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Skin Sensitizers Induce Antioxidant Response Element Dependent Genes: Application to the *In Vitro* Testing of the Sensitization Potential of Chemicals

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Tests for skin sensitization are required prior to the market launch of new cosmetic ingredients and *in vitro* tests are needed to replace the current animal tests. Protein reactivity is the common feature of skin sensitizers and it is a crucial question whether a cellular *in vitro* assay can detect protein reactivity of diverse test chemicals. The signaling pathway involving the repressor protein Keap1 and the transcription factor nuclear factor-erythroid 2–related factor 2, which binds to the antioxidant response element (ARE) in the promoter of many phase II detoxification genes, is a potential cellular marker because Keap1 had been shown to be covalently modified by electrophiles which leads to activation of ARE-dependent genes. To evaluate whether this regulatory pathway can be used to develop a predictive cellular *in vitro* test for sensitization, 96 different chemicals of known skin sensitization potential were added to Hepa1C1C7 cells and the induction of the ARE-regulated quinone reductase (QR) activity was determined. In parallel, 102 chemicals were tested on the reporter cell line AREc32, which contains an eightfold repeat of the ARE sequence upstream of a luciferase gene. Among the strong/extreme skin sensitizers 14 out of 15 and 30 out of 34 moderate sensitizers induced the ARE-dependent luciferase activity and in many cases this response was paralleled by an induction of QR activity in Hepa1C1C7 cells. Sixty percent of the weak sensitizers also induced luciferase activity, and the overall accuracy of the assay was 83 percent. Only four of 30 tested non-sensitizers induced low levels of luciferase activity, indicating a high specificity of the assay. Thus, measurement of the induction of this signaling pathway provides an interesting *in vitro* test to screen for the skin sensitization potential of novel chemicals.

Key Words: **skin sensitization; *in vitro* testing; antioxidant response element.**

The risk of skin sensitization is a critical issue in the development of novel ingredients for cosmetic products. The current model of choice is the local lymph node assay (LLNA) in mice, in which the cellular proliferation in the draining lymph nodes is measured after repeated topical application of the test compound onto the ears (Basketter *et al.*, 2002; Gerberick *et al.*, 2004a, 2007a). Results are expressed as EC3 values indicating the % concentration, which induces a three-fold increase in cellular proliferation. However, with the forthcoming ban on animal testing for cosmetic ingredients in the EU, there is a pressing need for alternative tests which make animal testing obsolete.

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 modified peptides are then displayed by dendritic cells in the draining lymph nodes where they trigger a specific T-cell mediated immune response (reviewed in Smith and Hotchkiss, 2001). One promising possibility to predict skin sensitization based on *in vitro* data is therefore the evaluation of the chemical reactivity of a test compound toward peptides and proteins (Divkovic *et al.*, 2005; Gerberick *et al.*, 2004b, 2007b).

A completely different direction of research has focused on cellular responses to sensitizers such as gene expression changes measured with gene-chip analysis (Ryan *et al.*, 2004) or reverse transcription-PCR (RT-PCR) (Gildea *et al.*, 2006), altered expression of surface markers detected with flow cytometric analysis (e.g., Hulette *et al.*, 2005; Sakaguchi *et al.*, 2006), or changes in cytokine levels (Coquette *et al.*, 2003). The analytical endpoints selected in these approaches were either empirical in nature or based on markers which are known to be upregulated upon emigration of Langerhans cells from the skin.

The elicitation phase of skin sensitization is a very specific immune reaction, with hapten-specific T cells as effector cells. However, during the induction phase of the sensitization, which is simulated with almost all *in vitro* tests currently under development, this specificity does not yet exist. The key questions then are: How should during the unspecific induction phase of sensitization the dendritic cells (or any other cells proposed for *in vitro* tests) be able to recognize structurally highly different allergens? And how should they discriminate them from irritants to yield a universal response (be it at the RNA or protein level) which can then be used to develop

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a predictive cell-based *in vitro* test? This theoretical questions have received astonishingly little attention in the discussion on and the search for molecular endpoints of utility for *in vitro* test development.

Skin sensitizers have a high chemical and physicochemical diversity, yet as pointed out above they have a unique feature in common in that they in principle share an intrinsic protein/peptide reactivity, or are believed to be metabolized to reactive molecules in the skin (prohaptens) (reviewed by Smith and Hotchkiss, 2001). Therefore, the cellular test system optimally would recognize this unifying feature, that is, be able to rate reactivity in order to have a broad applicability. Interestingly, a cellular sensor mechanism which recognizes various electrophiles has recently been discovered (Dinkova-Kostova *et al.*, 2005; Wakabayashi *et al.*, 2004). The sensor protein Keap1 (Kelch-like ECH-associated protein 1) contains highly reactive Cys residues. Covalent modification of crucial Cys residues by small molecules leads to 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 (mainly genes coding for phase II detoxifying enzymes) having an antioxidant response element (ARE) in their promoter sequence (Dinkova-Kostova *et al.*, 2005). Thus, the theoretically needed prerequisite, namely that cells do have a sensor mechanism to recognize intrinsic reactivity of molecules with diverse structures, is indeed found.

In this study we used two model systems: (1) the ARE-regulated quinone reductase (QR) activity in Hepa1C1C7 cells and (2) the ARE-regulated luciferase activity in the cell line AREc32, which contains an eightfold repeat of the ARE sequence upstream of a luciferase reporter gene (Wang *et al.*, 2006). These models were used to assess activation of the Keap/Nrf2/ARE regulatory pathway by a collection of 102 different chemicals of known skin sensitization potential. We report a good sensitivity to identify moderate, strong, and extreme allergens especially for the *in vitro* test with the AREc32 cell line and a high specificity of the assay.

TABLE 1
Summary Results for QR Induction in the Hepa1C1C7 Cell Line by Chemicals of Known Skin Sensitization Potential

Sensitization class	Number of chemicals tested	QR induction above 1.25 threshold (positive)[a]	No QR induction (negative)
Extreme	5	3	2
Strong	7	3	4
Moderate	32	23	9
Weak	20	3	16
Very weak/none	30	3	28
Total sensitizers[b]	**64**	**32**[c]	**32**
Total nonsensitizers	**30**	**3**	**27**
Result not clear-cut, compounds excluded from Cooper statistics	2		
Total compounds tested	96		

[a]Chemicals with significant induction of QR activity (at least 25% above background in at least one test concentration in two out of two or three out of four repetitions).

