 Utility of Rat Liver S9 Fractions to Study Skin-Sensitizing Prohaptens in a Modified KeratinoSens Assay

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Prohaptens are chemicals, which may cause skin sensitization after being converted into electrophilic molecules by skin enzymes. Aroclor-induced rat liver S9 fractions represent the metabolic activation system most commonly used in in vitro toxicology. This system contains much higher enzyme activities compared with those reported in skin, but it may still serve as a surrogate system to study the potential of chemicals to act as prohaptens. To test this concept, the luciferase induction in KeratinoSens reporter cells treated with chemicals in presence and absence of S9 fractions was measured. Suspected prohaptens such as methylisoegenol, eugenol, or trans-anethole gave no, or only weak, gene induction in absence of S9 fractions, and a significantly enhanced luciferase induction in presence of S9, proving their prohapten status. Direct-acting haptenes like 2,4-dinitrochlorobenzene or cinnamic aldehyde gave a reduced response in presence of S9. We evaluated whether this metabolic activation assay might be implemented in a tiered screening strategy to counter-screen negatives in the KeratinoSens assay to enhance sensitivity. To this aim, all chemicals classified negative were retested with this activation step. Among the 77 chemicals found as correct-negatives, 73 were also negative in presence of metabolic activation, thus this counter-screen would reduce specificity only slightly. However, this comprehensive screening showed that only a small fraction of the known skin sensitizers need activation by the S9 system. Therefore, the KeratinoSens-S9 assay appears useful for the in vitro evaluation of specific classes of potential prohaptens and to mechanistically rationalize their prohapten status.

Key Words: skin sensitization; prohaptens; S9 fractions; in vitro testing; reporter gene; nuclear factor-erythroid 2-related factor 2.

Most skin sensitizers are reactive chemicals, which can modify skin proteins, thereby rendering them immunogenic. Yet, a minority of sensitizers are not electrophilic. The hapten hypothesis postulates that some of these molecules are transformed by enzymes in the skin into the true reactive haptenes (Barratt and Basketter, 1992; Karlberg et al., 2008). This hypothesis is broadly accepted, even if the proof remained indirect until recently. Typical suspected prohaptens include eugenol and derivatives, which are well-known substrates of P450 enzymes (Thompson et al., 1990), and α,β-unsaturated alcohols such as geraniol or cinnamic alcohol, which can be oxidized to the corresponding reactive aldehydes and which often cross-react with these (Cheung et al., 2003). In an early attempt to prove the prohapten theory, eugenol and isoeugenol were applied in presence of P450 inhibitors in the local lymph node assay (LLNA). However, the inhibitors boosted the response rather than to inhibit it (Scholes et al., 1994). Later, eugenol was shown to trigger enhanced leukocyte proliferation in presence of horseradish peroxidase (HRP) and H₂O₂ (Shvedova et al., 1999). Some recent studies further advanced our understanding: The putative prohaptens benzo(a)pyrene, carvone oxime, and paracetamol were tested in mice with a hypomorphic NADPH-cytochrome P450 reductase (Chipinda et al., 2011a). The lymphocyte proliferation was unaffected for direct-acting haptenes and reduced for prohaptens in these mice. A number of in vitro studies focused on the skin sensitizers carvone oxime and (4R)-1-methyl-6-methylene-4-(1-methylthylencyclohexene (further referred to as the “conjugated diene”) and formation of peptide-reactive metabolites by incubation with P450 enzymes was verified (Bergström et al., 2007; Nilsson et al., 2005b). The prohapten geraniol was shown to be transformed to sensitizing aldehydes and epoxides by P450 enzymes (Hagvall et al., 2008). Eugenol was reported to covalently react with peptides after activation by human liver microsomes (Ball et al., 2011) or by HRP (Gerberick et al., 2009).

These studies substantiate the theory that metabolic enzymes activate some prohaptens to reactive species, although limited evidence for P450-mediated activation of sensitizers in the skin is available. In an early study, P450 enzyme activity in microsomes obtained from skin, induction of this activity by Aroclor 1254, and P450 levels detected by CO difference spectra were quantified. P450 levels were found, if normalized to microsomal protein content, to be at around 5% of the levels found in the liver (Bickers et al., 1982). Later, expression in keratinocytes was confirmed at the messenger RNA (mRNA) level for CYP1A1, 1B1, 2B6, 2E1, and 3A, and several of these enzymes were also detected by immunohistochemistry and at the catalytic level (Baron et al., 2001). Expression at the mRNA level was studied in more detail...
(Bergström et al., 2007; Luu-The et al., 2009), and these studies showed that in the skin, phase I enzymes are expressed at a far lower level compared with phase II enzymes and that the mRNA levels are 5000 times lower compared with the liver. A recent study at the proteomic level indicated that skin expresses fewer P450 enzymes (van Eijl et al., 2012) than previously thought and that predominantly phase II enzymes are expressed in the skin. In primary keratinocyte-derived skin models, no detectable CYP1A/CYP2B/CYP3A activity was found (Jackh et al., 2011), but inducible CYP1A1 (Bonifás et al., 2010) and CYP2S1 (McNeilly et al., 2012) activities were again reported in the keratinocyte compartment. However, metabolic activity in the skin may not rely only on keratinocytes but rather be concentrated to specific minor cell types such as Langerhans cells (Modi et al., 2012). An important role for CYP1B1 as metabolic enzyme in dendritic cells was found in two studies (Baron et al., 1998; Ott et al., 2009).

