



A new in vitro method for identifying chemical sensitizers combining peptide binding with ARE/EpRE-mediated gene expression in human skin cells

James M. McKim Jr., Don J. Keller III & Joel R. Gorski

To cite this article: James M. McKim Jr., Don J. Keller III & Joel R. Gorski (2010) A new in vitro method for identifying chemical sensitizers combining peptide binding with ARE/EpRE-mediated gene expression in human skin cells, Cutaneous and Ocular Toxicology, 29:3, 171-192, DOI: [10.3109/15569527.2010.483869](https://doi.org/10.3109/15569527.2010.483869)

To link to this article: <http://dx.doi.org/10.3109/15569527.2010.483869>



Published online: 24 May 2010.



Submit your article to this journal [↗](#)



Article views: 287



View related articles [↗](#)



Citing articles: 37 View citing articles [↗](#)

RESEARCH ARTICLE

A new *in vitro* method for identifying chemical sensitizers combining peptide binding with ARE/EpRE-mediated gene expression in human skin cells

James M. McKim, Jr.¹, Don J. Keller, III¹, and Joel R. Gorski²

¹CeeTox Inc., Kalamazoo, Michigan, USA, and ²NAMSA (North American Science Associates, Inc.), Northwood, Ohio, USA

Abstract

Allergic contact dermatitis (ACD) is a significant safety concern for developers of cosmetic, personal care, chemical, pharmaceutical, and medical device products. The guinea pig maximization test (GMPT) and the murine local lymph node assay (LLNA) are accepted methods for determining chemical sensitization. Recent legislative initiatives in Europe require the development of new *in vitro* alternatives to animal tests for chemical sensitization. The aim of this project was to develop an *in vitro* screening method that uses a human skin cell line (HaCaT), chemical reactivity, and gene expression profiling to identify positive and negative responses, to place chemicals into potency categories of extreme/strong (ES), moderate (M), weak (W), and nonsensitizers (N), and to provide an estimate of corresponding LLNA values. The method and processing algorithm were developed from a training set of 39 chemicals possessing a wide range of sensitization potencies. Three cationic metals, chromium (Cr), nickel (Ni), and silver (Ag), were also evaluated in this model. Chemical reactivity was determined by measuring glutathione (GSH) depletion in a cell free matrix. Three signaling pathways (Keap1/Nrf2/ARE/EpRE, ARNT/AhR/XRE, and Nrf1/MTF/MRE) that are known to be activated by sensitizing agents were monitored by measuring the relative abundance of 11 genes whose expression is controlled by one of these 3 pathways. Final exposure concentrations were based on toxicity and solubility. A range-finding experiment was conducted with each compound to determine cytotoxicity and solubility. Six exposure concentrations (0.1 to 2,500 μ M) and an exposure time of 24 hours were used in the final experiments. Glutathione depletion alone did not provide the accuracy necessary to differentiate potency categories. However, chemical reactivity combined with gene expression profiles significantly improved the *in vitro* predictions. A predicted toxicity index (PTI) was determined for each test chemical. A comparison of LLNA values with PTI values revealed an inverse relationship. The large variation in LLNA data for compounds in the same potency category makes direct extrapolation from PTI to LLNA difficult. To challenge the system, 58 additional compounds were submitted in a blinded manner. Compounds placed into ES and M categories were considered positive, whereas compounds classified as W or N were considered negative. Accuracy was approximately 84%, with a sensitivity of 81% and a specificity of 92%. The model correctly identified 2 of 3 cationic metals as positive. In conclusion, the method described here demonstrates a valuable *in vitro* method for identifying chemicals and metals that induce skin sensitization.

Keywords: Skin sensitization; *in vitro* methods; nonanimal methods; safety testing; chemicals; peptide binding; gene expression

Introduction

The potential for a novel chemical to cause allergic contact dermatitis (ACD) through the process known as chemical sensitization is a significant concern for

companies that are developing new compounds to be used in cosmetic, personal care, chemical, pharmaceutical, and medical device products. In Europe, 2 legislative initiatives make it necessary to begin identifying nonanimal methods for safety testing. The

Address for Correspondence: James M. McKim, Jr., PhD, CeeTox, Inc., 4717 Campus Drive, Kalamazoo, MI 49008, USA. Phone: 269-372-3351; E-mail: jmckim@ceetox.com

(Received 06 April 2010)

ISSN 1556-9527 print/ISSN 1556-9535 online © 2010 Informa UK Ltd
DOI: 10.3109/15569527.2010.483869

<http://www.informahealthcare.com/cot>

first involves the testing required for the registration, evaluation, authorization, and restriction of chemicals (REACH), and the second is the seventh amendment to the cosmetics directive, which provides for a ban on the use of animals to identify skin sensitizers by 2013 (1). These events are driving the development of new *in vitro* methods that can characterize the safety of new products. *In vitro* cell-based models may provide a rapid, inexpensive, and robust means of identifying chemical sensitizers. The ultimate success of an *in vitro* approach to predict *in vivo* effects is dependent on how closely the cell model and the endpoints measured mimic the *in vivo* route of exposure, target organ, and the molecular mechanisms involved with the response.

Allergic contact dermatitis, or chemical hypersensitivity, is the result of sensitization and requires an intact immune system to occur. Key events in this process include haptenation and the stimulation of lymphocyte proliferation (2). These events occur in 2 phases, induction and elicitation. Induction involves an initial exposure of the chemical or hapten to the skin, protein binding, transport to the local lymph nodes, and proliferation of lymphocytes. Chemicals that possess electrophilic centers or that can be metabolically changed to electrophiles are typically more potent allergens than chemicals without these properties. Electrophilicity (reactivity) is the basis for the peptide binding assay for detection of chemical sensitizers described by Gerberick and colleagues (3,4).

Once the sensitizing chemical binds to a protein carrier, the dendritic cells of the skin, known as Langerhans cells (LCs), take up the protein-chemical complex and process it. The LCs then transport the complex to local draining lymph nodes. During transport, the LCs undergo maturation processes that produce a change in gene expression profiles (5). Interactions between the loaded dendritic cells and T-lymphocytes stimulate proliferation and maturation of the cells into primed effector T-lymphocytes. Elicitation occurs when the primed T-cells mount an exaggerated response upon subsequent exposure to the sensitizing agent (2,6).

Although the guinea pig maximization test (GPMT) is accepted and widely used, the current model of choice for evaluating sensitization properties is the murine local lymph node assay (LLNA). The LLNA is quantitative, and it is less expensive and uses fewer animals than the GPMT. The LLNA has been shown to be predictive for the identification of skin-sensitizing chemicals and metals (2,4,7,8). The LLNA measures events that occur following an initial exposure to the test chemical and has been an important test used by many companies to identify skin sensitizers (2,4).

Most *in vitro* methods for detecting chemical sensitizers have focused on biochemical and molecular changes that occur in the cells during the sensitizing or induction phase. In order for a chemical to induce a true contact sensitization reaction, it must penetrate the skin, bind to proteins, and be recognized as an antigen by the immune system (3). There is a strong correlation between chemical reactivity and skin sensitization (3,9–11). In 1936, Landsteiner and Jacobs first presented the idea that if a chemical is capable of binding to cellular proteins, then it also has the potential of causing ACD (9). The relationship between chemical reactivity and protein binding led to the development of an *in vitro* screening model that identifies peptide reactivity as a means of predicting contact allergens (3,4,12).

Many biochemical processes occur in keratinocytes exposed to chemical sensitizers. One of these processes is activation of the signaling pathway that controls the expression of several cytoprotective genes through the antioxidant/electrophilic response element ARE/EpRE (13). This unique cytoprotective pathway was discovered and characterized during research designed to understand polycyclic aromatic hydrocarbon (PAH) toxicity (14,15). PAHs induce cytochrome P450 (CYP)1A genes via interaction with ARNT/AhR/XRE, and are metabolized to highly reactive intermediates, which then interact with the ARE/EpRE pathway to induce the expression of several cytoprotective proteins (16–18). The ARE/EpRE signaling pathway and the transcription factors that control it have been described as an intrinsic sensor for electrophiles (Figure 2). The link between chemical sensitizers and induction of genes controlled by the ARE/EpRE signaling pathway is based on the knowledge that most chemicals classified as sensitizers are also reactive. These reactive compounds (electrophiles) bind to the inhibitory factor known as Kelch-like ECH-associated protein 1 (Keap1). Nrf2 is a nuclear transcription factor that activates the ARE/EpRE pathway. Under normal conditions, Keap1 binds Nrf2 and targets it for proteosomal degradation (19,20). However, when reactive compounds bind Keap1, it is displaced from Nrf2, which allows Nrf2 to accumulate in the nucleus, where it forms a heterodimer with other basic leucine zipper proteins (bZIPs) before binding to the ARE/EpRE motif. The association of Nrf2 with the ARE/EpRE induces the expression of cytoprotective phase 2 genes, such as nicotinamide adenine dinucleotide phosphate oxidase (NADPH)-quinone oxidoreductase 1 (NQO1), aldo-ketoreductase (AKR1C2), glutathione S-transferase (GST), and glutamate cysteine ligase catalytic unit (GCLC) (13,14,21,22).