[b]In bold, the summary figures used for the calculation of the cooper statistics.

[c]Cooper statistics: sensitivity 50.0%; specificity 90.0%; positive predictivity 91.4%; negative predictivity 45.8%; accuracy 62.7%.

MATERIALS AND METHODS

All fragrance chemicals are commercial qualities obtained from Givaudan Schweiz AG, Geneva, Switzerland. All other test chemicals were purchased from Fluka/Sigma/Aldrich, Buchs, Switzerland. The chemical and trivial names, the structures, along with CAS-numbers and LLNA data of all the test chemicals are summarized in Table 1 in the supporting information. Many of the chemicals used in this study are moderate to extreme skin sensitizers, and any skin contact with these chemicals should be avoided.

AREc32 is a stable cell line derived from the human MCF7 breast carcinoma cell line. The generation of the cell line was described by Wang *et al.* (2006) and the cell line has been licensed from CRX biosciences, Dundee, Scotland. AREc32 cells were maintained in Dulbecco's modified Eagle's medium containing glutamax (Gibco/Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum and 500 μg/ml G418. Hepa1C1C7 cells were obtained from ATCC (European distributor, LGC Promochem, France) and were cultured in Dulbecco's modified Eagle's medium without nucleotides and deoxynucleotides (Gibco/Invitrogen) supplemented with 10% fetal calf serum. Both cell lines were grown at 37°C in the presence of 5% CO_2.

Test chemicals were dissolved in acetonitrile or dimethyl sulfoxide (DMSO) at a concentration of 100mM. They were further diluted in culture medium to a final concentration of either 10 or 2.5mM, and then serially diluted in culture medium containing an equal concentration of solvent in order to keep the solvent level constant at each test concentration. AREc32 cells were seeded in 96-well plates at a density of 50,000 cells per well in 180 μl of growth medium. Test chemicals were added 40 h later dissolved in 20 μl of growth medium. Final solvent concentration was 0.25% in all experiments, unless a concentration range up to 1000μM was tested: in this case solvent levels were at 1%. Cells were washed with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} 24 h after compound addition and then lysed by the addition of 20 μl of passive lysis buffer (Promega AG, Wallisellen, Switzerland). Luciferase activity was initiated by adding 50 μl of the luciferase assay substrate dissolved in luciferase assay buffer (both from Promega) to the cell lysate. Alternatively, 50 μl of assay reagent was made up according to the following recipe: 20mM tricine; 2.67mM $MgSO_4$; 0.1mM ethylenediaminetetraacetic acid; 33.3mM dithiotreitol; 270μM coenzyme A; 470μM luciferin potassium salt (Synchem, Kassel, Germany); 530μM adenosine triphosphate; pH 7.8. Luciferase activity was measured with the GloMax luminometer (Promega).

Hepa1C1C7 cells were seeded in 96-well plates at a density of 50,000 cells per well and treated with test chemicals as described for the AREc32 cells. Twenty-four hours after addition of the compounds, the QR activity was determined as described by Kang and Pezzuto (1992). Briefly, the cells were lysed by addition of a digitonin solution. A reaction mixture was added, which contained menadione as a QR substrate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP+ (oxidized nicotinamide adenine dinucleotide phosphate), and flavin adenine dinucleotide (oxidized) as electron donating system and 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), bovine serum albumin, Tween20, and a Tris-buffer. QR reduces menadione to menadiol and the QR-specific activity is determined by measuring the NADPH dependent, menadiol-mediated reduction of MTT to a blue formazan dye. In both the QR and the luciferase assays, *tert*-butyl-hydroquinone was included as a positive reference chemical in each assay plate.

Cytotoxicity of the compounds for both cell lines was tested in parallel assays run under equal conditions and with equal test concentrations. Twenty-four hours after test chemical addition 27 µl of a 5 mg/ml solution of MTT in PBS was added to the growth medium, cells were incubated for further 4 h at 37°C and then the growth medium was discarded. Cells were lysed for 24 h by the addition of 200 µl of 10% sodium dodecyl sulfate (SDS), and then the optical density of the reduced formazan dye was measured at 600 nm. Data are expressed as IC50 values (inhibitory concentration reducing viability by 50%).

The screening on the AREc32 cell line was repeated three or four times, with duplicate analysis for each chemical at each test concentration in each repetition. In the first two repetitions four concentrations (2, 10, 50, and 250µM) were tested. In the third and fourth repetition, six binary dilutions covering the maximal noncytotoxic doses for each test chemical were selected. Wherever possible, tests up to a maximal dose of 1000µM were performed in these repetitions. For chemicals with contradictory results, further repetitions were made to clarify whether they are indeed ARE-inducers or not. The screening with the Hepa1C1C7 cell line was repeated twice, with duplicate analysis at four concentrations (2, 10, 50, and 250µM) in each experiment. Based on these experiments, for each test chemical (1) the average maximal induction of gene activity (I_{max}), (2) the concentration range for maximal induction (CI_{max}), and (3) the average concentration inducing significantly enhanced gene activity above a certain threshold (EC 1.25 for QR and EC1.5 for luciferase activity) were determined. The latter calculations were performed with log-linear extrapolation from the values above and below the induction threshold (as for the EC3 value determination in the LLNA and with the formula described in Gerberick *et al*., 2007a). A chemical was rated positive, if it induced significantly enhanced gene activity above the threshold indicated above at any of the tested concentrations and either in all repetitions made or in three out of four or four out of five repetitions.

LLNA data on novel fragrance materials were determined under standard conditions as defined in the Organization for Economic Co-operation and Development guideline 429 and the data were previously published by Natsch *et al*. (2007). Further LLNA data were either taken from the general literature summarized in Gerberick *et al*. (2004a,b, 2005) or from the Research Institute on Fragrance Materials RIFM. The literature references for all the original LLNA studies are added to Table 1 in the supporting information. To rate the chemicals, EC3 values were expressed in millimolar to give a better comparison between chemicals, although the primary LLNA data are always reported on a percentage (wt/vol) basis. The sensitization class in Table 1 is given based on the scheme of Kimber *et al*. (2003). V. weak/none is indicated for chemicals with EC3 > 30% due to data set inadequacies (several chemical considered nonsensitizers have not been tested at > 25–50% in the LLNA).