These somewhat contradictory reports on key enzymatic activities in the skin hamper the definition of an experimentally founded enzymatic activation system for in vitro studies in dermatotoxicology. An exception is the “enriched P450 skin cocktail” (Bergström et al., 2007), which is based on mRNA data. In the absence of more empirical data, two pragmatic approaches were proposed: In a modified peptide reactivity assay, an oxidative system based on H$_2$O$_2$ and HRP was used to activate prohaptens, and enhanced peptide depletion for a number of prohaptens was reported (Troutman et al., 2011). S9 liver fractions from Aroclor-induced rats were integrated into the activation assay with THP-1 cells and the prohaptens benzo(a)pyrene, 7,12-dimethylbenz[a]anthracene, carvone oxime, cinnamic alcohol, and isoeugenol were shown to stimulate cell surface markers in presence but not in absence of S9 (Chipinda et al., 2011b). This approach, based on experience in activating promutagens in in vitro assays (Billington et al., 2010; Jagger et al., 2009), appears promising, but data on only two non-sensitizers were reported. Therefore, we cannot estimate whether this approach will not lead to an oversensitive system.

We have described the KeratinoSens screening system to test chemicals for their skin sensitization potential based on activation of a luciferase gene under control of an antioxidant response element (ARE) (Emter et al., 2010). This assay has been evaluated for intra- and interlaboratory reproducibility (Natsch et al., 2011), and results on 220 chemicals were submitted to the European Center for the Validation of Alternative Methods to animal testing (ECVAM) for evaluation of the method. Based on these screenings, the applicability domain of the assay could be defined and “blind spots” of the method were identified. Thus, a number of suspected prohaptens according to Kern et al. (2010) summarized in Supplementary table S1 were correct-positives in the assay, indicating that this keratinocyte-based system does have intrinsic metabolic capacity to activate some prohaptens. On the other hand, the suspected prohaptens in Table 1 are borderline positive or negative in the assay, thus indicating that it lacks sufficient metabolic activity to strongly activate certain phenolic prohaptens. Here, we thus developed a KeratinoSens assay with an added rat S9 metabolic component and tested a large number of putative prohaptens and compared the response to correct-negative nonsensitizers.

### MATERIALS AND METHODS

**Chemicals.** Fragrance chemicals were all obtained from Givaudan Schweiz AG. Other chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland). Carvone oxime was kindly provided by Prof A. T. Karliberg. The conjugated diene was synthesized from L-carvone according to the published method (Nilsson et al., 2005). The chemicals tested were selected based on the following rationale: Putative prohaptens in Table 1 were chosen either (1) based on literature (Bergström et al., 2007; Chipinda et al., 2011a,b; Nilsson et al., 2005a,b) or (2) based on structural alerts and the fact that they were either false-negative or only borderline positive (dihydroeugenol, 1-naphtho) in previous screenings in the KeratinoSens assay. In addition, all the chemicals that were tested negative (both false-negative and correct-negative) in our previous publications (Emter et al., 2010), in the studies conducted for ECVAM prevalidation and in Natsch et al. (2013), were retested, to evaluate which negatives in the KeratinoSens assay are activated by the S9 system.

**Standard testing of chemicals in the KeratinoSens cell line.** The stable engineered cell line KeratinoSens containing the functional ARE in the promoter of the human AKR1C2 gene (Lou et al., 2006) upstream of a luciferase gene was described before (Emter et al., 2010). Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing glutamates (Gibco/Invitrogen) supplemented with 9% fetal calf serum (FCS) and with 500 µg/ml G418 at 37˚C in the presence of 5% CO$_2$. The same standard operating procedure (SOP) for testing the chemicals as described before, with few modifications, was used. 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 and no phenol red. For treatments without S9, a stock solution of 0.2mM NADPH, 1mM D-Glucose-6-phosphate, and 1% FCS in clear DMEM was prepared. For treatments with S9, the same stock solution was prepared and further enriched with 4% of S9 fractions (LS9 SD Aroclor 1254, lyophilized, from male Sprague Dawley rat liver, M22-01L2, Trinova, Giessen, Germany). Both solutions were filtered (0.22 µm Millipore Express membrane).

Test chemicals were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 200mM. They were serially diluted in DMSO to obtain six final concentrations in the range between 0.1 and 200mM. Because all chemicals had been tested before in the standard assay, these concentrations could be chosen to cover five nontoxic concentrations. The DMSO solutions were diluted 25-fold in the two stock solutions with/without S9 fractions. Then, 50 µl were added to the cells in different wells. Final solvent and S9 concentration were thus at 1%. In each experiment, each chemical was tested in triplicate at the six concentrations. Eugenol as positive control was always included in each test plate, and each plate contained three control wells with cells and solvent for both the ± S9 treatment. 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 replaced with 100 µl PrestoBlue reagent (Invitrogen, Zug, Switzerland) diluted 10-fold in clear DMEM to determine cytotoxicity. Plates were incubated for 30 min at 37˚C and 5% CO$_2$. The fluorescence at 560 excitation and 590nm emission was determined and plates were washed with 200 µl PBS. Cells were then lysed with 20 µl Passive Cell Lysis Buffer (Promega Duebendorf) at room temperature for 30min, and plates were 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.