Natsch and Emter recognized that chemical sensitizers might be identified based on their ability to

A. Cell viability Assay (employing tetrazolium salt [MTT] method)

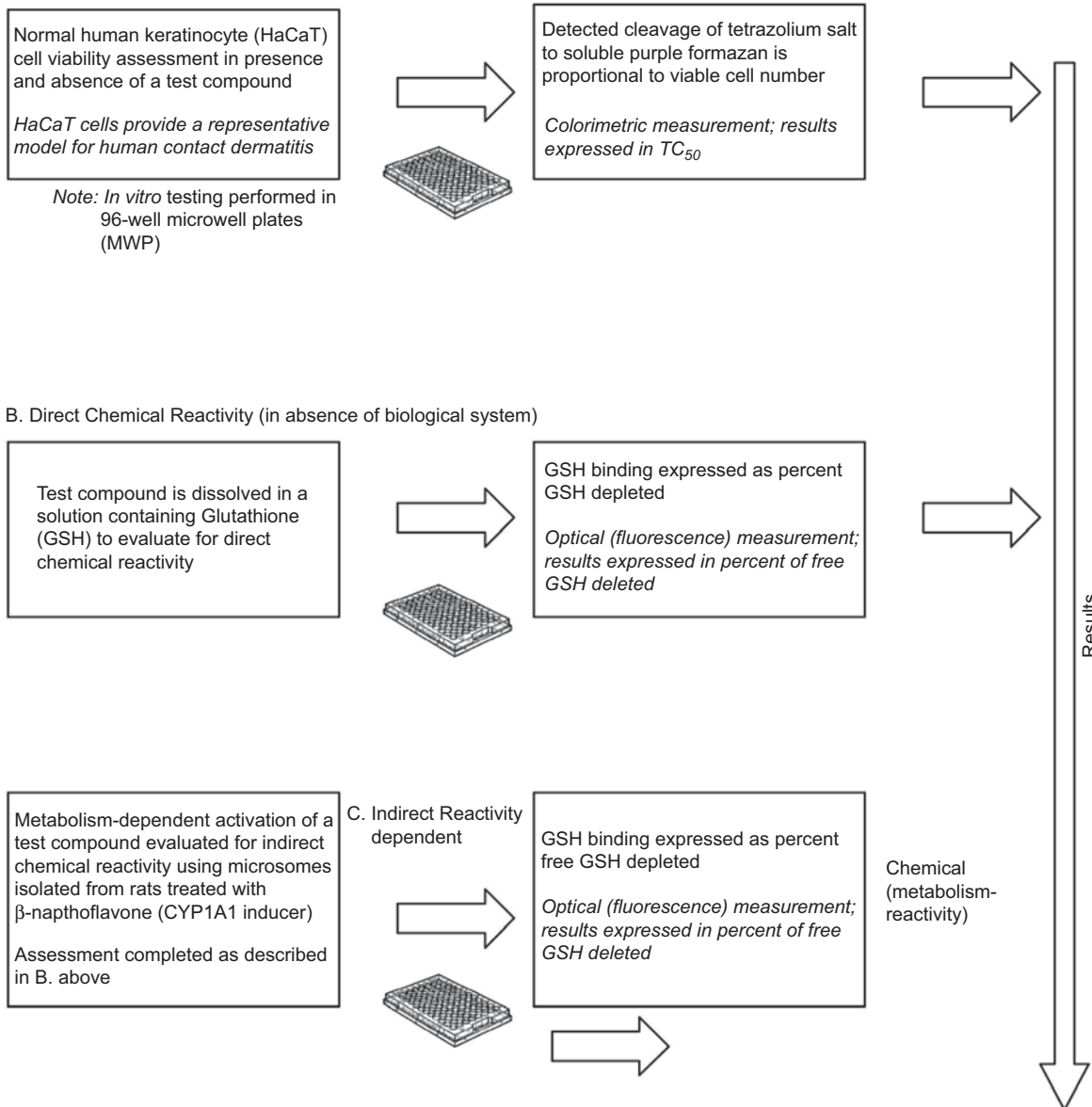


Figure 1. Overview of the CeeTox *in vitro* method for identifying chemical sensitizers. MTT=3-[4,5-dimethylthiazol-2-yl] 2,5diphenyltetrazolium bromide; TC_{50} =concentration that resulted in a viability of 50%; GSH=glutathione; CYP=cytochrome P450; CCD=charge-coupled device; qRT-PCR=quantitative real-time polymerase chain reaction.

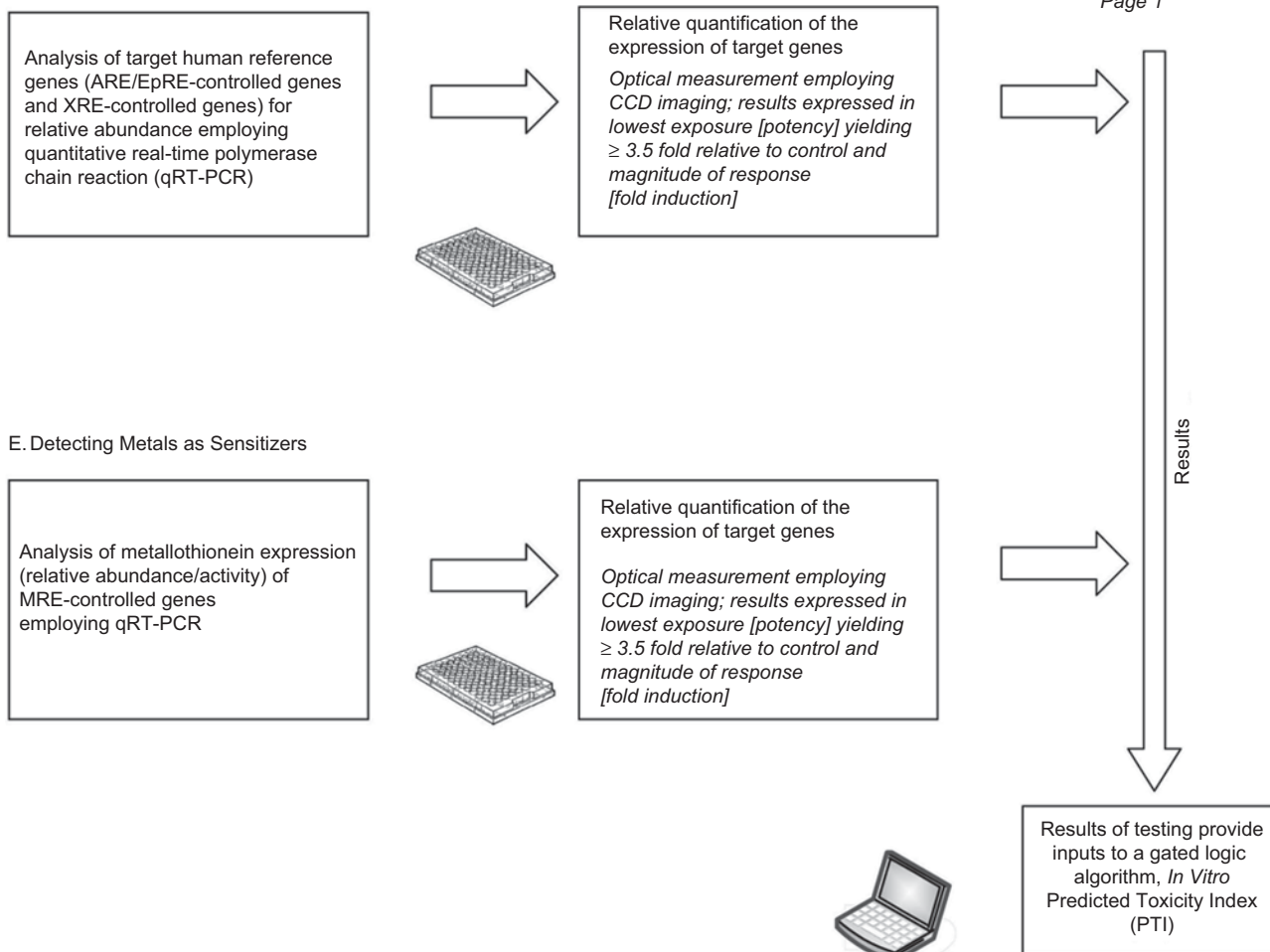
Figure 1. continued on next page

induce genes controlled by the Keap1/Nrf2/ARE/EpRE signaling pathway (23). They proposed an *in vitro* screening model and tested this with 2 cell types. The first was the mouse hepatoma (Hepa1c1c7) cell line in which 96 chemicals with varying potency for sensitization were tested by measuring the expression of a single ARE/EpRE-controlled gene (NQO1). The results showed high specificity, but low sensitivity, and the system could not be used to separate chemicals based on potency. The second cell model was derived from the human mammary carcinoma

cell line (MCF-7) by stably transfecting into the cell multiple copies of the ARE promoter. The resultant AREc32 cell line was used to test 102 chemicals for sensitization. This model yielded better statistical results with both sensitivity and specificity greater than 80%. Some disadvantages of this model include the use of nonskin cells, the role of metabolic activation, the use of a single gene marker, the inability to predict potency, and the use of a culture system that requires a high degree of solubility in order to achieve adequate tissue exposure.

Figure 1. Continued.

D. Measuring Gene Expression



PTI Score is the sum of the weighted values determined values for viability, reactivity and gene expression

Data collected for each compound include:

1. Viability
2. Direct reactivity
3. Metabolism-dependent reactivity
4. ARE/EpRE-controlled genes
5. XRE-controlled genes
6. MRE-controlled genes
7. Concentration-response
8. Magnitude of response

Biochemical events that occur in response to dermal contact with chemical sensitizers can also be identified and measured in dendritic cells. In one study by Hooyberghs et al., CD34+ progenitor-derived dendritic cells from human cord blood were used as the test system (24). Sensitization was determined by analyzing the expression of 13 marker genes. In this study, 10 sensitizers and 11 nonsensitizers were tested under 9 different exposure scenarios. Subsequent analysis of the data revealed a strong concordance between the *in vitro* method and *in vivo* data. The maturation of dendritic cells during the induction phase requires the release of cytokines. Trompezinski

et al. evaluated the expression of inflammatory cytokines and measured the maturation of dendritic cells isolated from human monocytes (25). Although promising, the use of dendritic cells has several disadvantages, including donor variation and difficulty in isolation and culture. This model also appeared to lack the resolution necessary to assign potency categories for chemical sensitizers.

The aim of the present study was to develop an improved *in vitro* method of identifying chemicals that are skin sensitizers by combining chemical reactivity, cytotoxicity, concentration-response, gene expression, and a gated algorithm for processing