RESULTS

Induction of QR Activity by Skin Sensitizers

A subset of 96 of the chemicals listed in Table 1 in the supporting information was screened for the induction of QR in the Hepa1C1C7 cells. Table 1 lists the summary of the results, and Table 2 in the supporting information lists the detailed results for the individual compounds. On average, the relative variation between duplicates was only 3.7%, and a 25% enhanced expression was statistically significant in all cases. This value was therefore selected as the threshold to classify a chemical to be a QR-inducer. Table 2 lists the average maximal induction (I_{max}), the concentration for maximal induction (CI_{max}), and the EC 1.25 value (extrapolated concentration yielding 25% enhanced QR activity). Based on this threshold, 29 of the 44 tested moderate, strong, and extreme sensitizers significantly induced QR expression at least 25% above the level in control cells in both repetitions. Among the weak sensitizers, only three out of 20 chemicals also induced QR activity (Table 1). Among the nonsensitizers according the LLNA, only geraniol, 2-hexenol, and 6-methyl-coumarin induced QR activity. This leads to the following Cooper statistics (Cooper *et al*., 1979): sensitivity 50.0%, specificity 90.0%, and an accuracy of 62.7%. The dose–response curves of QR induction by five sensitizing fragrance chemicals are shown in Figure 1.

These results with QR induction indicate that many skin sensitizers can be recognized by screening for the enhanced expression of a gene which is under the control of an ARE regulatory sequence, but the sensitivity of the QR-assay is clearly not yet satisfactory. The dynamic range of this assay is also rather low, with a relatively high background level of QR activity and a maximal induction of 3.15-fold in the case of safranal.

Induction of ARE-Regulated Luciferase Activity

A comprehensive screening using the AREc32 cell line was made on all the 102 test chemicals shown in Table I. Because this cell line stably carries a luciferase reporter gene under the control of eight copies of the ARE sequence and because reporter gene activity allows very sensitive detection with a low background signal, this cell line was chosen with the aim to obtain an improved sensitivity. The average relative variance between duplicates in this assay was 8.3%, and a luciferase expression of 50% above background values was statistically significant in all cases. Therefore, the threshold of 50% enhanced expression was selected as representative of significant induction. EC1.5 values were calculated accordingly with log-linear extrapolation (Gerberick *et al*., 2007a). Very similar results were also obtained with linear extrapolation. With this threshold, the assay was positive for 14 out of the 15 strong and extreme sensitizers and for 31 out of the 35 moderate sensitizers. Among the weak sensitizers 12 out 20 did induce luciferase activity significantly, whereas only four out of 30 nonsensitizers did induce the reporter gene activity. The calculated sensitivity of the assay is therefore 81.4%, the specificity is 86.6%, the positive predictivity is 93.4%, the negative predictivity is 66.6%, and the overall accuracy is 83.0%. Table 2 lists the detailed results for each test chemical and Table 3 shows the summary results used for calculating the Cooper statistics. Besides the EC1.5 values, the maximal induction I_{max}, and the concentration range CI_{max} where this maximal induction was achieved is given in Table 2. The levels of induction are very different for different sensitizers, with some chemicals inducing the luciferase activity by 1.5- to twofold and others inducing the luciferase activity up to 40-fold above the background level. The dose–response curves for a few typical sensitizers and the irritant SDS are shown in Figure 2.

Cytotoxicity for the AREc32 cells was assessed for all compounds in parallel assays. For the majority of compounds,

TABLE 2
Induction of Luciferase Activity in AREc32 Cells by 102 Chemicals of Known Skin Sensitization Potential