In some experiments, viability was determined in parallel using the Neutral red uptake (NRU) assay and by cellular ATP content determination. For the NRU assay, cells were washed once with PBS and then incubated with DMSO supplemented with 50 µg/ml Neutral red and 25mM HEPES for 3 h. After three washes with PBS, cells were lysed in acidic alcohol (50% ethanol, 1% acetic acid). Lysates were rocked for 10 min, and the absorbance of Neutral red was measured at 540 nm. To determine cellular ATP content, 5 µl of the cell lysate...
used for luminescence measurement was mixed with 50 µl luciferase substrate lacking ATP but supplemented with 1.25 µg/ml recombinant luciferase. Luminescence was measured to quantify the ATP concentration. To study the effect of P450 inhibitors, the S9 fractions were diluted in cell culture medium and preincubated with the inhibitors for 1 h. Then, the mixture of the S9 with inhibitors and the test substance were added to the cells simultaneously. All tests were done in at least three independent runs, each with triplicate analysis at six concentrations in all three runs. For most chemicals, the assay was run in the same assay plates with and without S9 for a direct comparison. Statistical comparisons between treatments with S9 fractions versus treatments without S9 fractions were done with Student’s t test (two-tailed; two-sample equal variance).

Because all the nonsensitizers had been tested before without S9, a fraction of the screening on negatives in the classical assay was conducted only in presence of S9 and the data compared with the historical values for the assays without S9.

For treatments without S9, both the viability and the luciferase determinations were normalized to solvent controls without S9, whereas for all treatments with S9, data were normalized to the treatment receiving solvent and S9. For each test chemical, the average maximal induction of gene activity ($I_{\text{max}}$) in presence and absence of S9 activation was calculated. The average concentration inducing significantly enhanced gene activity of 100% above control values (EC2) was determined. The latter calculations were performed with linear extrapolation from the values above and below the induction.
Screening Prohaptens in vitro, an observation we cannot explain mechanistically. The dose-response curves for some of these chemicals, along with acetaminophen and carvoxime.

RESULTS

Assay Optimization

First, the optimal conditions to perform the S9 activation assay were defined. The following parameters were tested: (1) use of Aroclor-induced S9 fractions versus noninduced fractions; (2) contact time between S9, chemicals, and the cells; and (3) the concentration of S9 to be added. In these optimization steps, the putative prohaptens eugenol and methylisoeugenol were tested. S9 fractions from noninduced rat liver did enhance the response to methylisoeugenol, but not for eugenol, hence all further work was done with Aroclor-induced fractions. Whereas in cell-based assays for genotoxicity, a 3-h contact time with chemicals and the S9 fractions is sufficient, the S9 fractions had to be present for at least 24 h in order to significantly increase the luciferase response in presence of eugenol (data not shown). In a further comparison, the medium with test agent and S9 was left in contact with the cells for 48 h as in the standard KeratinoSens assay, or it was changed after 24 h and replaced by medium without test agent and S9 for a 24-h postincubation period. Because the cells survived the S9 treatment with a 48-h contact time, this option was chosen. Finally, different dilutions of the S9 mix (0.25, 0.5, 1, and 2%) were tested. A level of 1% was required to yield a clearly enhanced response in presence of eugenol (data not shown). In a further comparison, the medium with test agent and S9 was left in contact with the cells for 48 h as in the standard KeratinoSens assay, or it was changed after 24 h and replaced by medium without test agent and S9 for a 24-h postincubation period. Because the cells survived the S9 treatment with a 48-h contact time, this option was chosen. Finally, different dilutions of the S9 mix (0.25, 0.5, 1, and 2%) were tested. A level of 1% was required to yield a clearly enhanced response in presence of eugenol (data not shown). In a further comparison, the medium with test agent and S9 was left in contact with the cells for 48 h as in the standard KeratinoSens assay, or it was changed after 24 h and replaced by medium without test agent and S9 for a 24-h postincubation period. Because the cells survived the S9 treatment with a 48-h contact time, this option was chosen. Finally, different dilutions of the S9 mix (0.25, 0.5, 1, and 2%) were tested. A level of 1% was required to yield a clearly enhanced response in presence of eugenol (data not shown).

Response to Typical Haptens

The basic idea was to test chemicals for potential activation only if they are rated as nonsensitizing by the standard KeratinoSens assay, and thus correct-positive haptens were not tested routinely. Nevertheless, the response to two typical correct-positive haptens (2,4-dinitrochlorobenzene [DNCB] and cinnamic aldehyde) and the putative prehapten isoeugenol was first analyzed (Figs. 1 and 2, Table 2). These three chemicals induced the luciferase response in presence and in absence of S9, but especially for the DNCB and cinnamic aldehyde, the dose response for gene induction and cytotoxicity is shifted toward higher concentrations, indicating a potential of S9 fractions to detoxify or bind some of these haptens.