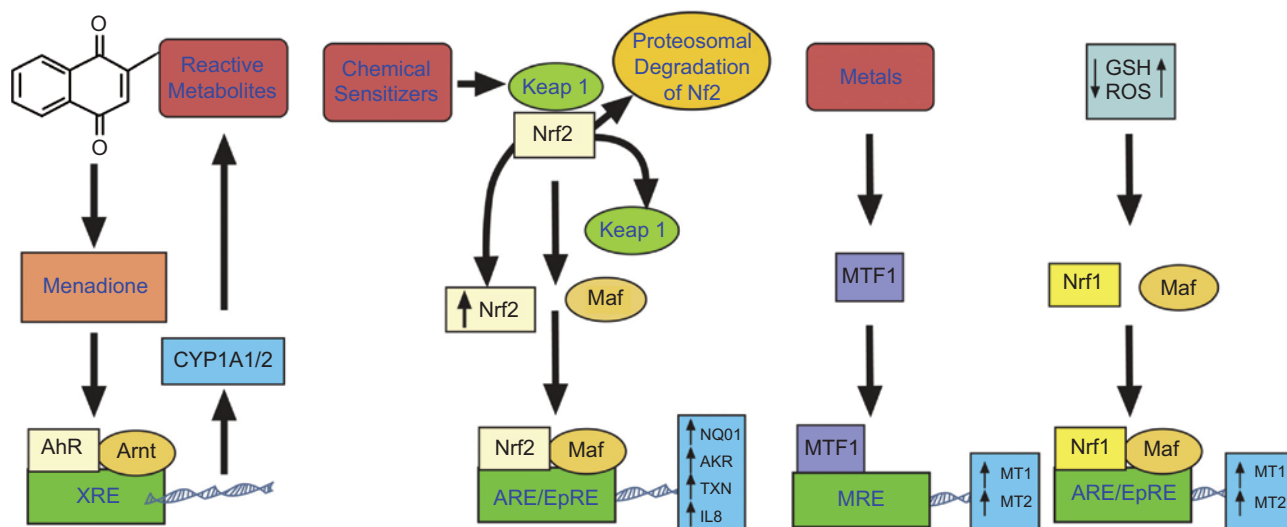


Figure 2. Three signaling pathways associated with sensitization. Chemicals that cause allergic contact dermatitis are in a class known as sensitizers. A key step in the sensitization process is the binding of the chemical hapten to cellular protein. Chemicals can possess direct reactivity or they can be metabolized to reactive intermediates. An important enzyme in this metabolism is cytochrome P450 (CYP)1A1. Compounds that possess planar cyclic structures can bind to the cytosolic receptor AhR, which in turn binds to ARNT. This complex can then bind deoxyribonucleic acid (DNA) at the XRE site to induce the expression of CYP1A1. The biochemical mechanisms involved with chemical sensitization include Keap1/Nrf2/ARE/EpRE. Keap1 bound to Nrf2 targets Nrf2 for degradation by proteasomes. Electrophiles or reactive oxygen species can bind to Keap1 and displace or prevent them from binding to Nrf2. The result is a buildup of Nrf2 in cytosol and movement to the nucleus where it activates the ARE/EpRE promoter and the induction of several cytoprotective genes. Small and large MafF proteins form dimers with Nrf2 and enable binding to DNA. Some Maf proteins, such as MafK, are stimulatory, whereas others prevent the activation of ARE-mediated expression. Several metal cations are also sensitizers. Metallothionein (MT) proteins 1 and 2 can be induced by oxidative stress and by metals. The transcription factor (MTF1) can be activated by metals and Nrf1 can also be activated to induce MT. Induction of MT may therefore indicate the presence of metal cations and potential metal sensitizers.

these data (Figure 1). The information obtained would not only identify positive and negative compounds, but also group them by potency category. The system was initially established with a set of 39 chemicals with varying sensitization potencies, and then challenged with a unique set of 58 additional chemicals. A total of 97 chemicals were evaluated in this model.

Materials and methods

Chemicals

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the QuantiTect SYBR Green RT-PCR and QuantiTect Primer assays from QIAGEN (Germantown, MD, USA). Ribonucleic acid (RNA) isolation was done using QIAGEN's RNeasy-96 kit along with QIAGEN's RNase-free DNase set. Triethanolamine (TEAM), dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl] 2,5diphenyltetrazolium bromide (MTT), 5,5'-dithiobis(2-nitro-benzoic acid) (DTNB), glutathione (GSH), and yeast GSH reductase were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). Cell culture reagents, Dulbecco's

modified eagle media (DMEM) and 100x penicillin/streptomycin, were purchased from Sigma-Aldrich Corporation. Human keratinocyte (HaCaT) cells were obtained from Amara Biosystems GmbH (Cologne, Germany). NADPH, tetrazolium salt, was obtained from Calbiochem (Gibbstown, NJ, USA).

Direct chemical reactivity

The chemical reactivity of each test compound in the absence of a biological system was assessed by measuring GSH binding. A stock solution (500 mM) of each test compound was prepared in DMSO. The final reaction mixture consisted of the following at the final concentrations indicated: Sodium phosphate (20 mM), pH 7.4, test compound (20 mM), and GSH (100 μ M) were prepared in black 96-well micro plates with each compound run in triplicate. The reactions were incubated for 24 hours at room temperature on a plate shaker essentially as described by Gerberick et al. (3). After 24 hours, metaphosphoric acid (MPA) was added to a final concentration of 2.8% in each well and the plate was shaken for 2 minutes at room temperature. Next, 50 μ L of reaction mix was removed and added to a clear 96-well plate along with 10 μ L

of 1.5 M TEAM. After 2 minutes of additional shaking, 150 μ L of GSH assay buffer (0.7 mM DTNB, 0.25 mM NADPH, and 0.6 U/mL GSH reductase) was added to the wells. The plates were shaken for an additional 2 minutes and then read using a Packard α HT Fusion plate reader at 415 nm as described by Griffith et al. (26). The mean absorbance for the test compound, corrected for background control values, was divided by the mean absorbance of the vehicle controls to obtain the percentage of free GSH depleted.

Indirect chemical reactivity

Metabolism-dependent activation of a test chemical was evaluated with microsomes isolated from rats that had been treated with naphthoflavone, a potent CYP1A1 inducer (XenoTech, Lenexa, KS, USA). The final reaction buffer contained the following at the final concentrations indicated: sodium phosphate buffer (20 mM), pH 7.4, test chemical (100 μ M, final DMSO at 0.1%), microsomes (0.1 mg/mL), NADPH (1 mM), and GSH (0.05 μ M). The reactions were carried out at 37°C for 24 hours. Quantitation of reactivity was done according to the method of Griffith et al. (26) as described above, and the percentage depletion of GSH relative to control was calculated.

Cells

The HaCaT cells were cultured in DMEM supplemented with 1% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin). Cells were grown at 37°C under 5% carbon dioxide (CO_2) with humidity (27). The cells were seeded into 96-well culture plates at a density of 12,000 cells per well in 100 μ L of culture medium and allowed to equilibrate for 48 hours. Following the equilibration period, the medium was removed and replaced with medium containing the test compounds. Following a 24-hour exposure period, the cells were harvested and processed for analysis.

Preparing test chemicals for exposure to cells

Compound names, Chemical Abstracts Service (CAS) numbers, and sources are listed in Table 2. For each chemical, a stock solution of 500 mM was prepared in DMSO on the day of use. Stock solutions were serially diluted in DMSO to obtain 200x final stock. This stock solution was used to prepare the targeted exposure concentrations in culture media. The final DMSO concentration was 0.5%.

Solubility

Compound solubility was checked by adding 150 μ L of the final dosing solution in culture media to clear, flat-bottom 96-well culture plates. Light scattering in the solutions was measured using a Nepheloskan Ascent nephelometer (LabSystems, Helsinki, Finland). Compounds with a scattering greater than 3 times that of vehicle control were considered insoluble. The plate was incubated for 24 hours at 37°C with 5% CO_2 . The solubility was again tested to ensure that solubility was stable over the 24-hour exposure period.

MTT viability assay

Viability of control and treated cells was assessed using the MTT assay. Briefly, MTT was dissolved in complete HaCaT medium to a concentration of 0.5 mg/mL. The MTT assay was performed as described previously (28). Digitonin (1 mM) was used as a positive control for maximal cell death. Final absorbance values were determined at 570 nm and 650 nm with a Packard α HT Fusion plate reader.

RNA isolation

Ribonucleic acid was isolated using QIAGEN's RNeasy 96 kit combined with the vacuum/centrifugation protocol as described by the manufacturer. The final RNA preparation was quantified using a NanoDrop ND-8000 spectrophotometer (Wilmington, DE, USA). The concentration of eluted RNA varied from 5 to 20 ng/ μ L with an A_{260} to A_{280} ratio of greater than 1.6.

Measuring gene expression

Selection of control genes was done through a series of experiments in which cells were treated with media only and with media + 1% DMSO (29,30). First, RNA isolated from treated HaCaT cells was run on a Human Reference Gene Panel, 96 (Roche Applied Science, Mannheim, Germany). The Human Reference Gene Panel has 19 human reference genes. Genes that showed less than 2-fold change in their relative abundance were considered acceptable to use as control genes. Four housekeeping genes were selected and included with sample runs to normalize gene induction. QuantiTect Primer assays for the responding genes were obtained from QIAGEN. The candidate genes were run using the primer assays and QIAGEN's QuantiTect SYBR Green qRT-PCR kit. Results from the RT-PCR reaction using both media-only and

Table 1. Chemicals used to develop the predictive toxicity index (PTI).

Compound	MTT Assay			Predicted Toxicity Index (PTI)										
	Viability (TC50uM)	% GSH Depleted	NQO1	AKRIC2	TXN	IL8	CYP1A1	ALDH3A	HMOX	MAFF	GCLC	MT1A	MT2A	Index (PTI)
Diphenylcyclopropenone	10	44	NC	NC	"++"	NC	NC	NC	"+++++"	"+++++"	NC	"+"	"++++"	8
5-Chloro-2-methyl-4-isothiazolin-3-one	20	10	"+"	NC	NC	"++"	NC	NC	NC	NC	NC	NC	NC	5
p-Benzoquinone	200	70	"+++"	"++++"	NC	NC	"++++"	"++++"	NC	"++"	NC	NC	NC	7
1-Chloro-2,4-dinitrobenzene	20	75	"++++"	"++"	NC	"+"	"++"	NC	NC	"++"	NC	NC	"++"	8
Formaldehyde	2500	81	NC	"++"	"+"	NC	"++"	NC	NC	NC	"++++"	NC	NC	6
Metol	100	73	NC	"++++"	"+"	"++"	"++++"	"++++"	"++++"	NC	NC	NC	"+"	7
Fluorescein-5-isothiocyanate	1000	ND	"+++"	"++++"	"++++"	NC	"++++"	"++++"	NC	"++++"	NC	NC	"+"	4
1,4-Phenylendiamine	1000	48	NC	"++++"	NC	"++++"	NC	NC	"++++"	NC	NC	NC	NC	6
Propyl gallate	500	63	NC	"++++"	NC	NC	"++++"	"+"	"++++"	"++++"	NC	NC	NC	8
2-Aminophenol	1000	12	"+"	"++++"	NC	"++++"	"++++"	"++++"	"++++"	NC	NC	NC	NC	7
Phthalic anhydride	2500	88	NC	NC	NC	NC	"++"	NC	NC	"++"	NC	NC	NC	6
trans-2-Hexenal	500	89	NC	"++++"	NC	NC	"++"	NC	"++++"	"++"	NC	NC	NC	6
Citral	500	4	NC	"++++"	NC	NC	"++"	NC	"++++"	"++"	NC	NC	NC	5
3-Dimethylaminopropylamine	>2500	9	"+++"	"++++"	"++++"	NC	NC	"++++"	"++++"	"++++"	NC	NC	"+"	5
Mercaptobenzothiazol	>2500	-1	NC	"+"	NC	NC	"++++"	"+"	NC	NC	"++++"	NC	NC	4
1,2-Benzisothiazolin-3-one	100	74	"+"	NC	"++++"	NC	NC	"++++"	NC	"++++"	NC	NC	"++++"	6
2-Hydroxyethylacrylate	100	94	"++"	"++"	NC	NC	"++"	NC	NC	NC	NC	NC	NC	5
1-Phenyl-1,2-propanedione	1000	-1	NC	"++++"	NC	NC	"++++"	"+"	"++++"	"++++"	NC	NC	NC	4
Isoeugenol	1000	92	"++++"	"++++"	NC	NC	"++++"	NC	"++++"	NC	NC	NC	"++"	5
2-Bromotetradecanoic acid	100	9	NC	"++"	NC	NC	"++"	"++++"	"++"	NC	NC	"+"	"++"	4
Diethyl maleate	300	92	"++++"	"++++"	NC	"++"	NC	"++++"	"++++"	"++"	NC	"+"	"++"	6
Diethyl sulfate	>2500	7	NC	"++"	NC	NC	"++"	"++++"	"++"	"++"	"++"	NC	NC	4
1-Perillaldehyde	100	-5	NC	NC	NC	"++"	NC	NC	NC	"++"	NC	NC	NC	1
2-Nonynoic acid, methyl ester	500	7	"++++"	"++++"	"++"	NC	NC	"++++"	"++++"	"++"	"++"	NC	"++"	5
trans-2-Decenal	100	12	NC	"++"	"++"	"++"	NC	"++"	"++++"	"++++"	NC	NC	NC	4
Phenylacetaldehyde	50	9	NC	"++"	NC	NC	NC	"++"	NC	"++++"	NC	"++"	"++++"	4
Benzyl cinnamate	2500	-6	"+"	NC	"++"	NC	"++++"	"++"	NC	"++"	NC	"++"	"++++"	3
Hydroxycitronellal	100	-2	NC	NC	"++"	NC	NC	NC	"++"	"++++"	NC	"++"	"++++"	1
Farnesal	500	-7	NC	"++"	NC	NC	NC	NC	NC	NC	"++++"	NC	"++"	1
Phenyl benzoate	2500	-6	NC	"++++"	"++++"	"++"	NC	NC	"++"	"++"	NC	NC	NC	3
Eugenol	2500	-1	NC	"++++"	NC	"++"	"++++"	"++"	"++++"	NC	NC	NC	NC	2
d-Limonene	500	-2	NC	NC	NC	NC	"++++"	NC	"++++"	NC	NC	NC	NC	0
Sodium dodecyl sulfate	500	69	"+"	"++"	"++"	"++"	"++++"	"++"	NC	"++"	NC	"++"	"++"	6