No.	Test compound	EC3 value in LLNA (mM)	Sensitization class[a]	Cytotoxicity (IC50)[b]	Induction of ARE-regulated luciferase activity				
					I_{max}[c]	CI_{max}[d]	EC1.5[e]	N[f]	Rating[g]
1	Oxazolone	0.14	Extreme	> 1000	2.3	1000	214.6	3/3	+
2	Diphenylcyclopropenone	0.15	Extreme	21.57	20.0	8–10	1.1	4/4	+
3	5-Chloro-2-methyl-4-isothiazolin-3-one	0.60	Extreme	10.29	7.2	8–10	<< 2	3/3	+
4	*p*-Benzoquinone	0.93	Extreme	> 250	19.6	31–50	2.1	3/3	+
5	1-Chloro-2,4-dinitrobenzene	1.98	Extreme	7.79	12.3	4–10	1.4	4/4	+
6	Fluorescein-5-isothiocyanate	3.60	Strong	> 1000	2.2	500–1000	275.6	3/3	+
7	Lauryl gallate	8.88	Strong	40.50	2.8	8–50	4.8	3/3	+
8	1,4-Hydroquinone	9.09	Strong	70.57	28.6	31–50	1.6	3/3	+
9	Glutaraldehyde	10.00	Strong	267.64	17.7	50–125	20.3	4/4	+
10	Phthalic anhydride	10.80	Strong	> 1000	1.3		n.i.	0/3	–
11	1,4-Phenylendiamine	14.81	Strong	380.00	12.7	62–250	11.6	4/4	+
12	Propyl gallate	15.09	Strong	> 1000	8.3	1000	3.4	4/4	+
13	Metol	22.67	Strong	28.66	32.4	10–16	1.9	3/3	+
14	2-Amino-phenol	36.36	Strong	> 250	23.3	31–50	1.5	3/3	+
15	1-Phenyl-1,2-propanedione	87.72	Moderate	> 1000	27.5	1000	114.0	3/3	+
16	1-Naphtol	90.15	Moderate	756.57	2.4	62–250	15.8	3/3	+
17	5,6,7-Trimethyl-(2E)-2,5-octadien-4-one	96.39	Moderate	281.16	38.7	31–50	2.6	3/3	+
18	1-Spiro[4.5]dec-7-en-7-yl-4-penten-1-one	100.92	Moderate	778.39	16.7	1000	25.5	3/3	+
19	2-Mercaptobenzothiazol	101.64	Moderate	> 1000	10.9	250–500	32.0	3/3	+
20	Isoeugenol	109.76	Moderate	> 1000	60.2	500	17.9	4/4	+
21	2-Bromotetradecanoic acid	110.75	Moderate	250.00	3.1	62–125	54.8	3/3	+
22	2-Hydroxy-ethyl-acrylate	120.56	Moderate		15.2	50–62	5.6	3/3	+
23	Diethyl maleate	122.09	Moderate	> 250	41.0	250	2.3	3/3	+
24	Benzyl-salicylate	127.19	Moderate	> 1000	3.6	250	18.1	3/3	+
25	2-Nonynoic acid, methyl ester	148.81	Moderate	250.00	19.5	62–250	0.7	3/3	+
26	1,2-Benzisothiazolin-3-one	152.12	Moderate	43.59	6.6	10–31	1.9	3/3	+
27	4-Vinyl-pyridine	152.38	Moderate	200.83	13.8	50–62	2.9	3/3	+
28	Galbanone	156.25	Moderate	133.40	8.1	62–250	24.2	3/3	+
29	*trans*-2-Decenal	162.34	Moderate	175.72	11.6	62–250	56.2	3/3	+
30	2-Methyl-2*H*-isothiazolin-3-one	165.22	Moderate	85.52	12.5	50–62	2.6	3/3	+
31	*trans*-Anethole	182.43	Moderate	> 1000	0.9		n.i.	0/3	–
32	3-Aminophenol	210.53	Moderate	> 1000	5.0	1000	68.5	3/3	+
33	Diethyl sulfate	214.01	Moderate	> 1000	1.2		n.i.	0/3	–
34	3-Dimethyl-amino-1-propylamine	215.69	Moderate	> 1000	5.4	1000	156.4	3/3	+
35	Formaldehyde	233.33	Strong	376.77	4.1	50–62.5	34.5	3/4	+
36	2,4-Dimethyl-3-cyclohexene-1-carboxaldehyde	233.81	Moderate	> 1000	8.0	500	227.1	3/3	+
37	Cinnamic aldehyde	234.85	Moderate	289.19	31.6	50–250	19.0	3/3	+
38	Phenylacetaldehyde	250.00	Moderate	> 1000	9.8	250	47.5	3/3	+
39	Benzylideneacetone	253.42	Moderate	332.38	27.5	50–125	9.4	3/3	+
40	α-Methyl cinnamic aldehyde	308.2	Moderate	> 1000	23.3	500	46.8	3/3	+
41	Citral	328.95	Moderate	> 1000	9.8	1000	64.0	3/3	+
42	β-Damascone	348.96	Moderate	> 250	26.7	31–250	1.5	3/3	+
43	3,4-Dihydrocoumarin	378.38	Moderate	> 1000	0.9		n.i.	0/3	–
44	Dihydroeugenol	409.64	Moderate	400.84	3.4	125–250	46.3	3/3	+
45	2-Methoxy-4-methyl-phenol	420.29	Moderate	> 1000	1.4		n.i.	0/4	–
46	2-Phenyl-propionic aldehyde	440.56	Moderate	445.35	2.3	250	120.8	4/5	+
47	2-Methyl-3-(4-(2-methylpropyl)phenyl)-propanal	441.18	Moderate	> 1000	3.1	1000	185.4	2/4[h]	+
48	Sodium dodecyl sulfate	484.43	non-sensitizer	243.24	1.2		n.i.	0/3	–
49	Safranal	499.33	Moderate	> 500	32.9	500	12.0	3/3	+
50	Farnesal	531.82	Weak	988.41	2.9	500	108.3	3/3	+
51	Perillaldehyde	540.00	Moderate	662.55	42.1	250–500	37.0	3/3	+
52	α-Hexyl cinnamic aldehyde	555.56	Weak	302.61	3.9	125–250	36.1	3/3	+
53	*trans*-2-Hexenal	561.22	Moderate	562.71	29.7	62–250	11.6	3/3	+
54	6-(1-Methylpropyl)quinoline	605.41	Weak	141.31	2.0	125–500	55.9	4/5	+
55	3-Methyl-(5*Z*)-5-cyclotetradecen-1-one	738.74	Weak	188.74	1.1		n.i.	0/3	–
56	Benzyl cinnamate	773.11	Weak	853.84	8.8	500	26.8	3/3	+

