane-containing broccoli sprout extracts. Cancer Lett. 240, 243–252.

Durchdewald, M., Beyer, T.A., Johnson, D.A., Johnson, J.A., Werner, S., auf dem Keller, U., 2007. Electrophilic chemicals but not UV irradiation or reactive oxygen species activate Nrf2 in keratinocytes in vitro and in vivo. J. Invest. Dermatol. 127, 646–653.
Enoch, S.J., Roberts, D.W., Cronin, M.T., 2009. Electrophilic reaction chemistry of low molecular weight respiratory sensitizers. Chem. Res. Toxicol. 22, 1447–1453.
Freudenberg, M.A., Esser, P.R., Jakob, T., Galanos, C., Martin, S.F., 2009. Innate and adaptive immune responses in contact dermatitis: analogy with infections. G. Ital. Dermatol. Venereol. 144, 173–185.
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. Am. J. Contact Dermat. 11, 3–18.
Gerberick, G.F., Ryan, C.A., Kern, P.S., Dearman, R.J., Kimber, I., Patlewicz, G.Y., Basketter, D.A., 2004a. A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. Contact Dermat. 50, 274–288.
Gerberick, G.F., Vassallo, J.D., Bailey, R.E., Chaney, J.G., Morrall, S.W., Lepoittevin, J.P., 2004b. Development of a peptide reactivity assay for screening contact allergens. Toxicol. Sci. 81, 332–343.
Gerberick, F., Aleksic, M., Basketter, D., Casati, S., Karlberg, A.T., Kern, P., Kimber, I., Lepoittevin, J.P., Natsch, A., Ovigne, J.M., Rovida, C., Sakaguchi, H., Schultz, T., 2008. Chemical reactivity measurement and the predictive identification of skin sensitisers. ATLA Altern. Lab. Anim. 36, 215–242.
Gildea, L.A., Ryan, C.A., Foertsch, L.M., Kennedy, J.M., Dearman, R.J., Kimber, I., Gerberick, G.F., 2006. Identification of gene expression changes induced by chemical allergens in dendritic cells: opportunities for skin sensitization testing. J. Investig. Dermatol. 126, 1813–1822.
Hulette, B.C., Ryan, C.A., Gildea, L.A., Gerberick, G.F., 2005. Relationship of CD86 surface marker expression and cytotoxicity on dendritic cells exposed to chemical allergen. Toxicol. Appl. Pharmacol. 209, 159–166.
Jowsey, I.R., Basketter, D.A., Westmoreland, C., Kimber, I., 2006. A future approach to measuring relative skin sensitising potency: a proposal. J. Appl. Toxicol. 26, 341–350.
Kim, H.J., Barajas, B., Wang, M., Nel, A.E., 2008. Nrf2 activation by sulforaphane restores the age-related decrease of TH1 immunity: role of dendritic cells. J. Allergy Clin. Immunol. 121.
Lepoittevin, J.P., 2006. Metabolism versus chemical transformation or pro- versus prehaptens? Contact Dermat. 54, 73–74.
Lou, H., Du, S., Ji, Q., Stolz, A., 2006. Induction of AKR1C2 by phase II inducers: identification of a distal consensus antioxidant response element regulated by NRF2. Mol. Pharmacol. 69, 1662–1672.
Natsch, A., 2010. The Nrf2-Keap1-ARE toxicity pathway as a cellular sensor for skin sensitizers—functional relevance and a hypothesis on innate reactions to skin sensitizers. Toxicol. Sci. 113, 284–292.
Natsch, A., Emter, R., 2008. Skin sensitizers induce antioxidant response element dependent genes: application to the in vitro testing of the sensitization potential of chemicals. Toxicol. Sci. 102, 110–119.
Natsch, A., Gfeller, H., 2008. LC-MS-based characterization of the peptide reactivity of chemicals to improve the in vitro prediction of the skin sensitization potential. Toxicol. Sci. 106, 464–478.
Natsch, A., Gfeller, H., Rothaupt, M., Ellis, G., 2007. Utility and limitations of a peptide reactivity assay to predict fragrance allergens in vitro. Toxicol. in Vitro 21, 1220–1226.
Natsch, A., Emter, R., Ellis, G., 2009. Filling the concept with data: integrating data from different in vitro and in silico assays on skin sensitizers to explore the battery approach for animal-free skin sensitization testing. Toxicol. Sci. 107, 106–121.
Python, F., Goebel, C., Aeby, P., 2007. Assessment of the U937 cell line for the detection of contact allergens. Toxicol. Appl. Pharmacol. 220, 113–124.
Python, F., Goebel, C., Aeby, P., 2009. Comparative DNA microarray analysis of human monocyte derived dendritic cells and MUTZ-3 cells exposed to the moderate skin sensitizer cinnamaldehyde. Toxicol. Appl. Pharmacol. 239, 273–283.
Roberts, D.W., Natsch, A., 2009. High throughput kinetic profiling approach for covalent binding to peptides: application to skin sensitization potency of Michael acceptor electrophiles. Chem. Res. Toxicol. 22, 592–603.
Roberts, D.W., Patlewicz, G., Kern, P.S., Gerberick, F., Kimber, I., Dearman, R.J., Ryan, C.A., Basketter, D.A., Aptula, A.O., 2007. Mechanistic applicability domain classification of a local lymph node assay dataset for skin sensitization. Chem. Res. Toxicol. 20, 1019–1030.
Ryan, C.A., Gildea, L.A., Hulette, B.C., Dearman, R.J., Kimber, I., Gerberick, G.F., 2004. Gene expression changes in peripheral blood-derived dendritic cells following exposure to a contact allergen. Toxicol. Lett. 150, 301–316.
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. Toxicol. in Vitro 20, 774–784.
Sakaguchi, H., Ashikaga, T., Kosaka, N., Sono, S., Nishiyama, N., Itagaki, H., 2007. The in vitro skin sensitization test; human cell line activation test (h-CLAT) using THP-1 cells. Abstracts of the 44th Congress of the European Societies of Toxicology. Toxicol. Lett. 172 (Supplement 1), S93.
Sakaguchi, H., Ashikaga, T., Miyazawa, M., Kosaka, N., Ito, Y., Yoneyama, K., Sono, S., Itagaki, H., Toyoda, H., Suzuki, H., 2009. The relationship between CD86/CD54 expression and THP-1 cell viability in an in vitro skin sensitization test—human cell line activation test (h-CLAT). Cell Biol. Toxicol. 25, 109–126.
Van Och, F.M.M., Van Loveren, H., Van Wolfswinkel, J.C., Machielsen, A.J.C., Vandebriel, R.J., 2005. Assessment of potency of allergenic activity of low molecular weight compounds based on IL-1? and IL-18 production by a murine and human keratinocyte cell line. Toxicology 210, 95–109.
Wakabayashi, N., Dinkova-Kostova, A.T., Holtzclaw, W.D., Kang, M.I., Kobayashi, A., Yamamoto, M., Kensler, T.W., Talalay, P., 2004. Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. Proc. Natl. Acad. Sci. U. S. A. 101, 2040–2045.
Xiu, J.W., Hayes, J.D., Wolf, C.R., 2006. Generation of a stable antioxidant response element-driven reporter gene cell line and its use to show redox-dependent activation of Nrf2 by cancer chemotherapeutic agents. Cancer Res. 66, 10983–10994.
Zhang, X., Chen, X., Song, H., Chen, H.Z., Rovin, B.H., 2005. Activation of the Nrf2/antioxidant response pathway increases IL-8 expression. Eur. J. Immunol. 35, 3258–3267.