Table 1. continued on next page

Table 1. Continued

Compound	MTT Assay Viability (TC50 μ M)	%GSH Depleted	NQO1	AKR1C2	TXN	IL8	CYP1A1	ALDH3A	HMOX	MAFF	GCLC	MT1A	MT2A	Predicted Toxicity Index (PTI)
1-Butanol	2500	10	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	0
Salicylic acid	>2500	-5	NC	"+"	"+"	NC	NC	"+"	NC	"+"	NC	"+"	NC	1
Benzoic acid	>2500	0	NC	NC	"+"	NC	NC	NC	NC	NC	NC	"+"	"+"	0
Glycerol	>2500	4	NC	NC	NC	NC	NC	NC	"+"	NC	NC	"+"	"+"	1
Vanillin	>2500	-4	NC	"++++"	NC	"+"	"+"	"+"	NC	NC	NC	NC	"+"	1
Linalool	>2500	-2	NC	"+"	"+"	NC	"++++"	NC	NC	NC	"+"	NC	NC	2

Compounds used to develop the method described (training set) are shown. Extreme/strong chemicals are shown in orange, moderate in green, weak in blue, and nonsensitizers in gray. Cell viability is expressed as a concentration that resulted in a viability of 50% (TC₅₀). If a TC₅₀ could not be determined, the TC₅₀ was expressed as greater than (>) the highest concentration tested. Reactivity is shown in column 3 as percentage of glutathione (GSH) depleted. MTT = 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide; NQO1 = nicotinamide adenine dinucleotide phosphate oxidase (NADPH)-quinone oxidoreductase; AKR1C2 = aldoketoreductase; TXN = thioredoxin; IL8 = interleukin 8; CYP1A1 = cytochrome P450 1A1; ALDH3A = aldehyde dehydrogenase 3A; HMOX1 = heme-oxygenase 1; MAFF = V-maf musculoaponeurotic fibrosarcoma oncogene homolog F; GCLC = glutamate cysteine ligase catalytic subunit; MT1 = metallothionein 1; MT2 = metallothionein 2; NC = no change relative to controls. The exposure concentrations tested ranged from 0.1 to 2,500 μ M. Six concentrations were used for each chemical. If viability was less than 50% gene expression, data were not used at those concentrations in the final analysis. Three consecutive exposure concentrations must meet the viability criterion. If less than 3 concentrations could be used, testing was repeated at a lower concentration range. For typical experiments, a range-finding experiment was performed in order to select final exposure concentrations that would result in high viability. The numerical values under each gene heading represent fold induction relative to vehicle controls. The predictive toxicity index (PTI) values in green represent category misses, but not false negative or false positive. The 2 red cells represent false negative (perrilaldehyde) or false positive (sodium dodecyl sulfate). Optimization of the analysis algorithm corrected the false positive response for sodium dodecyl sulfate. The plus signs represent the type of gene response: + = minimum acceptable response (≥ 3.4), ++ = multiple concentration response at concentrations ≥ 500 μ M, +++ = clear concentration response but values ≤ 10 -fold, ++++ = clear concentration response at concentrations below 500 μ M with responses ≥ 10 -fold, and NC = no change.

media/DMSO samples were evaluated and the 4 housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA (RRN18S), beta-2-microglobulin (B2M), and transferrin receptor (TFRC), which showed good slope (-2.5 to -4.0), low standard deviation in the crossing point (CP), fewer than 3 cycles between sample CPs, and no difference between DMSO-treated and untreated conditions, were selected as the controls for normalizing target gene expression.

The abundance of messenger RNA (mRNA) for 11 target genes was determined relative to an average amount of mRNA determined from 3 of the 4 housekeeping genes monitored. QuantiTect Primer assays for the following 11 target genes were obtained from QIAGEN: 1) NQO1, 2) AKR1C2, 3) thioredoxin (TXN), 4) interleukin 8 (IL8), 5) aldehyde dehydrogenase 3A (ALDH3A), 6) heme-oxygenase 1 (HMOX1), 7) musculoaponeurotic fibrosarcoma (MafF), 8) GCLC, 9) CYP1A1, 10) metallothionein 1 (MT1), and 11) metallothionein 2 (MT2).

Detecting metals as sensitizers

Metallothionein (MT) expression is controlled by the presence of cationic metals such as zinc, nickel, silver, cadmium, and chromium. In addition, MT expression is controlled by Nrf1/MafF and the metal response element (MRE). Therefore, changes in the relative abundance of MT mRNA can be used as a sensor for the presence of sensitizing metals.

Statistical analyses

The accuracy as determined by sensitivity and specificity was determined by standard statistical methods described by Altman and Bland (31) and Zhou et al. (32).

Results

Cell system

In this study, normal human keratinocytes (HaCaT) were selected as a representative cell model for human contact dermatitis. The biochemical and molecular properties of these cells have been well characterized and the cells are inexpensive and easy to culture. The cells possess CYP activity and, in particular, the CYP1A1 gene and enzyme are fully functional (33,34). The presence of constitutive and inducible CYP1A1 enzymes is important to this study because it is this

group of metabolizing enzymes that is most often linked to the production of reactive intermediates. The Keap1/Nrf2/ARE/EpRE signaling system is also present in these cells. This makes them ideal for monitoring genes related to cell stress and cellular protection (35,36).

Tiered data acquisition

A total of 97 test chemicals were selected from published chemical lists. These chemicals represent a wide range of sensitization potency (Tables 1 and 2). In order to gain resolution among potency groups, and reduce false-negative and false-positive results, multiple assays were combined. The data for each test compound were collected in the following order: viability, direct reactivity, metabolism-dependent reactivity, ARE/EpRE-controlled genes, XRE-controlled genes, and MRE-controlled genes (Figure 2). Viability was reported as a concentration that resulted in a viability of 50% (TC_{50}) when possible. Reactivity data were expressed as percentage of free GSH depleted. Changes in target gene mRNA were evaluated based on the lowest exposure concentration that produced an induction ≥ 3.5 -fold relative to controls and the magnitude of the response. A training set of 39 compounds was used to develop the method and analytical process for detecting skin sensitizers (Table 1). Once developed, the system was challenged with 58 additional compounds submitted in a blinded manner (Table 2). The first 39 chemicals in Table 2 were used in the development of the assay (no PTI value), but were not included in the final evaluation and statistical analysis.

Cell viability

Exposure concentrations that produce acute cytotoxicity cannot be used to monitor changes in gene expression. Acceptable exposure concentration ranges were identified using range-finding experiments. Exposure concentrations that resulted in viability that was $\leq 50\%$ were not included in the final analysis. These data are summarized in Table 2.

Chemical reactivity

Direct chemical reactivity measures the capacity of the test compound to bind reduced GSH in a cell-free aqueous buffered solution. In general, chemicals that caused GSH depletion could be divided into 3 groups (Table 2): those compounds that depleted $>40\%$ of the free GSH (extreme/strong), those that

Table 2. Challenging the predictive power with 58 new chemicals.