TABLE 2—*Continued*

No.	Test compound	EC3 value in LLNA (mM)	Sensitization class[a]	Cytotoxicity (IC50)[b]	Induction of ARE-regulated luciferase activity				
					I_{max}[c]	CI_{max}[d]	EC1.5[e]	N[f]	Rating[g]
57	Eugenol	792.68	Weak	> 1000	2.3	500–1000	38.2	4/5	+
58	Benzyl-benzoate	801.9	Weak	> 1000	2.0	250–1000	25.9	3/3	+
59	Lyral	814.29	Weak	1000.00	4.6	500	98.3	3/3	+
60	Phenyl benzoate	863.64	Weak	> 1000	3.6	1000	193.5	2/3[h]	+
61	Lilial	931.37	Weak	500.00	1.0		n.i.	0/3	−
62	Cyclopropanecarboxylic acid, 2-[1-(3,3-dimethylcyclohexyl)ethoxy]-2-methylpropyl ester	939.19	Weak	210.31	1.1		n.i.	0/3	−
63	1-(Cyclopropylmethyl)-4-methoxybenzene	1030.86	Weak	> 1000	0.9		n.i.	0/3	−
64	6-Ethyl-3-methyl-6-octen-1-ol	1135.29	Weak	171.05	0.9		n.i.	0/3	−
65	Ambrettolide	1142.86	Weak	392.66	0.8		n.i.	0/3	−
66	Cyclamen aldehyde	1173.68	Weak	124.92	1.8	50–125	55.7	2/3	+/−
67	2,3-Butanedione	1279.07	Weak	> 1000	4.8	1000	125.1	3/3	+
68	Hydroxycitronellal	1337.21	Weak	> 1000	0.9		n.i.	0/3	−
69	Methyl atrarate	1433.67	Weak	371.16	1.5	250		1/3	−
70	Estragole	1442.86	Weak	605.91	2.4	500	297.8	2/4[h]	+
71	Cinnamic alcohol	1567.16	Weak	> 1000	2.1	1000	308.9	3/3	+
72	Benzocaine	> 3026	Weak sensitizer	> 1000	3.0	500–1000	51.4	3/3	+
73	Isopropyl myristate	1629.63	V. weak/none	> 1000	1.0		n.i.	0/3	−
74	1,1,3-Trimethyl-3-phenylindane	1834.75	V. weak/none	283.55	1.2		n.i.	0/3	−
75	Linalool	1948.05	V. weak/none	> 1000	1.1		n.i.	0/3	−
76	2-Methyl-butanoic acid-hexyl-ester	2941.65	V. weak/none	> 1000	1.1		n.i.	0/3	−
77	Geraniol	3701.30	V. weak/none	415.25	1.3		n.i.	0/3	−
78	2,3-Dihydro-2,3,3-trimethyl-1*H*-Inden-1-one	5747.00	V. weak/none	> 1000	1.0		n.i.	0/3	−
79	Hexenol-2-trans	6000.00	V. weak/none	> 1000	1.5	500	294.0	1/3	−
80	Pyridine	9000.00	V. weak/none	> 1000	1.1		n.i.	0/3	−
81	Ethylene brassylate	> 1111	V. weak/none	346.92	1.1		n.i.	0/3	−
82	Methyl salicylate	> 1315	V. weak/none	> 1000	1.1		n.i.	0/3	−
83	6-Methyl-coumarin	> 1562	V. weak/none	> 1000	4.4	1000	69.3	3/4	+
84	Hedione	> 1769	V. weak/none	> 1000	1.1		n.i.	0/3	−
85	4-Hydroxybenzoic acid	> 1811	V. weak/none	> 1000	1.2		n.i.	0/3	−
86	Limonene	> 2205	V. weak/none	194.10	1.3		n.i.	0/3	−
87	Diethyl phthalate	> 4504	V. weak/none	> 1000	1.9	1000	614.4	4/5[h]	+
88	Benzaldehyde	> 2358	V. weak/none	> 1000	1.1		n.i.	0/3	−
89	1-Butanol	> 2702	V. weak/none	> 1000	1.1		n.i.	0/3	−
90	Propyl-paraben	> 2776	V. weak/none	958.96	2.4	125–250	34.8	3/4	+
91	Lactic acid	> 2777	V. weak/none	> 1000	1.1		n.i.	0/3	−
92	Salicylic acid	> 1812	V. weak/none	> 1000	1.1		n.i.	0/3	−
93	Methyl paraben	> 3289	V. weak/none	> 1000	2.5	1000	233.7	4/5[h]	+
94	Vanillin	> 3289	V. weak/none	> 1000	1.2		n.i.	0/3	−
95	4-Methoxyacetophenone	> 3333	V. weak/none	> 1000	1.7	500–1000	449.3	2/4	+/−
96	Phenyl ethyl alcohol	> 4098	V. weak/none	> 1000	1.1		n.i.	0/3	−
97	Benzyl alcohol	> 4629	V. weak/none	> 1000	1.0		n.i.	0/3	−
98	6-Methoxy-2,6-dimethyl octanal	> 5376	V. weak/none	> 1000	1.1		n.i.	0/3	−
99	Glycerol	> 10,869	V. weak/none	> 1000	1.1		n.i.	0/3	−
100	Propylene glycol	> 13,157	V. weak/none	> 1000	1.0		n.i.	0/3	−
101	Benzoic acid		Nonsensitizer	> 1000	1.0		n.i.	0/3	−
102	Benzenesulfonic acid		Nonsensitizer	> 1000	1.2		n.i.	0/3	−

[a]Sensitization class according the LLNA, only for SDS and benzocaine classification according to human data were taken.

[b]IC50 indicates concentration reducing cell viability of AREc32 by 50% after 24 h as measured with the MTT assay.

[c]I_{max}: Fold induction of luciferase activity over background, average of the maximal stimulation observed at any test concentration in the three repetitions, for chemicals for which maximal induction was observed at > 250μM, only data for repetitions including highest test concentrations were taken for average calculations.

[d]CI_{max}: Concentration for maximal stimulation, a range is given where the maximal stimulation was observed at different test concentrations in different repetitions.

[e]Average concentration inducing luciferase activity 50% over background, concentration values obtained by log-linear extrapolation.

[f]Number of repetitions with significant luciferase induction/number of repetitions made.

[g]Rating used in summary results in Table 3; + indicates that a compound was rated as ARE inducing based on evidence from all repetitions.

[h]Chemicals rated positive, but significant induction only in repetitions with maximal test concentration of 1000μM, not if tested up to 250μM only.

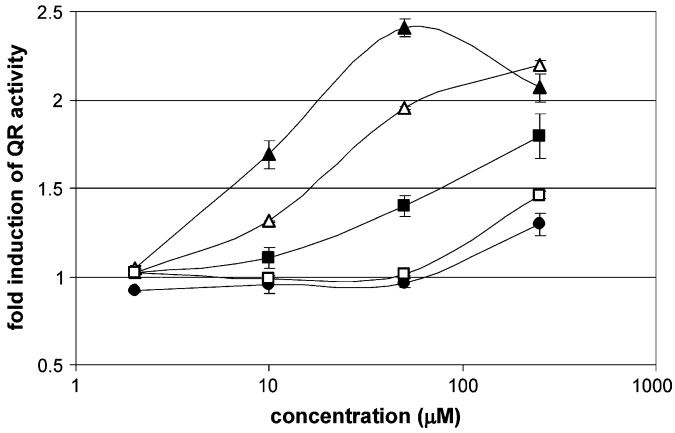

FIG. 1. Dose–response curve of QR induction in the Hep1C1C7 cell line by five fragrance chemicals with skin sensitizing potential. Given are results from one representative experiment. Note: a logarithmic scale was applied to the *x*-axis. Diethyl maleate, filled triangle; isoeugenol, open triangle; cinnamic aldehyde; filled square; 2-phenylacetaldehyde, open square; benzyl-salicylate, filled circle.