Response to Putative Prohaptens

In Table 1, a number of putative prohaptens are listed, which are negative in the standard KeratinoSens assay or, for some chemicals, gave borderline positive results in the previous screening in the standard assay (dihydroeugenol, naphtol, and the conjugated diene). For all the chemicals in Table 1, a clearly enhanced response in presence of S9 fractions was observed, which confirms their prohapten status and the possibility to become activated by typical enzymes contained in the S9 fractions. The induction above the background was at least twofold at noncytotoxic concentrations in presence of S9. In Figures 1 and 2, the dose-response curves for some of these chemicals are shown. Interestingly, as for the direct-acting hapten, the cytotoxicity dose response is also shifted toward higher concentrations, indicating that the S9 system has for some prohaptens at the same time a detoxifying effect (leading to reduced cytotoxicity) and an activating effect (leading to increased luciferase response). The only exception from this rule is 1-naphthol, which became more toxic in presence of the S9 fractions. Interestingly, in some cases, the viability is even increased over the control in treatments with S9 fractions. To rule out that the viability determinations are affected by interactions between the oxidation-sensitive Resazurin dye (contained in the PrestoBlue assay) and the S9 fractions, seven chemicals were retested and viability determinations were performed in parallel with the PrestoBlue, the NRU, and the ATP content assay. No significant difference in the resulting IC50 values (Supplementary table S2) and in the dose-response curves were noted between the different tests (Supplementary fig. S1). For methylisoeugenol, again a relatively enhanced viability in S9 treatments was noted by all three methods (Supplementary fig. S1), an observation we cannot explain mechanistically.

The typical promutagens benzo(a)pyrene and 7,12-dimethylbenz[a]anthracene had been reported positive in the LLNA at very low concentrations, and benzo(a)pyrene was tested in detail in the studies of Chipinda et al. (2011a,b). These chemicals, along with the related promutagen methylcholantrene, were therefore also tested (Table 3). An enhanced maximal gene induction was observed for benzo(a)pyrene and methylcholantrene, whereas the response was not enhanced for 7,12-dimethylbenz[a]anthracene, which is already positive in the standard KeratinoSens assay at very low concentrations.

Not all putative prohaptens induced the luciferase in presence of S9. The four chemicals in Table 4 remain false-negative in presence of S9 fractions. These included the two 1,3-substituted compounds resorcinol and 3-aminophenol, as well as acetaminophen and carvoxime.
The results for the KeratinoSens assay in presence (filled symbols) and absence (open symbols) of extrinsic metabolic activation: The putative pro-
haptens eugenol (a and b), methylisoeugenol (c and d), trans-anethole (e and f), and the putative prehapten isoeugenol (g and h) were tested. The experiments were repeated in nine independent runs (six runs for isoeugenol). Luciferase induction and relative viability as determined with the PrestoBlue assay are indicated. Statistically significant differences between the two treatments are indicated with *p < 0.05, **p < 0.005, and ***p < 0.0005.
FIG. 2. The results for the KeratinoSens assay in presence (filled symbols) and absence (open symbols) of extrinsic metabolic activation: Data for methyl salicylate (a and b) as nonsensitizer, the conjugated diene (c and d) as putative prohaptens, and cinnamic aldehyde (e and f) and DNCB (g and h) as direct-acting hapten were shown. The experiments were repeated in three independent runs (nine runs for the conjugated diene). Luciferase induction and relative viability as determined with the PrestoBlue assay are indicated. Statistically significant differences between the two treatments are indicated with *p < 0.05, **p < 0.005, and ***p < 0.0005.
Next to the chemicals discussed above, we found a number of false-negative chemicals in the KeratinoSens assay, which are not suspected as prohaptens, and a large number of correct-negatives. It is especially the latter group that needs a careful evaluation if a nonphysiological (i.e., not skin-derived) metabolic activation system is used because, in theory, Aroclor-induced rat liver might activate a wide range of chemicals, which are nonsensitizing due to insufficient metabolism in the skin. We thus screened the chemicals previously found to be negative in the classical KeratinoSens assay and evaluated whether any of these chemicals induce the luciferase above a threshold of twofold.

In total, 76 correct-negative chemicals were rescreened in the S9 activation assay. The 19 chemicals tested as

### TABLE 2

<table>
<thead>
<tr>
<th>Name</th>
<th>LLNA EC3 (%)</th>
<th>I_max (fold induction)</th>
<th>EC2 (µM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Chloro-2,4-dinitrobenzene</td>
<td>0.04</td>
<td>13.2</td>
<td>2.6</td>
<td>6.4</td>
</tr>
<tr>
<td>Cinnamic aldehyde</td>
<td>3.1</td>
<td>10.0</td>
<td>41.4</td>
<td>160.6</td>
</tr>
<tr>
<td>Isoeugenol</td>
<td>1.8</td>
<td>21.2</td>
<td>150.6</td>
<td>489.4</td>
</tr>
</tbody>
</table>

### TABLE 3

<table>
<thead>
<tr>
<th>Name</th>
<th>LLNA EC3 (%)</th>
<th>I_max (fold induction)</th>
<th>EC2 (µM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzo(a)pyrene</td>
<td>&lt;1.25&lt;sup&gt;a&lt;/sup&gt;, &lt;&lt;0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.23</td>
<td>1.60</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>7,12-Dimethylbenz[a]anthracene</td>
<td>0.006</td>
<td>10.9</td>
<td>&lt;3.91</td>
<td>36.4</td>
</tr>
<tr>
<td>Methylcholanthrene</td>
<td>strong sensitizer&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.2</td>
<td>3.2</td>
<td>ni&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note. ni, no induction.