Compound	CAS#	LLNA mM	Potency Category	Solubility (uM)	MTT Assay Viability (TC50 uM)	Direct Chemical Reactivity (%GSH Depleted)	Predicted Toxicity Index (PTI)
Training Set of 39							
Diphenylcyclopropenone	886-38-4	0.15	Extreme	>100	10	43.7	
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	0.60	Extreme	>20	20	9.9	
p-Benzoquinone	106-51-4	0.93	Extreme	>200	200	69.5	
1-Chloro-2,4-dinitrobenzene	97-00-7	1.98	Extreme	>20	20	74.5	
Formaldehyde	50-00-0	233.33	Strong	>2500	2500	81	
Metol	55-55-0	22.67	Strong	>2500	100	73	
Fluorescein-5-isothiocyanate	3326-32-7	3.60	Strong	>1000	1000	interference	
1,4-Phenyldiamine	106-50-3	14.81	Strong	>1000	1000	47.9	
Propyl gallate	121-79-9	15.09	Strong	500	500	63.1	
2-Aminophenol	95-55-6	36.36	Strong	>1000	1000	11.7	
Phthalic anhydride	85-44-9	10.80	Strong	>1000	2500	87.9	
trans-2-Hexenal	6728-26-3	561.22	Moderate	>500	500	88.6	
Citral	5392-40-5	328.95	Moderate	>250	500	4.2	
3-Dimethylaminopropylamine	109-55-7	215.69	Moderate	>2500	>2500	9	
2-Mercaptobenzothiazol	149-30-4	101.64	Moderate	>2500	>2500	-0.5	
1,2-Benzisothiazolin-3-one	2634-33-5	152.12	Moderate	>1000	100	74.1	
2-Hydroxyethylacrylate	818-61-1	120.56	Moderate	>100	100	94	
1-Phenyl-1,2-propanedione	579-07-7	87.72	Moderate	>2500	1000	-1	
Isoeugenol	97-54-1	109.76	Moderate	>2500	1000	92.4	
2-Bromotetradecanoic acid	10520-81-7	110.75	Moderate	>100	100	9.4	
Diethyl maleate	141-05-9	122.09	Moderate	>2500	300	92.4	
Diethyl sulfate	64-67-5	214.01	Moderate	>2500	>2500	6.5	
1-Perillaldehyde	18031-40-8	540.00	Moderate	>500	100	-4.9	
2-Nonynoic acid, methyl ester	111-80-8	148.81	Moderate	>250	500	6.6	
trans-2-Decenal	3913-81-3	162.34	Moderate	>100	100	12.4	
Phenylacetaldehyde	122-78-1	250.00	Moderate	>100	50	8.8	
Benzyl cinnamate	103-41-3	773.11	Weak	>1000	2500	-6.1	
Hydroxycitronellal	107-75-5	1337.21	Weak	>2500	100	-2.4	
Farnesal	19317-11-4	531.82	Weak	>2500	500	-7.2	
Phenyl benzoate	93-99-2	863.64	Weak	>2500	2500	-5.5	
Eugenol	97-53-0	792.68	Weak	>2500	2500	-0.8	
d-Limonene	5989-27-5	>2205	Very Weak/non	>200	500	-1.6	
Linalool	78-70-6	1948.05	Very Weak/non	>2500	>2500	-1.6	
Sodium dodecyl sulfate	151-21-3	484.43	non sensitizer	>500	500	68.5	
1-Butanol	71-36-3	>2702	Very Weak/non	>1000	2500	9.9	
Salicylic acid	69-72-7	>1812	Very Weak/non	>2500	>2500	-4.9	
Benzoic acid	65-85-0		non sensitizer	>2500	>2500	-0.2	
Glycerol	56-81-5	>10869	Very Weak/non	>2500	>2500	4.2	
Vanillin	121-33-5	>3289	Very Weak/non	>2500	>2500	-4.3	
Blinded Set 1							
Lauryl gallate	1166-52-5	8.88	Strong	1000	100	-10.2	6.5
Glutaraldehyde	111-30-8	10.00	Strong	>2500	500	93.6	7
4-Vinylpyridine	100-43-6	152.38	Moderate	>2500	500	94.8	5
Benzyl-salicylate	118-58-1	127.19	Moderate	>2500	500	-4	5
Benzylideneacetone	122-57-6	253.42	Moderate	>2500	500	80	3.5
1-Naphthol	90-15-3	90.15	Moderate	>2500	1000	0.8	4.5
3-Aminophenol	591-27-5	210.53	Moderate	>2500	1000	1	4
Benzocaine	94-09-7	>3026	Weak	>2500	>2500	-5.4	2.5
Benzyl-benzoate	120-51-4	801.90	Weak	1000	2500	-3	3

Table 2. continued on next page

Table 2. Continued.

Compound	CAS#	LLNA mM	Potency Category	Solubility (uM)	MTT Assay Viability (TC50 uM)	Direct Chemical Reactivity (%GSH Depleted)	Predicted Toxicity Index (PTI)
Lyrar	31906-04-4	814.29	Weak	>2500	500	1.1	1.5
2,3-Butanedione	431-03-8	1279.07	Weak	>2500	>2500	8.1	2
Ambrettolide	123-69-3	1142.86	Weak	>2500	>2500	-5.4	3.5
Geraniol	106-24-1	3701.30	Very Weak/non	>2500	>2500	-1.1	1.5
Pyridine	110-86-1	9000.00	Very Weak/non	>2500	>2500	14.5	2.5
Isopropyl myristate	110-27-0	1629.63	Very Weak/non	>2500	>2500	-4.4	1.5
Hedione	24851-98-7	>1769	Very Weak/non	>2500	>2500	2.9	1
4-Hydroxybenzoic acid	99-96-7	>1811	Very Weak/non	>2500	>2500	-4.5	1
Ethylene brassylate	105-95-3	>1111	Very Weak/non	>2500	>2500	-1.2	0
Methyl salicylate	119-36-8	>1315	Very Weak/non	>2500	>2500	-5	0
Blinded Set 2							
Oxazolone	15646-46-5	0.14	Extreme	>2500	2500	91.5	7
Hydroquinone	123-31-9	9.09	Strong	>2500	500	88.9	6
3,4-Dihydrocoumarin	119-84-6	378.38	Moderate	>2500	1000	12.8	4
3-Dimethylaminopropylamine	109-55-7	215.69	Moderate	>2500	>2500	9	3
Cinnamic aldehyde	104-55-2	234.85	Moderate	>2500	250	22.3	5
β -Damascone	23726-91-2	348.96	Moderate	>2500	250	19.9	5
trans-Anethole	4180-23-8	182.43	Moderate	>2500	>2500	-2.8	5
Lyrar	31906-04-4	814.29	Weak	>2500	500	1.1	3
Estragole	140-67-0	1442.86	Weak	>2500	>2500	-3.7	3
Cyclamen aldehyde	103-95-7	1173.68	Weak	>2500	500	-4.1	2
6-(1-Methylpropyl)quinoline	65442-31-1	605.41	Weak	>2500	250	-2.9	3
Methyl Cyclopropanecarboxylate	2868-37-3	939.19	Weak	>2500	>2500	-3.1	2
Cinnamic alcohol	104-54-1	1567.16	Weak	>2500	2500	-2.8	2
Propyl-paraben	94-13-3	>2776	Very Weak/non	>2500	2500	-2.7	1.5
Methylcoumarin	92-48-8	>1562	Very Weak/non	>2500	>2500	-1.3	1
Propylene glycol	57-55-6	>13157	Very Weak/non	>2500	>2500	6.5	0
Benzenesulfonic acid	98-11-3		non sensitizer	>2500	>2500	1.7	2
4-Methoxyacetophenone	100-06-1	>3333	Very Weak/non	>2500	>2500	0.1	2
Benzaldehyde	100-52-7	>2358	Very Weak/non	>2500	>2500	-0.9	1
Methyl paraben	99-76-3	>3289	Very Weak/non	>2500	>2500	-0.8	3
Blinded Set 3							
1-Spiro[4.5]dec-7-en-7-yl-4-penten-1-one	224031-70-3	100.92	Moderate	>2500	500	8.3	4
Galbanone	56973-85-4	156.25	Moderate	>2500	2500	11.3	4
2-Phenylpropionic aldehyde	93-53-8	440.56	Moderate	>2500	2500	-1.7	3
Dihydroeugenol	2785-87-7	409.64	Moderate	>2500	2500	-2.4	4
Methylisothiazolinone	2682-20-4	165.22	Moderate	>2500	500	11.3	4
2-Methyl-3(4-(2-methylpropyl)phenyl)-propanal	6658-48-6	441.18	Moderate	>2500	500	2.6	3
Dimethyl-3-cyclohexene-1-carboxaldehyde	27939-60-2	233.81	Moderate	>2500	>2500	-2.3	2
α -Methyl cinnamaldehyde	101-39-3	308.20	Moderate	>2500	2500	7.1	4
Safranal	116-26-7	499.33	Moderate	>2500	250	20.8	4
Lilial®	80-54-6	931.37	Weak	>2500	2500	0.7	1.5
1-(cyclopropylmethyl)-4-methoxy benzene	16510-27-3	1030.86	Weak	>2500	500	-2.9	1
Methyl atrarete	4707-47-5	1433.67	Weak	>2500	2500	-3.5	4
α -Hexyl cinnamaldehyde	101-86-0	555.56	Weak	>2500	>2500	-4.7	3
trans-2-Hexenol	928-95-0	6000.00	Very Weak/non	>2500	>2500	7.2	3
Diethyl Phthalate	84-66-2	>4504	Very Weak/non	>2500	250	-3.4	1

Table 2. continued on next page

Table 2. Continued.

Compound	CAS#	LLNA mM	Potency Category	Solubility (uM)	MTT Assay Viability (TC50 uM)	Direct Chemical Reactivity (%GSH Depleted)	Predicted Toxicity Index (PTI)
Phenyl ethyl alcohol	60-12-8	>4098	Very Weak/non	>2500	>2500	-1.2	3
1,1,3-Trimethyl-3-phenylindane	3910-35-8	1834.75	Very Weak/non	500	250	5.3	4
Benzyl alcohol	100-51-6	>4629	Very Weak/non	>2500	>2500	-2.1	3
Hexyl 2 methyl butanoate	10032-15-2	2941.65	Very Weak/non	>2500	>2500	-2.5	2.5

The screening method was developed based on data obtained from the 39 chemicals shown in Table 1. To test the approach, 58 additional chemicals were analyzed in 3 blinded groups. All 3 groups were analyzed prior to unmasking the chemical names. The color code indicates extreme/strong = orange, moderate = green, weak = blue, and nonsensitizers = gray. The data from the blinded sets were used to calculate overall sensitivity and specificity. The cells in the predictive toxicity index (PTI) column highlighted in green represent false negatives (6) and false positives (3). Sodium dodecyl sulfate highlighted in yellow was a false positive in the LLNA. The statistical analysis can be found in Figure 7. CAS = Chemical Abstracts Service; LLNA = local lymph node assay; MTT = 3-[4,5-dimethylthiazol-2-yl] 2,5diphenyltetrazolium bromide; TC₅₀ = concentration that resulted in a viability of 50%; GSH = glutathione.

had values <40% (moderate), and those that showed no measurable GSH depletion (weak/nonsensitizers). Extreme sensitizers (e.g., benzoquinone) have strong electrophilic centers and as such bind nearly all of the free GSH, while in comparison, nonsensitizers like glycerol, with no electrophilic centers, have no detectable binding (Figure 3; Table 2). Exceptions to these designations occurred (Table 2), underscoring the importance of multiple endpoints for *in vitro* screening. Prohaptens can be metabolized to active intermediates that can bind proteins. An important enzyme in this process is CYP1A. Compounds that fell into the nonsensitizer category were also evaluated as possible prohaptens with a metabolically competent system. 2-Amionophenol showed less than 15% depletion without metabolism and more than 30% depletion in the presence of phase I metabolizing enzymes. Test chemicals that increased CYP1A1 mRNA 10-fold or more relative to vehicle controls were flagged by the algorithm as potential prohaptens. These chemicals along with their chemical structures are shown in Figure 4.