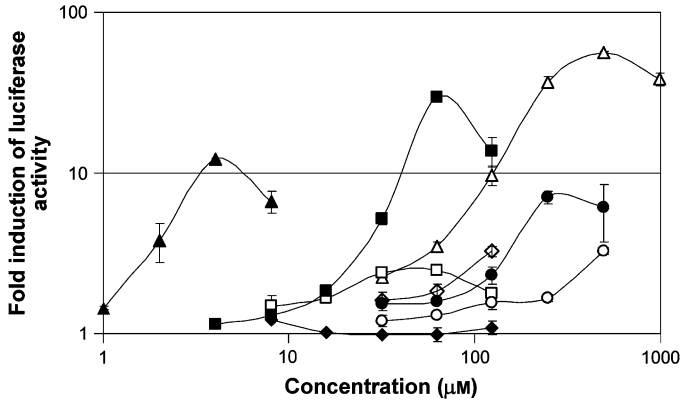

FIG. 2. Dose–response curve of ARE-dependent luciferase induction in the AREc32 cell line by some reference sensitizers and SDS. Given are results from one representative experiment, only values from noncytotoxic concentrations are shown. Note: a logarithmic scale was applied to both axes. 1-Chloro-2,4-dinitrobenzene, filled triangle; isoeugenol, open triangle; 2-phenylacetaldehyde, filled circle; lyral, open circle; glutaraldehyde, filled square; 1,4-phenylendiamine, open square; α-hexyl cinnamic aldehyde; open diamond; SDS, filled diamond.

the EC 1.5 value giving significant luciferase induction was 5- to 50-fold lower than the IC50 value. For many compounds, the luciferase induction increased in a dose-dependent manner with higher test concentrations (Fig. 2) and for many compounds this increase continued up to partly cytotoxic concentrations. Therefore, the concentration (CI_{max}) for maximal gene induction was for several compounds (e.g., compounds **3**, **5**, **7**, **18**, **25**, **28**, and **29**) at a concentration with a partial cytotoxicity. At completely cytotoxic concentrations, the luciferase expression dropped below the control levels.

TABLE 3
Summary Results for Luciferase Induction in the AREc32 Cell Line by Chemicals of Known Skin Sensitization Potential

Sensitization class	Number of chemicals	Luciferase induction above 1.5 threshold (positive)[a]	No luciferase induction (negative)
Extreme	5	5	0
Strong	10	9	1
Moderate	35	31	4
Weak	20	12	8
Very weak/none	30	4	26
Total sensitizers[b]	**70**	**57**[c]	**13**
Total nonsensitizers	**30**	**4**	**26**
compounds excluded from analysis	2		
Total compounds	102		

[a]Chemicals with significant induction of ARE-regulated luciferase activity (at least 50% above background in at least one test concentration in three out of three or three out of four repetitions, see Table 2).

[b]In bold, the summary figures used for the calculation of the cooper statistics.

[c]Cooper statistics: sensitivity 81.4%; specificity 86.6%; positive predictivity 93.4%; negative predictivity 66.6%; accuracy 83.0%.

As indicated above, tests were either run with 0.25% or 1% acetonitrile or DMSO (DMSO was only used for chemicals with poor solubility in acetonitrile). There was only a marginal difference in background luciferase activity in cells treated with 0.25% Acetonitrile (ACN) (average light emission for all experiments during 2-s integration = $3.72 \times 10^5 \pm 2 \times 10^5$ relative light units [RLU]) or 1% ACN ($3.14 \times 10^5 \pm 2 \times 10^5$ RLU). The positive control *t*-butyl-hydroquinone significantly induced luciferase activity in all assays with similar induction at the two dosing regimes: at 1% solvent and binary dilutions the average induction for *t*-butyl-hydroquinone compared with solvent control was 20.3-fold at 31μM and 41.4-fold at 62μM, whereas it was 31.5-fold at 50μM on the average in the experiments with 0.25% solvent.

Correlation of ARE Induction with EC3 Values

Based on the Cooper statistics given above, the AREc32 test appears to be an interesting approach for hazard identification, but beside hazard identification, the LLNA is also very helpful for hazard characterization. We next evaluated whether the levels of gene induction and the threshold concentrations EC1.5 can also help for hazard characterization, namely whether there is a correlation with the EC3 values derived from the LLNA.

This additional analysis was performed on all the 61 chemicals which were identified as significant inducers of ARE-dependent luciferase activity. The Spearman rank correlation was first calculated for the LLNA EC3 values against the maximal induction of luciferase activity (I_{max}): the correlation coefficient is −0.396 and the p value is 0.0016. Calculation of the Spearman correlation of the LLNA EC3 values versus the EC1.5 values from the AREc32 assays led to

a correlation coefficient of 0.562 and a p value of 0.000002. Thus, there is a significant positive correlation between LLNA EC3 values and ARE EC1.5 values, and a significant but less strong negative correlation between LLNA EC3 and maximal ARE induction.

In general, Michael acceptors would be predicted as particularly strong sensitizers with the AREc32 assay, thus introducing a certain bias when comparing all chemicals against each other. Nevertheless, within structural classes and among structurally related compounds, the ranking is even more interesting: thus, the isothiazolinones **3**, **26**, and **30** would be correctly ranked based on the EC1.5 values. Also based on EC1.5 values, the aminophenol **14** would correctly be predicted as a stronger sensitizer than the related compound **32**, isoeugenol would correctly be rated stronger than eugenol, and cinnamic aldehyde stronger than α-methyl-cinnamic aldehyde and α-hexyl-cinnamic aldehyde. Based on I_{max}, for example, 1-phenyl-1,2-propanedione would be rightly rated stronger than 2,3-butanedione.