<sup>a</sup>From Chipinda et al. (2011a,b).

<sup>b</sup>From Ashby et al. (1995).

<sup>c</sup>See Scholes et al. (1994).

<sup>d</sup>Only in one repetition above twofold induction and at cytotoxic dose.

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**Screening a Wide Range of Chemicals That Are Negative in the Classical KeratinoSens Assay**

Next to the chemicals discussed above, we found a number of false-negative chemicals in the KeratinoSens assay, which are not suspected as prohaptens, and a large number of correct-negatives. It is especially the latter group that needs a careful evaluation if a nonphysiological (i.e., not skin-derived) metabolic activation system is used because, in theory, Aroclor-induced rat liver might activate a wide range of chemicals, which are nonsensitizing due to insufficient metabolism in the skin. We thus screened the chemicals previously found to be negative in the classical KeratinoSens assay and evaluated whether any of these chemicals induce the luciferase above a threshold of twofold.

In total, 76 correct-negative chemicals were rescreened in the S9 activation assay. The 19 chemicals tested as
correct-negatives in our previous publication (Emter et al., 2010) are listed in Table 5, none of these chemicals triggered significantly enhanced luciferase activity in presence of S9. Supplementary table S3 lists another 53 chemicals tested as correct-negatives in presence and absence of S9. None of these chemicals induced luciferase above a threshold of twofold, but some gave a weakly enhanced response between 1.5- and two-fold. Four of the 76 chemicals negative in the LLNA and in the standard KeratinoSens assay became positive due to S9 activation, and these are listed in Table 6.

Among the 19 false-negative chemicals in the KeratinoSens assay for which we did not suspect a prohapten status, the three chemicals in Table 7 were activated by S9. The 16 chemicals in Supplementary table S4 remain false-negative in presence of S9 fractions. They include especially typical amine-reactive acyl transfer agents that are negative in KeratinoSens but can reliably be identified with a parallel peptide-reactivity screening with a peptide containing a Lys residue (Lalko et al., 2012; Natsch, 2010), but there are also nine chemicals for which we find neither a positive result in KeratinoSens with or without S9 nor direct peptide reactivity. Most of these latter chemicals are weak in the LLNA.

Inhibition of the S9-Dependent Response to Prohaptens by P450 Inhibitors

In order to evaluate whether the S9-dependent enhanced induction of the luciferase signal is due to P450 enzymes contained in the S9 fractions, the experiments were repeated for methylisoeugenol, dihydroeugenol, and 1-naphtol in absence and presence of different P450 inhibitors (see Supplementary table S5 and Supplementary fig. S2). In parallel experiments, fluorogenic P450 substrates were tested, in order to validate successful inhibition of ethoxyresorufin-deethylase (EROD), metoxyresorufin-demethylase (MROD), and pentoxyresorufin-dealkylase activity (PROD) under the chosen experimental conditions. The inhibitor concentrations were chosen to be above published IC$_{50}$ values and below the level affecting cellular viability or luciferase readout as determined in preliminary experiments. The Cyp1A1 inhibitor α-naphtoflavone at 0.625 µM had no effect on the luciferase induction but did also not affect EROD, MROD, or PROD activity neither. At 2.5 µM, it enhanced the luciferase signal, while inhibiting EROD and MROD, indicating that Cyp1A1 is not involved in activation of the selected prohaptens, but rather in their detoxification. The Cyp1A2 inhibitor Fluvoxamine and the Cyp3A4 inhibitor Ketoconazole strongly inhibited the luciferase induction by dihydroeugenol and 1-naphtol and had partial effects in the case of methylisoeugenol. These two inhibitors strongly inhibited PROD and to a lesser extend EROD, with no effect on MROD. In addition, we tested piperonyl butoxide because this compound is a reported inhibitor of the metabolism of eugenol at least if tested at 1mM (Thompson et al., 1990). At the concentrations tested, it had neither effect on the luciferase readout nor on O-dealkylation reactions.