Signaling pathways and gene expression

Chemical sensitizers are known to induce the expression of several cytoprotective proteins. This study evaluated genes under the control of 3 separate, but related, signaling pathways. These included the Keap1/Nrf2/ARE/EpRE (NQO1, AKR1C2, TXN, IL-8, ALDH3A, HMOX1, GCLC), the ARNT/AhR/XRE (CYP1A), and the Nrf1/MTF/MRE (MT1 and MT2) pathways. The expression of each gene was evaluated based on the following 3 criteria: 1) concentration where a significant increase (≥3.5-fold) was first observed, 2) concentration-response, and 3) the magnitude of the response. The presence of a clear concentration-response profile for a marker gene

increased the significance and potency weighting of the test chemical. Inducing a significant response at lower exposure concentrations also increased the significance of the response. For example, chemicals that produced induction only at concentrations ≥500 μM were considered to be weaker sensitizers than chemical that caused significant induction at 5 μM. The number of genes responding was also an important factor. Each marker gene was assigned a weighted response value ranging from + (low) to ++++ (high) based on the criteria above (Table 1). Extreme and strong sensitizers tended to induce more genes at lower exposure concentrations than weak or moderate sensitizers (Table 1). Figure 5 shows the frequency of marker genes induced in a concentration-dependent manner. Figure 6 provides an example of the differences in the gene induction profiles among chemicals with extreme/strong, moderate, and nonsensitizer potency categories. The lowest concentration where response was detected was selected for comparison. Note the number of genes responding, the magnitude of induction, and the potency. When these data are analyzed with reactivity data (GSH depletion), a higher degree of sensitivity is achieved. By developing a scoring system based on the expression profiles, it was possible to improve resolution between the potency categories of sensitization.

Detecting metals as sensitizers

Exposures to several metals known to cause sensitization were evaluated in this system. The presence of chromium, nickel, and silver produces marked induction of several ARE/EpRE genes and of MT2. This increase in MT2 mRNA signals the presence of metals (Table 3). The histogram in Figure 5 shows that MT2 was not upregulated by any of the 39 chemicals used in the training set, indicating that it may be a

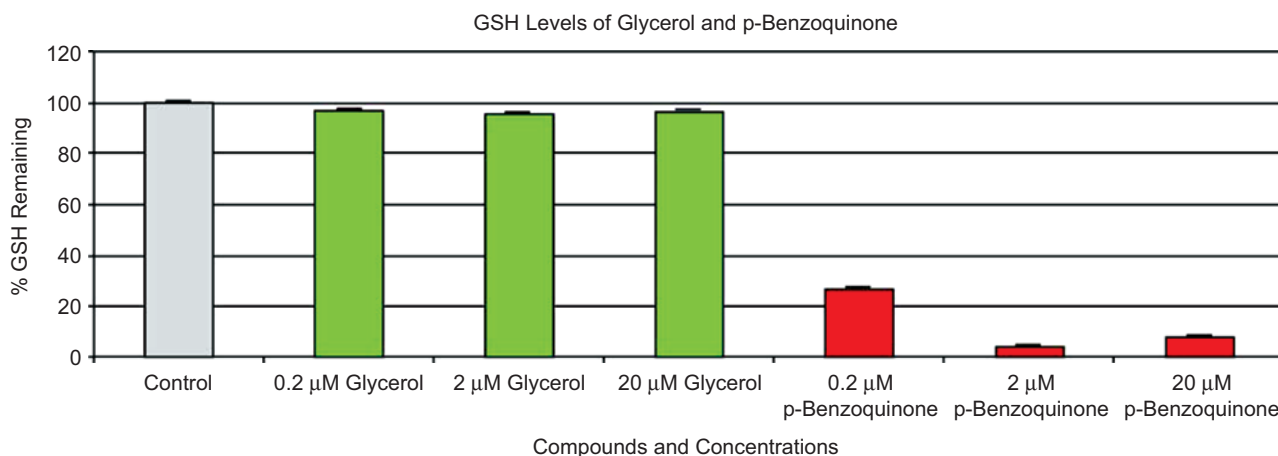


Figure 3. Glutathione (GSH) depletion assay for direct chemical reactivity. Reactive chemicals are also electrophiles that can bind with high affinity to nucleophiles. The tripeptide GSH is the primary source of protection from reactive chemicals and oxidative stress. GSH binding in the absence of enzymes indicates chemical reactivity. p-Benzoquinone, an extreme sensitizer, significantly depleted GSH, whereas the nonsensitizer glycerol had no measurable effect on GSH levels.

good intracellular sensor for the presence of cationic metals.

Developing a gated logic algorithm

In order to analyze the large amount of data resulting from this multiple endpoint approach, and to develop a means of making decisions on a test compound's potency category, it was necessary to develop an algorithm to process the data. This algorithm is based on the following factors: cell viability, chemical reactivity, the number of Nrf2 genes with mRNA ≥ 3.5 -fold, the magnitude of response (fold induction), and the exposure concentration that produced the response (potency). Each of these factors was assigned a gate threshold and a series of "if, then" statements. The result of each gate test was a weighted value. The sum of the weighted values resulted in the final predicted toxicity index (PTI) score (see equation below):

$$\text{PTI score} = \sum V \left\{ \begin{array}{l} (\omega_i (R) + \omega_i (G_{\text{ARE}}) + \omega_i (G_{\text{XRE}})) \\ + \omega_i (G_{\text{MRE}}) \end{array} \right\}$$

where V = viability, R = reactivity, G = gene expression, and ω_i = weighting factor.

The final measure of response was the *in vitro* predicted PTI value. The PTI value ranged from 0 to 8, with 0–1 indicating nonsensitizers, 2–3 weak sensitizers, 4–5 moderate sensitizers, and 6–8 strong and extreme sensitizers. The PTIs determined for each chemical in the training set are shown in Table 1. When there were incorrect predictions, adjustments were made to the algorithm. The initial algorithm was developed with

this initial training set of 39 compounds (Table 1). The sensitization potencies and LLNA values were from Natsch and Emter (23).

Challenging the model

In order to test the model developed with the chemicals listed in the training set (Table 1), an additional 58 compounds were evaluated in a blinded manner in 3 separate groups (Table 2). Samples were selected by the analytical laboratory, and each chemical received a code for identification. All data were gathered in the analytical laboratory. The data for all 3 groups were then sent to a different location for analysis. The compounds are identified in Table 2 as blinded sets 1 to 3. This means that the group processing the final data and assigning the potency category was blinded to the sample ID and the selection process. The potency categories were assigned by the algorithm and analyzed to determine sensitivity and specificity (Table 2; Figure 7). For this analysis non- and weak sensitizers were considered negative, whereas moderate and strong/extreme sensitizers were considered positive. This separation is based on LLNA data for the test compounds where there is clear overlap between LLNA values and non- and very weak potencies categories. In other words, there is not a clear break point in LLNA data that marks the end of one potency category and the beginning of another. The predicted values were then sent back to the analytical laboratory where the sample codes were unmasked. The logic system developed was able to place test chemicals into 4 potency categories: extreme/strong, moderate, weak, and non-sensitizers (Figure 8).

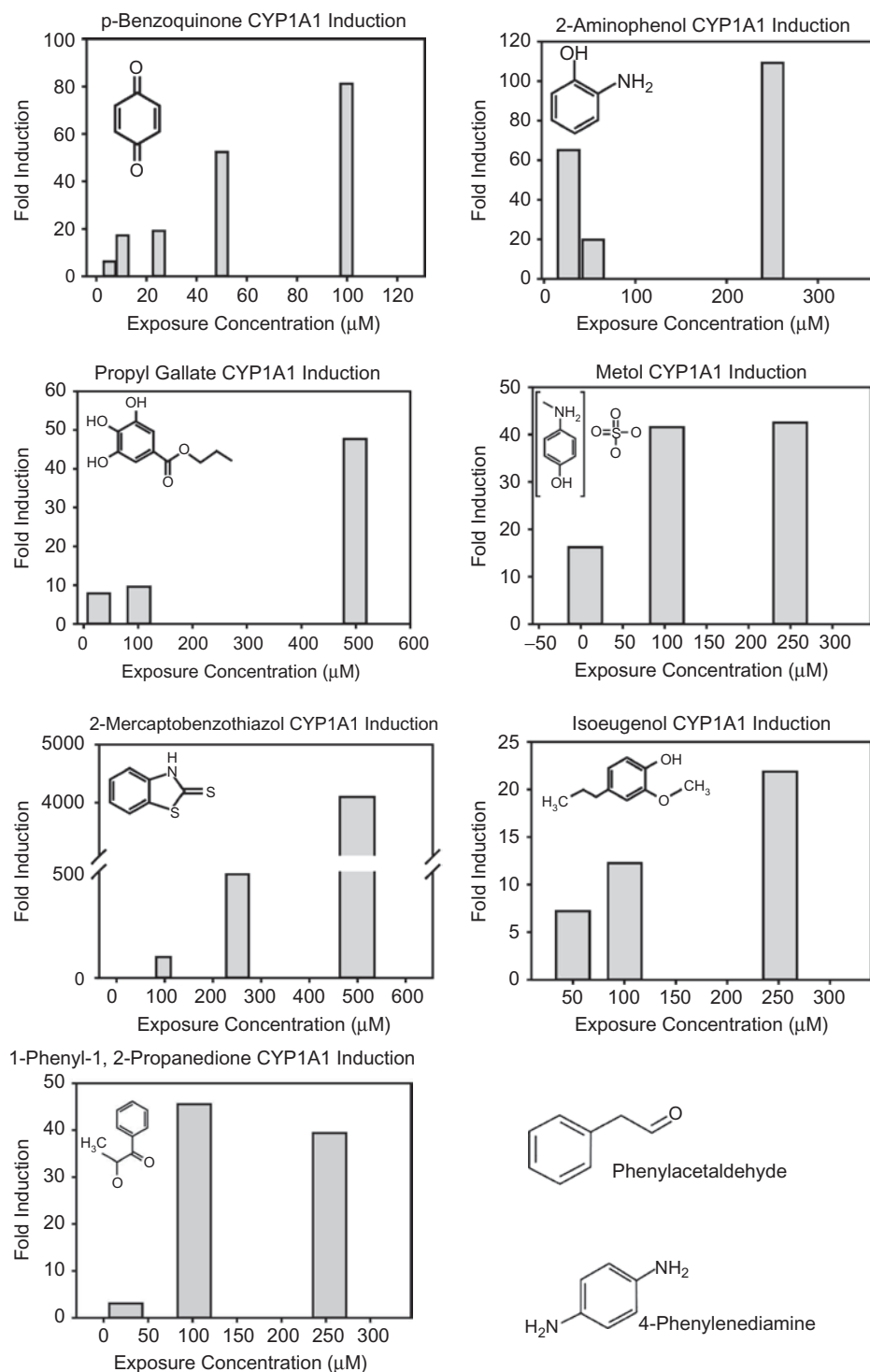


Figure 4. Compound structure and cytochrome P450 (CYP)1A1 induction. Compounds that induce CYP1A1 have similar chemical and structural characteristics. CYP1A1 and CYP1A2 convert nonreactive polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines and amides into reactive intermediates. Chemicals that induce CYP1A1 can be metabolized to reactive intermediates, which can then bind proteins and induce sensitization. PAHs are well known to induce their own metabolism (65). CYP1A1 induction can be an indication that the chemical is also a prohapten, because prohaptenes by definition require metabolic activation, and many sensitizers are PAHs. The compounds shown all have similar properties and they all induced CYP1A1 expression. Phenylacetaldehyde and 4-phenylenediamine have structures that are similar to the other chemicals shown; however, they do not induce CYP1A1 expression. These compounds are strong sensitizers, but did not induce CYP1A1. These chemicals are not metabolized by CYP1A, but are rapidly metabolized in cytosol, and therefore would not be expected to induce CYP1A1. Thus, while induction usually indicates sensitization, a lack of induction does not indicate a nonsensitizer.