DISCUSSION

This study was based on the following hypothesis: if a cellular model should be able to respond to a wide variety of structurally diverse skin sensitizers and yield a homogenous biological readout useful for the development of a predictive *in vitro* assay, then the cellular model must somehow be able to detect the reactivity of the diverse sensitizing chemicals toward proteins, because this is, to the current knowledge, the only feature all skin sensitizers have in common (Smith and Hotchkiss, 2001). Indeed, a cellular regulatory pathway, which responds to electrophiles, has been identified (Dinkova-Kostova, *et al*., 2005; Wakabayashi *et al*., 2004). We therefore investigated this regulatory pathway as a potential screening target to predict skin sensitizers *in vitro*. The results of our study clearly show that the majority of the skin sensitizers indeed activate this pathway. This activation occurs at subcytotoxic doses (in most cases the EC1.5 value for significant ARE induction is 5- to 50-fold lower than the IC50 concentration reducing cell viability by 50%; see Table 2). This is particularly interesting for *in vitro* testing: most currently developed *in vitro* tests for sensitizers rely on testing compounds at partly cytotoxic concentrations (Coquette *et al*., 2003; Hulette *et al*., 2005) and thus test conditions which may make differentiation between an unspecific cellular stress reaction on the one hand and a more specific reaction to the sensitizing properties of chemicals rather difficult. Indeed, Hulette *et al*. (2005) had shown that around partly cytotoxic concentrations also the irritant SDS induces the cellular marker CD86 in many cases.

Most of the molecules reported in the literature as activators of ARE-dependent genes are α,β-unsaturated carbonyl compounds (Michael acceptors), isothiocyanates and oxidizable diphenols (Dinkova-Kostova *et al*., 2005). The results of our study show that indeed many skin sensitizers belonging to these structural classes do induce this regulatory pathway. However, further skin sensitizers from other structural classes did activate the ARE-dependent luciferase activity (e.g., several aldehydes, amines, α-diketones), indicating that the method is even more broadly applicable and gives an even better sensitivity than initially expected. Particularly interesting in this respect are for example compounds such as formaldehyde, glutaraldehyde, eugenol, isoeugenol, lyral, 2-phenyl-propionic aldehyde, phenylacetaldehyde, 2,3-butanedione, and 1-phenyl-1,2-propanedione. All these skin sensitizers induced ARE-dependent gene expression, although they are, to the current knowledge, not typical ARE-inducers based on their structure, and many of them are only weak to moderate allergens which may be difficult to predict with other cell-based *in vitro* assays. Overall, the results of this study therefore show a good positive predictivity and a high sensitivity.

Among the false negatives are several aldehydes with no α,β-unsaturation. However, for this class of aldehydes accurate mathematical models have been described to rate their sensitization potential (Roberts *et al*., 2006) and such modeling could complement the proposed *in vitro* assay. Another false negative is phthalic anhydride. This strong sensitizer has been shown to deplete a Lys-peptide exclusively, and not to react with a Cys-peptide (Gerberick *et al*., 2007b). Its specificity to NH_2 groups could be the reason why it does not react with KeapI and therefore does not activate ARE-dependent gene activity. For another structural class, LLNA data were not correctly predicted: the macrocyclic and linear musks **55**, **62**, and **65** are weak sensitizers according to the LLNA, but no human sensitization to this widely used class of fragrance compounds has been recorded, and they have no structural alert. Given their relatively high EC3 values, they could therefore rather be false positives in the LLNA, which might be due to an irritation rather than a sensitization reaction. The result for SDS is also interesting in this context: this nonsensitizing skin irritant is known to be false positive in the LLNA (Basketter *et al*., 2006), but it would correctly be classified as nonsensitizer in an ARE-based assay.

Any false-positive LLNA result in the set of test chemicals reduces the measured sensitivity of the assay. In the future, it will be important to use an official reference list of chemicals for validating *in vitro* skin sensitization methods, which does not contain compounds with such putative false-negative results, in order to correctly assess the sensitivity and the prediction power of the method. As currently no accepted list of test chemicals for assay validation is available, we included a large series of fragrance molecules, preservatives, and other cosmetic ingredients in our test set, as the most critical requirement for *in vitro* tests is detection of potential sensitizers among these chemical groups which are regularly applied topically to the skin in the general population. In the published data sets (Gerberick *et al*., 2004a, 2005), there are many LLNA

data for halogenated and/or alkylating compounds. These compounds are mainly used as industrial intermediates, and in the general population exposure of the skin to these chemicals is very low. Therefore, we included only few representatives of these chemical classes.

The specificity of the method was very good. Among the 30 negatives in the LLNA included in the study, only methyl- and propyl-paraben, diethyl phthalate, and 6-methyl-coumarine slightly but significantly induced ARE-dependent gene activity. Methyl- and propyl-paraben are negative in the LLNA, but these compounds are well documented but rare sensitizers in humans (0.6% of dermatitis patients are sensitive; Basketter *et al*., 2006). Thus, their current classification as false positives in the ARE assay is not absolute. Diethyl phthalate was repeatedly positive in the ARE-based assay, however, significant induction was observed only at a test concentration of 1000μM.

The observed low incidence of true false positives (i.e., the high specificity of the assays) both when screening for QR induction and when screening for ARE-dependent luciferase activity is critical if this or a related *in vitro* assays would be integrated in a scheme with a battery of assays used in parallel as proposed by Jowsey *et al*. (2006): with such parallel testing in multiple assays, each test may have an overlapping applicability domain to identify sensitizers. The sum of positive test results should then cover all important classes of sensitizers, but at the same time each assay should only yield no or only very few false positives, as otherwise, the sum of the false positives finally may rate almost any chemical a skin sensitizer.

Besides identifying skin sensitizers (hazard identification), the LLNA provides a measure of potency of sensitizers, which is critical for risk assessment and for determining appropriate use levels of novel compounds especially in cosmetic applications. *In vitro* assays proposed as replacements of the LLNA should therefore be able to give also a measure of potency. The current data show that most strong and extreme sensitizers gave higher induction of ARE activity at lower concentration as compared with the weak sensitizers, and Spearman rank correlation analysis showed a highly significant association of high I_{max} and low EC1.5 values from the AREc32 cell–based assay with the low EC3 valued from the LLNA. However, the correlation is not yet sufficiently accurate to use this test as stand-alone test to directly rate the sensitizers. Particularly of interest will be combination of the data with results from the peptide reactivity assay proposed by Gerberick *et al*. (2004, 2007b), which is currently the most advanced test with most available *in vitro* data. Inspecting the published peptide reactivity data (Gerberick *et al*., 2004b, 2007b; Natsch *et al*., 2007), it is evident many chemicals would be rated as sensitizers by both assays. However, the current assay could correctly classify the following sensitizers, which were nonreactive in the peptide-binding assay: α-hexyl-cinnamic aldehyde, 3-aminophenol, and benzyl-benzoate. On the other hand, phthalic anhydride and oxazolone are examples of compounds which are better predicted in the peptide reactivity assay then in the ARE-based assay. Integrating the data from the current work with both the published peptide reactivity data and predicted skin penetration rates will thus be the subject of our future research.