Stability of the Readout and Batch Effects

In each run, eugenol was included as positive control at two concentrations both in presence and in absence of S9. Runs were considered valid only if eugenol gave an enhanced response above a threshold of 2 at 1000µM. Figure 3 shows
TABLE 5
KeratinoSens Result for Nonsensitizers in Presence and Absence of S9*

<table>
<thead>
<tr>
<th>Name</th>
<th>LLNA EC3 (%)</th>
<th>Iₘₐₓ (fold induction)</th>
<th>EC2 (µM)</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No S9</td>
<td>With S9</td>
<td>No S9</td>
</tr>
<tr>
<td>Sodium lauryl sulphate</td>
<td>14</td>
<td>3.39</td>
<td>1.62</td>
<td>ni</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>&gt; 25</td>
<td>0.87</td>
<td>1.11</td>
<td>ni</td>
</tr>
<tr>
<td>Methyl salicylate</td>
<td>&gt; 20</td>
<td>1.10</td>
<td>1.32</td>
<td>ni</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>&gt; 25</td>
<td>1.08</td>
<td>0.99</td>
<td>ni</td>
</tr>
<tr>
<td>Diethyl phthalate</td>
<td>&gt; 100</td>
<td>0.84</td>
<td>1.45</td>
<td>ni</td>
</tr>
<tr>
<td>Glycerol</td>
<td>&gt; 100</td>
<td>1.19</td>
<td>1.00</td>
<td>ni</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>&gt; 20</td>
<td>0.95</td>
<td>1.20</td>
<td>ni</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>&gt; 20</td>
<td>1.11</td>
<td>0.89</td>
<td>ni</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>&gt; 20</td>
<td>1.14</td>
<td>1.11</td>
<td>ni</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>&gt; 25</td>
<td>0.94</td>
<td>0.75</td>
<td>ni</td>
</tr>
<tr>
<td>Sulfanilic acid</td>
<td>NC</td>
<td>0.96</td>
<td>0.89</td>
<td>ni</td>
</tr>
<tr>
<td>Tartaric acid diammonium salt</td>
<td>&gt; 25</td>
<td>0.94</td>
<td>0.75</td>
<td>ni</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>&gt; 50</td>
<td>1.17</td>
<td>0.83</td>
<td>ni</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>&gt; 50</td>
<td>1.21</td>
<td>1.29</td>
<td>ni</td>
</tr>
<tr>
<td>Dextran</td>
<td>NC</td>
<td>1.13</td>
<td>0.89</td>
<td>ni</td>
</tr>
<tr>
<td>Chlorobenzene</td>
<td>&gt; 25</td>
<td>1.16</td>
<td>1.48</td>
<td>ni</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>&gt; 25</td>
<td>1.17</td>
<td>1.09</td>
<td>ni</td>
</tr>
<tr>
<td>Phenol</td>
<td>NC</td>
<td>0.79</td>
<td>0.99</td>
<td>ni</td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>&gt; 50</td>
<td>0.94</td>
<td>1.15</td>
<td>ni</td>
</tr>
</tbody>
</table>

Note. ni, no induction.
*Shown are results for the chemicals previously found correct-negative in the KeratinoSens assay (Emter et al., 2010). Results on a more extensive screening on negatively rated chemicals are shown in Supplementary tables.
NC, negative, maximal test concentration not given.
Induction only at cytotoxic concentrations.

TABLE 6
Chemicals That Become False-Positive in Presence of S9 Fractions

<table>
<thead>
<tr>
<th>Name</th>
<th>LLNA EC3 (%)</th>
<th>Iₘₐₓ (fold induction)</th>
<th>EC2 (µM)</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No S9</td>
<td>With S9</td>
<td>No S9</td>
</tr>
<tr>
<td>N,N-Diethyl-3-methylbenzamide (DEET)</td>
<td>&gt; 60</td>
<td>0.92</td>
<td>3.60</td>
<td>ni</td>
</tr>
<tr>
<td>6-Methoxynaphthalene-2-carbaldehyde</td>
<td>&gt; 20</td>
<td>2.16</td>
<td>3.10</td>
<td>154*</td>
</tr>
<tr>
<td>Vanillin</td>
<td>&gt; 50</td>
<td>1.41</td>
<td>2.48</td>
<td>ni</td>
</tr>
<tr>
<td>3-Hydroxy-2-nitopyridine</td>
<td>&gt; 26</td>
<td>1.07</td>
<td>2.01</td>
<td>ni</td>
</tr>
</tbody>
</table>

Note. ni, no induction.
*Induction only at cytotoxic concentrations.
the summary from all the 74 valid runs for eugenol at the two test concentrations. To further evaluate the stability of the read-out and potential batch effects, some chemicals were retested with an independent batch of S9. In Supplementary fig. S3, the comparison of the results with the two batches is shown. For eugenol, methylisoeugenol, resorcinol, and dihydroeugenol, very similar results were obtained with the two batches, indicating no strong dependence on the S9 batch used.

### DISCUSSION

The main findings of this study indicate that a number of sensitizers, which were long considered as putative prohaptens, can be activated by Arochlor-induced rat liver S9 fractions and then induce an enhanced luciferase response at a lower concentration in the KeratinoSens assay. This stands in contrast to most nonsensitizers, for which no such activation was observed, and to the tested direct-acting haptens, for which the detoxifying action of S9 metabolism appears to be dominating. Thus, this work gives a further confirmation of the prohapten hypothesis, and the modified assay may serve as a further tool to investigate a potential prohapten status of a test chemical.