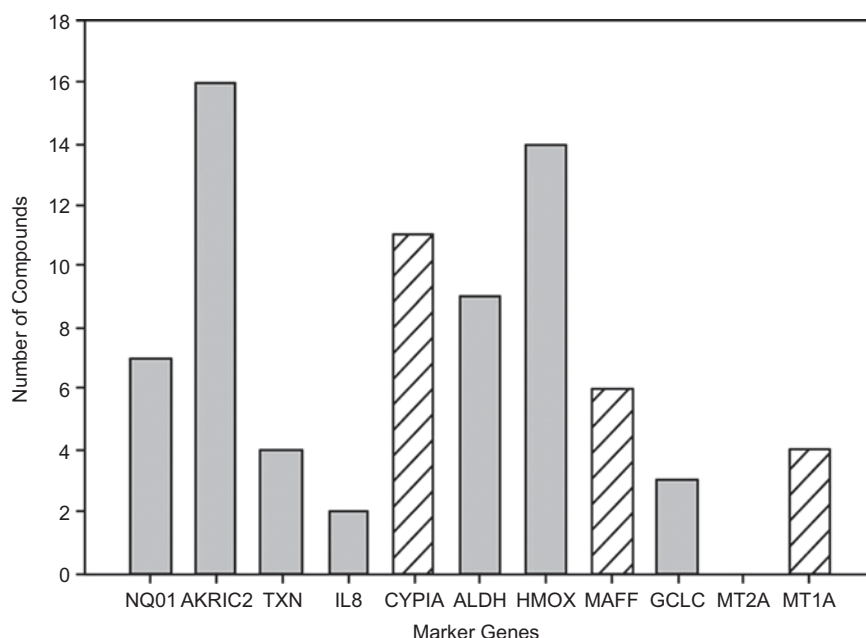


Figure 5. Frequency histogram of genes responding to the test chemicals in the training set. Genes controlled by the same signaling pathways do not respond in an identical manner. The figure shows the frequency of response following exposure to weak, moderate, strong, and extreme sensitizing chemicals. Marker genes that showed significant induction (≥ 3.5 -fold) and concentration-dependent response were counted as responders. The graph provides an indication of the genes that responded and the number of test chemicals in the training set of 39 (Table 2) that caused the response. In general, weak sensitizers and nonsensitizers were poor inducers. The low response by metallothionein (MT) genes supports their inclusion as markers for the presence of metal cations. Solid bars represent genes under control of ARE/EpRE and hatched bars represent genes under control of XRE or MRE.

Determining a relationship between the predicted toxicity index and the local lymph node assay

The LLNA concentration of test chemical required to provoke a 3-fold increase in lymph node cell proliferation (EC_3) values (in mM) were compared with the PTI values using a simple scatter plot analysis (Figure 9). A clear inverse linear relationship can be seen. Because of the large variation in LLNA EC_3 values within a potency category, it is difficult to accurately predict LLNA values from the PTI. The PTI value can be used to assign a range for expected LLNA results.

Discussion

The development and implementation of an *in vitro* method intended to replace current animal models for identifying chemical sensitizers require several key components. The *in vitro* cell model should mimic as closely as possible the *in vivo* tissue type. For the evaluation and risk assessment of chemicals that may produce ACD in humans, the ideal *in vitro* cell model would be human skin. Systems designed in tumor cell lines derived from other organs, such as liver, may not provide an accurate prediction of what actually occurs following a dermal exposure. The primary signaling

pathways and their feedback loops can vary among species and tissues. Significant differences between the *in vitro* exposure concentration and the actual *in vivo* exposure concentration in liver may mean that *in vitro* sensitization data collected in liver cell lines would not accurately reflect *in vivo* responses from a dermal exposure. The *in vitro* system should be able to predict not only whether a compound is positive or negative, but also its potency. The ability to detect metals that are sensitizers and also to evaluate mixtures is important in order for the *in vitro* assay to replace current animal tests. Ideally, an *in vitro* system should also provide concentration-response data that can be used to estimate the *in vivo* no-observable-effect level (NOEL).

A chemical with the ability to induce ACD is classified as a skin sensitizer. Once sensitized, a person can have a heightened response when exposed to the same chemical again. Allergic contact dermatitis is an immunological (type IV hypersensitivity) response that is mediated by T-cell processes. The *in vitro* model proposed in this study focuses on 2 key properties of chemical sensitizers. The first property is that in order for a small molecule to elicit an immune response, it must be absorbed through the skin and bind protein. Most chemical sensitizers are electron deficient (electrophilic). A compound may

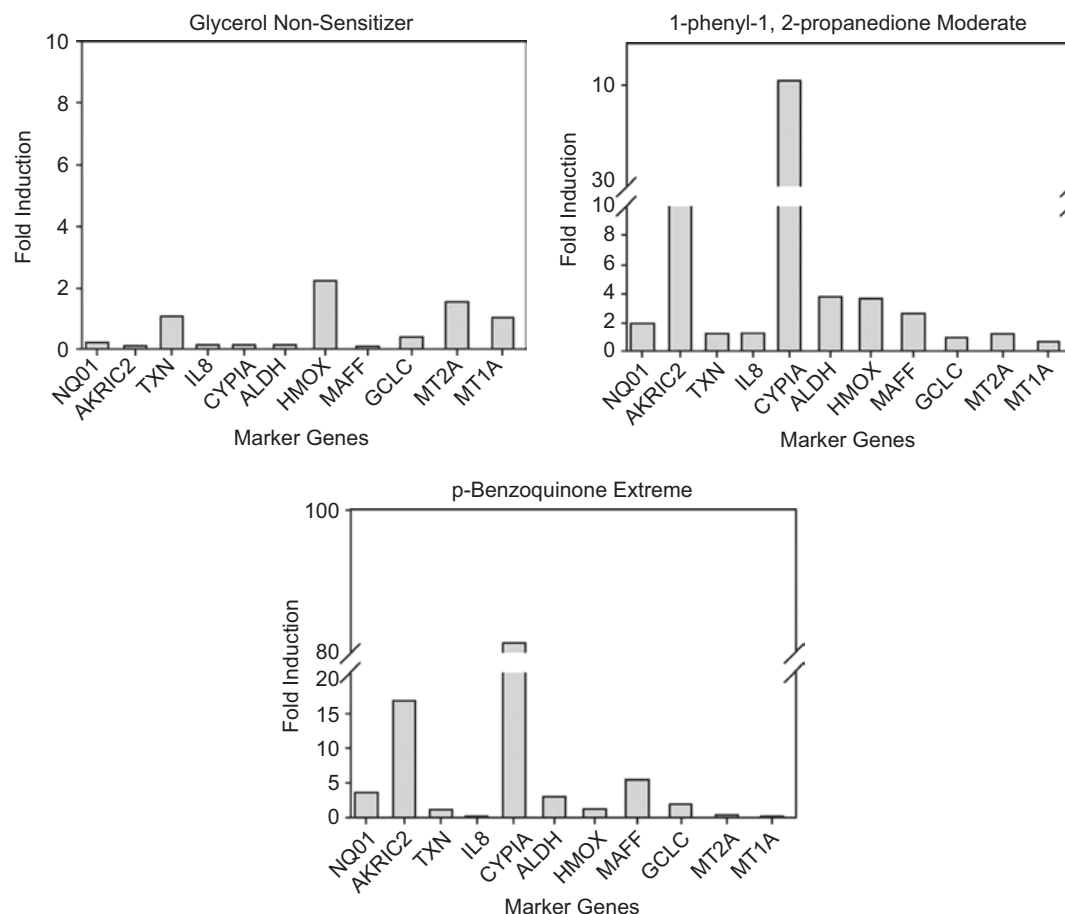


Figure 6. Induction profiles of non-, moderate, and extreme sensitizers. A key aspect of the screening method described is the induction of genes linked to known mechanisms of sensitization. To be predictive, chemicals with different sensitization potencies should produce different induction profiles. Differences in the number of genes responding, the lowest exposure concentration that causes induction, and the magnitude of induction are all affected by the potency of the test compounds.

be able to bind directly to a protein or it may require metabolic conversion, by enzymes in the skin, to a reactive form. The electrophilic nature of small-molecule sensitizers enables them to bind with the nucleophilic amino acids of proteins. Examples of chemicals that are electrophiles include those that possess halogen, aldehyde, ketone, or amide groups. Cations, such as nickel (Ni^{+2}) or chromium (Cr^{+3}), are also electrophiles and cause sensitization. In order for small chemicals with electrophilic properties to be sensitizers, they must bind to proteins with nucleophilic groups. Nucleophiles have one or more unshared pairs of electrons or are negatively charged ions. The side chains of some amino acids (including lysine, cysteine, and histidine) provide strong nucleophilic centers (12,37,38). In this study, the tripeptide GSH was used as a first-tier screen for reactivity. Chemicals that bind GSH are considered to be reactive, and the use of GSH as an indicator molecule for detecting skin sensitizers has been demonstrated (38,39). Although peptide binding can accurately

identify those chemicals that are sensitizers, it is difficult to discern chemical potency with this endpoint alone. Peptide binding data presented by Gerberick et al. and Natsch et al. demonstrated that peptides with different amino acids had different affinities for chemical sensitizers (3,39). Although there was good correlation between chemical-sensitizing potency and depletion of GSH and other probe peptides, clear resolution between potency categories was not apparent in these studies. Data presented in this study accurately identified sensitizers from nonsensitizers with a GSH depletion assay. In the initial training set, 88% of the positive sensitizers depleted GSH, whereas 85% of the 13 negative chemicals showed no GSH depletion. This means that 12% of the nonsensitizers showed no GSH depletion and would have been false negatives, while 15% of the negative chemicals did deplete GSH, which would have resulted in false positives. This assay was not sufficient to place chemicals into their potency categories. However, by combining GSH binding with gene expression profiles, the

Table 3. Evaluation of 3 cationic metals.