It has often been mentioned that a metabolic component is critical for sensitizer assays (e.g., Bergström *et al*., 2007) because many sensitizers are believed not to be reactive *per se*, but become reactive after metabolic activation in the skin (prohaptens). Because the target metabolites (i.e., the true sensitizers) are reactive, their isolation from a metabolic system is often difficult if not impossible, but if they are formed *in situ* within a reporter cell line, also short-lived metabolites may immediately react with KeapI and give a positive response without the need of metabolite isolation and subsequent reactivity determination. Chemicals which are considered prohaptens, and which were rated positive by the AREc32 assay included eugenol, isoeugenol, 1-naphtol, 2-aminophenol, and dihydroeugenol, and it is possible that these chemicals were activated by metabolic enzymes in the cell prior to modification of KeapI. In this respect the results of 2-hexenol, geraniol, and cinnamic alcohol are particularly interesting: these are classical prohaptens and it is assumed that geraniol and cinnamic alcohol are transformed in the skin to the corresponding aldehydes. These compounds were highly active in the Hepa1C1C7 model, but not or only weakly in the AREc32 cell line. This could be due to enhanced levels of aldehyde dehydrogenase in the liver cell line.

There are interesting connections between the results of this study and earlier results with cell-based assays for the *in vitro* identification of sensitizers: interleukin-8 (IL-8) levels were found to be elevated in reconstituted skin after challenge with strong sensitizers (Coquette *et al*., 2003), and a high ratio of IL-8/IL-1α was indicative of sensitization. We were able to reproduce this effect in keratinocyte cultures with several moderate and strong sensitizers (unpublished results). Aeby *et al*. (2004) and Bergström *et al*. (2007) reported enhanced levels of IL-8 messenger RNA (mRNA) in sensitizer-treated dendritic cells. In parallel, in the gene-chip study of Ryan *et al*. (2004), IL-8 mRNA was found to be increased by a sensitizer both when detected with gene-chip and RT-PCR analysis. Thus, it was repeatedly found that IL-8 expression is induced by sensitizers and interestingly, IL-8 formation has recently been shown to be under the control of Nrf2 (Zhang *et al*., 2005). Another gene, whose enhanced expression was identified by the gene-chip based screening and RT-PCR (Gildea *et al*., 2006; Ryan *et al*., 2004) as a robust marker for sensitizers is AKR1C2 coding for an aldo–keto reductase. Interestingly, this gene also contains an ARE sequence in its promoter (Lou *et al*., 2006). Finally, the genes coding for thioredoxin and thioredoxin reductase I were significantly upregulated by a sensitizer in the study of Ryan *et al*. (2004), and these genes where shown to be under the control of Nrf2 in

a gene-chip study comparing expression changes after addition of an ARE inducer to wild-type and Nrf2 deficient mice (Kwak *et al*., 2003). Therefore, several of the earlier identified cellular markers responding to sensitizer challenge are indeed under control of the Keap1/Nrf2/ARE pathway.

The Keap1/Nrf2/ARE pathway is mainly known as the target of many "chemo-preventive" constituents in food, as its induction leads to upregulation of detoxifying genes (Dinkova-Kostova *et al*., 2005; Wolf, 2001). The broad ability of sensitizers to induce this pathway indicates that sensitizers may also induce cellular defense mechanisms. This may be a reason that, despite the fact that many sensitizers are routinely used in topical applications, sensitization reactions only occur in a small proportion of the human population.

A wide variety of sensitizers induces ARE-dependent genes, yet a key final question remains: is this pathway only a useful tool for *in vitro* screening, or does it play a role in the sensitization process *in vivo*? In other words, what is the relevance of the results of this study for the skin sensitization process and is emigration of dendritic cells from skin in the sensitization phase indeed regulated by activation of this pathway? Certainly, further studies are needed to address this question, but one possible link is already emerging: activation and migration of dendritic cells involves induction of the expression of the chemokine receptor CCR7, a process which is under the control of mitogen-activated proteins kinases (MAPKs, Boisleve *et al*., 2004, 2005). Interestingly, induction of ARE-dependent genes by a variety of electrophiles has also been shown to be dependent on different MAPKs (Yeh and Yen, 2005, 2006; Yuan *et al*., 2006), and inhibitors of these kinases blocked Nrf2 translocation and Nrf2 induced gene expression. Thus, there is a crosstalk between these regulatory pathways which deserves further attention in the context of a mechanistic understanding of the induction phase of skin sensitization. It has also been shown, that polycyclic aromatic hydrocarbons activate macrophages, and that both ARE-activation and activation of MAPKs are involved in this process (Ng *et al*., 1998). Finally, although the presence of the KeapI–Nrf2 pathway has not yet been investigated in Langerhans cells, it was widely studied in cells of the monocytic lineage, both in peripheral blood derived mononuclear cells and in the monocytic cell line THP-1 (Rushworth *et al*., 2006). Interestingly, in THP-1 cells it is induced by the well-known contact allergen Ni(II) (Lewis *et al*., 2006).

If Nrf2 activation was indeed needed for Langerhans cell activation, Nrf2 knockout mice would be less sensitive to sensitizers or at least the migration of Langerhans cells after hapten painting of the skin would be reduced. If, on the other hand, the protective effect of the phase II gene products induced by this pathway is of higher importance, Nrf2 knockout mice may also have enhanced sensitivity for sensitizers as they cannot detoxify sensitizers efficiently. Thus, comparing the potency of a model sensitizer on wild-type and Nrf2 deficient mice might be an interesting area of further research to find out whether the observed ARE-dependent gene activation by sensitizers is more relevant for the active induction of the sensitization process or rather for the induced detoxification of these chemicals.

SUPPLEMENTARY DATA

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

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