Another key observation is that the number of chemicals activated by S9 fractions constitutes a relatively small minority within the typical test chemicals investigated for skin sensitization. Overall, this finding is in accordance with the work of Troutman et al. (2011) who developed a HRP/H$_2$O$_2$ system to activate potential prohaptens. In a data set of 70 chemicals, including a specific selection of 19 potential prohaptens, they found a clearly enhanced reactivity in presence of HRP for 10 chemicals (eugenol, 1-naphthol, dihydroeugenol, creosol, methyleugenol, aniline, pentachlorophenol, 3-aminophenol, 2-aminophenol, and lauryl gallate). We found S9 activation for the former five molecules, whereas 2-aminophenol and lauryl gallate are positive in KeratinoSens also in absence of S9. Aniline, pentachlorophenol, and 3-aminophenol were not activated by S9. Both the current work and the work of Troutman et al. (2011) indicate that not only the number of prohaptens in the tested data sets, which are activated by the enzymatic test systems, is limited but that the structural diversity of prohaptens activated by the two experimental systems is relatively narrow, too. Although in our data set mainly phenols and methoxybenzenes were activated, the chemicals activated in the data set of Troutman et al. (2011) include phenols, methoxybenzenes, and aromatic amines.

Considering all the data, a key question from a practical point of view is whether the S9 activation should be included in a testing battery to routinely test all chemicals that are negative in the classical KeratinoSens assay. Given the limited chemical

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**TABLE 7**

Chemicals Not Suspected as Prohaptens, Which Became Correct-Positive in the KeratinoSens With S9 Fractions

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>LLNA EC3 (%)</th>
<th>$I_{max}$ (fold induction)</th>
<th>EC2 ($\mu$M)</th>
<th>IC$_{50}$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N,N-Dibutylaniline</td>
<td>19.6</td>
<td>1.7</td>
<td>11.0</td>
<td>18.6</td>
</tr>
<tr>
<td>Benzoyl peroxide</td>
<td>0.004</td>
<td>1.6</td>
<td>5.3</td>
<td>288.7</td>
</tr>
<tr>
<td>Beryllium sulphate</td>
<td>0.68</td>
<td>1.4</td>
<td>5.6</td>
<td>16.3</td>
</tr>
<tr>
<td>N,N-Dibutylaniline</td>
<td></td>
<td>ni</td>
<td>46.2</td>
<td>&gt;62.5</td>
</tr>
<tr>
<td>Benzoyl peroxide</td>
<td></td>
<td>ni</td>
<td>288.7</td>
<td>446.6</td>
</tr>
<tr>
<td>Beryllium sulphate</td>
<td></td>
<td>ni</td>
<td>16.3</td>
<td>&gt;125</td>
</tr>
</tbody>
</table>

*Note. ni, no induction.*

**FIG. 3.** Results for the positive control eugenol in all the valid runs.
diversity of chemicals activated by the system, a pragmatic proposal would be to perform this testing only for chemicals that are negative in a first screening and that contain certain structural alerts. Thus, based on the findings from this study and from the study of Troutman et al. (2011), we would propose to only test in the KeratinoSens-S9 assay (or another assay with an additional activation system) chemicals with either phenolic groups and/or alkoxy groups attached to a benzene ring, aromatic amines, and conjugated dienes. This proposal is schematically illustrated in Figure 4. With such a more targeted screening, many relevant P450-activated prohaptens would most likely be detected, without generating many unspecific false-positives. We would still miss some more unusual prohaptens, like carvoxime, which probably would be better detected in a peptide reactivity assay including an enzymatic activation system (Bergström et al., 2007). Alternatively, a routine cellular assay specifically expressing CYP1B1 would be needed, as this is a key enzyme in dendritic cells (Baron et al., 1998), which was shown as important enzyme in carvoxime metabolism (Ott et al., 2009).

Certainly, the use of S9 fractions from activated rat liver is a nonphysiological and exaggerated test system, which (as reviewed in more detail in the introductory paragraphs) is not reflecting true metabolic activity reported in the skin. Therefore, we considered it important to screen a large selection of nonsensitizers to evaluate, whether this system does not generate a very high frequency of positive responses for nonsensitizers. On the positive side, among 76 correct-negatives tested, only 4 chemicals gave a positive result in presence of the S9 fractions. In addition, most phenolic nonsensitizers (3-hydroxy-4-nitrobenzoic acid, 4-hydroxybenzaldehyde, salicylic acid, methyl salicylate, 4-hydroxybenzoic acid, and phenol) remain negative in presence of S9 fractions, thus we do not observe an unspecific activation of all phenolic test chemicals, with the exception of a positive result for vanillin. This compound has the structural alert common to several of the prohaptens in Table 1 (o-methoxy-phenol) and therefore this result is not entirely surprising. On the other hand, there is a very low incidence of positive sensitization data for vanillin from diagnostic patch testing (12 cases in almost 6000 patients tested for vanillin), despite the very broad use in cosmetic products. The most surprising and intriguing result was found for NN-diethyl-3-methylbenzamide (DEET). This chemical is clearly a nonsensitizer with widespread human exposure (used in topical mosquito repellent formulations up to a level of 50%). Interestingly, it induced luciferase reproducibly in presence but not absence of S9. Thus, although the overall screening indicates that the exaggerated metabolic system does not generate unspecific activation of many chemicals, this specific case highlights the possibility of generating false-positive results for molecules with a good safety profile. The result for 3-hydroxy-2-nitropyridine is borderline, whereas 6-methoxynaphthalene-2-carbaldehyde activates the luciferase with and without S9, but the response is at noncytotoxic levels in presence of S9 only. In these two cases, we have no sensitization data other than the LLNA result available to compare with.

There are also some suspected prohaptens, which did not induce the luciferase in presence of S9. The two 1,3-substituted compounds resorcinol and 3-amino phenol are probably not typical P450 substrates and cannot form quinoid structures. More surprising is the result for acetaminophen, a known P450 substrate forming reactive metabolites (Patten et al., 1993). Although there are no reports on human sensitization, it was lately shown to be strongly positive in the LLNA (Chipinda et al., 2011a). The negative result for carvoxime is intriguing, too, because this chemical was shown to be activated by P450 preparations to form peptide-reactive metabolites (Bergström et al., 2007) of high sensitizing potential. However, a possible explanation for the lack of activity in the KeratinoSens-S9 assay is the fact that the reactive metabolites formed by S9 activation in the culture supernatant need to be sufficiently stable to migrate into the cytosol where they can activate the Kelch-like ECH-associated protein 1 sensor proteins. It had been reported that metabolites of carvone oxime are so highly reactive electrophiles and that they are not detectable in microsomal incubations (unless in the peptide-trapped form), and this was attributed to their rapid reaction with nucleophilic residues on microsomal proteins (Bergström et al., 2007). A similar explanation could also be true for the lack of activity of acetaminophen. To further improve the system, P450 enzymes would need to be expressed within the KeratinoSens cells, e.g., with P450 expression vectors. Another theoretical possibility, which we considered, is to induce the expression of endogenous P450 enzymes. Indeed, we could identify a phase I specific inducer (3,3’,4,4’,5-pentachlorobiphenyl) that induces EROD activity > 40-fold in KeratinoSens cells in the absence of luciferase induction. However, no increased response to the chemicals in Table 1 was detected in cells with induced EROD activity (our unpublished results).

Reproducible activation in presence of S9 was found for benzoyl peroxide, dibutylaniline, and beryllium sulphate, which have no typical structural alerts of prohaptens. Especially the case of beryllium sulphate is difficult to explain, as a prohapten mode of action for metal sensitizers was never postulated. However, as the mode of action for beryllium salts to trigger allergic reactions appears unknown, this result currently cannot be explained.
The clear difference between isoeugenol, activating the luciferase in absence of S9 and eugenol, requiring S9 for strong activation, confirms the different mode of action of these two structurally highly similar compounds. Isoeugenol is a prehapten modifying peptides upon spontaneous oxidation (Natsch and Gfeller, 2008), whereas eugenol appears to be a prohapten requiring metabolic activation to become peptide reactive (Ball et al., 2011). This different mode of action is also reflected in a lack of cross-sensitization to the two compounds in the majority of patients (Itoh, 1982) and a different potency of the two compounds in the human maximization tests (Marzulli and Maibach, 1980), Guinea pig maximization test, and the LLNA (Hilton et al., 1996) and a very different frequency of human patch test positive patients (Itoh, 1982). This different mode of action is also reflected in a lack of cross-sensitization to the two compounds in the majority of patients (Itoh, 1982) and a different potency of the two compounds in the human maximization tests (Marzulli and Maibach, 1980), Guinea pig maximization test, and the LLNA (Hilton et al., 1996) and a very different frequency of human patch test reactions (Uter et al., 2010). In all test systems, eugenol is clearly a weaker sensitizer compared with isoeugenol. A difference was also noted in a recent paper on coculture of THP-1 cells with HaCaT cells (Hennen et al., 2011). Whereas presence of HaCaT cells enhanced the response of THP-1 cells to eugenol, a reduced response to isoeugenol was found, probably due to detoxification. However, in that work the metabolic activation of eugenol by HaCaT cells was sufficient, although we find mostly negative results in most experiments with eugenol in absence of S9 for the HaCaT-derived KeratinoSens cell line, despite the ability of these cells to induce EROD activity and the observation that eugenol itself is an inducer for EROD activity (Hennen et al., 2011).

Results from inhibitor experiments indicate, that the CYP1A1 inhibitor α-naphthoflavone has no effect on the S9-triggered response, whereas the Cyp1A2 inhibitor Fluvoxamine and the Cyp3A4 inhibitor Ketoconazole partly inhibited the luciferase induction. Because the main focus of this study was on the development of KeratinoSens-S9 assay and to test how it could be integrated in a testing strategy, these experiments were not extended to further chemicals, but they indicate that P450 enzymes in S9 do play a role in the observed activation, and that further mechanistic studies with specific inhibitors could be performed to identify which enzymes are responsible for activation of different skin-sensitizing prohapten by S9 fractions.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci. oxfordjournals.org/.

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REFERENCES