Compound	Dose (μM)	NQO1	AKR1C2	TXN	IL8	CYP1A1	ALDH3A	HMOX1	MAFF	GCLC	MT2A	MT1A	PTI
Chromium (III) nitrate	0.1	1.0	3.0	1.7	0.3	1.5	2.0	3.5	1.3	2.3	12.6	1.8	4
	5	1.4	2.0	0.9	0.3	1.3	1.1	3.0	0.9	1.6	5.6	1.0	
	100	1.0	0.8	0.7	0.8	1.0	2.2	3.5	0.7	2.7	4.7	1.3	
	250	0.3	3.4	0.7	0.2	0.9	2.0	1.2	0.7	4.2	3.1	1.4	
	500	0.8	1.0	1.1	0.7	0.8	2.3	1.1	0.7	4.5	1.7	0.8	
	2,500	0.6	1.1	0.7	1.1	2.4	3.5	2.5	2.2	1.6	3.4	1.6	
Nickel sulfate	0.1	0.5	1.2	2.4	0.5	2.5	3.0	4.9	0.8	1.6	6.3	1.3	4
	5	0.5	1.4	1.7	0.4	1.3	1.7	1.8	0.9	4.3	2.7	1.2	
	100	1.1	1.4	0.4	1.6	1.8	2.2	2.1	1.1	2.1	4.7	1.0	
	250	0.9	1.8	2.2	2.3	1.2	2.4	1.1	0.9	5.9	3.4	1.2	
	500	0.5	2.3	2.1	0.2	4.7	2.9	4.5	0.8	5.1	3.9	0.9	
	2,500	2.2	1.8	1.5	0.6	1.1	8.9	30.2	1.7	9.3	13.3	1.1	
Silver nitrate	0.1	2.0	0.7	0.4	6.6	2.7	3.1	2.9	0.7	2.9	12.2	0.5	3
	5	1.5	2.4	0.3	15.8	0.5	5.6	2.8	1.2	2.5	13.3	1.1	
	100	10.9	16.2	0.6	148.6	3.1	0.3	2,825.1	5.4	5.0	80.7	5.4	
	250	0.9	3.2	1.9	0.0	16.5	0.7	25.9	0.3	0.9	2.7	690.8	
	500	0.8	2.8	1.8	0.0	66.9	0.5	11.7	0.6	0.7	2.4	237.1	
	2,500	13.4	83.7	4.8	17.3	6.9	0.4	11,404.9	5.2	4.8	34.7	87.5	

Chromium and nickel are known to cause skin sensitization reactions and can be detected with the local lymph node assay (LLNA) (8). Silver is a non- to very weak sensitizer. The metals were tested over a broad range of exposure concentrations for their effects on gene expression. Metallothionein genes (MT1 and MT2) were used as intracellular sensors for the presence of metals. Each exposure concentration was repeated in triplicate. Green highlighted cells represent genes that met the induction criteria for the algorithm. Gray highlighted cells represent data from cells with viability <50%. The assay assigned a predictive toxicity index (PTI) score of 3 (non- to weak sensitizer) to silver and a 4 (moderate sensitizer) to chromium and nickel. NQO1 = nicotinamide adenine dinucleotide phosphate oxidase (NADPH)-quinone oxidoreductase; AKR1C2 = aldoketoreductase; TXN = thioredoxin; IL8 = interleukin 8; CYP1A1 = cytochrome P450 1A1; ALDH3A = aldehyde dehydrogenase 3A; HMOX1 = heme-oxygenase 1; MAFF = V-maf musculoaponeurotic fibrosarcoma oncogene homolog F; GCLC = glutamate cysteine ligase catalytic subunit; MT2A = metallothionein 2a; MT1A = metallothionein 1a.

	+ In Vivo	- In Vivo	Totals	
P+	TP 25	FP 3	28	89% PPV
P-	FN 6	TN 33	39	85% NPV
Totals	31	36		
	Sensitivity = 81%			
	Specificity = 92%			

Figure 7. Determining sensitivity and specificity. The method described and developed with the 39 compounds (training set) was challenged by analyzing 58 new compounds in a blinded manner. The resulting predicted toxicity index (PTI) values were statistically analyzed to determine sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). *Sensitivity* is a measure of the assay's ability to correctly identify those compounds that are true sensitizers without regard to potency. This group included chemicals classified as moderate, strong, and extreme. *Specificity* is a measure of the assay's ability to correctly identify chemicals that are not sensitizers. This group includes weak and nonsensitization categories. Weak sensitizers are grouped with nonsensitizers because the local lymph node assay (LLNA) cannot reliably resolve these categories (see Figure 4). TP = true positive; FP = false positive; FN = false negative; TN = true negative.

resolution was improved and the incidence of false negatives was reduced.

The process of haptation is essential for a chemical to be fully immunogenic; however, protein binding can also initiate molecular signaling pathways that allow tissues to respond to potentially damaging electrophilic agents. Small molecules that are highly reactive, or that are metabolized to a reactive form, can induce the expression of cytoprotective proteins. Nrf2 is a bZIP in the cap and collar transcription factor family, which is characterized by a 45-amino-acid region known as the CNC domain. The Keap1/Nrf2/ARE/EpRE pathway appears to be a good intrinsic sensor system for electrophiles and reactive oxygen species. Genes controlled by this pathway are cytoprotective in their function. Because most chemical sensitizers are electrophiles, or can produce oxidative stress, activation of this signaling pathway has been proposed as a means of identifying dermal sensitizing agents (23). The work described here extends this initial work by evaluating the expression of many genes under the control of the ARE. In addition to Nrf2 linked genes, Nrf1 and linked genes were also monitored (40) were also monitored. The complexity of this signaling system and the cross-talk between systems means that in order

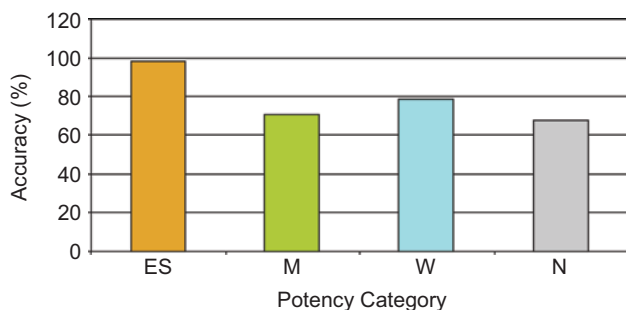


Figure 8. Accuracy of assigning potency categories. The assay method and the analysis algorithm described accurately assigned test chemicals to 4 potency categories: extreme/strong=ES, moderate=M, weak=W, and nonsensitizers=N. Predicted toxicity index (PTI) values 6–8=ES, 4–5=M, 2–3=W, and 0–1=N. Data were taken from the 3 blinded experiments (Table 2). The colors correspond to the potency categories in Table 2. The number of incorrect category predictions was subtracted from the total number of chemicals in a particular category. This yielded the number of correct predictions, which was divided by the total number of chemicals in the category to yield accuracy. For each chemical, potency was based on potency categories reported by Natsch and Emter (23).-

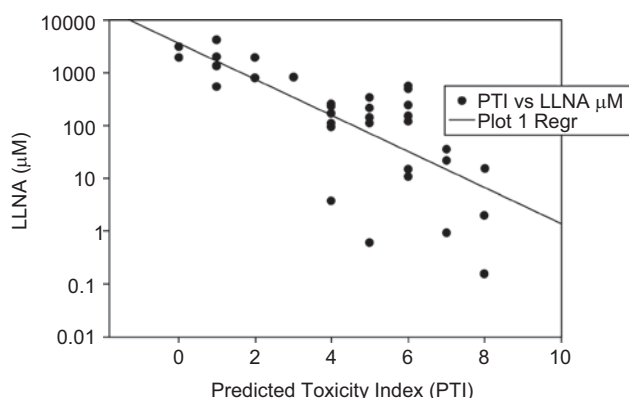


Figure 9. Relationship between local lymph node assay (LLNA) EC₃ values in micromolar and the predicted toxicity index (PTI). There is an inverse relationship between *in vivo* LLNA data and the *in vitro* PTI. A high degree of concordance between high PTI values and low LLNA values was observed; however, because of the large variation in published LLNA values within potency categories, an accurate prediction of an LLNA concentration of test chemical required to provoke a prediction of the amount of test chemical required to elicit a stimulation index of 3 (EC₃) is difficult using simple regression analysis. The potency categories for each chemical tested were based on Natsch and Emter (23).

for this model to be predictive, multiple components must be evaluated in a tiered approach to screening. The diagram in Figure 2 emphasizes the complexity and importance of these signaling systems.

An important factor to consider with *in vitro* models is cell exposure to the test material and the accessibility of nucleophilic amino acids for binding. The 2 primary factors that affect cellular uptake are aqueous solubility

and lipid solubility. The lipophilicity of chemicals can influence permeability. Compounds with higher octanol/water partition coefficient (LogP) values typically have greater dermal permeability (41), but the same compound administered orally may have lower systemic absorption. Another important consideration is metabolism. Metabolizing enzymes are considerably more abundant in liver than in the keratinocytes. In addition, the relative abundance of the CYP isoforms varies between tissue types. In keratinocytes, CYP2B6 and CYP1A1 are present constitutively and can be induced by chemical exposure. In contrast, CYP3A4 in skin is present at very low levels (34). In liver, most CYP isoforms are present and inducible, with CYP3A4 being the most abundant. The organ-specific differences in the type and abundance of chemical-metabolizing enzymes mean that significantly different metabolite profiles would be expected from cells derived from different tissue types. This is important when *in vitro* data are being used to predict an *in vivo* event that happens following dermal contact.

Although metabolizing enzymes, such as CYP, are present in keratinocytes, the actual amount or relative abundance of each isoform can vary significantly between tissues. *In vitro* systems can be subject to similar effects. Liver cell lines, such as Hepa 1C1C7, have a greater metabolizing capability than non-hepatic cells. As a result, the amount of metabolite formed in an *in vitro* liver cell model may be higher than the actual concentrations generated *in vivo* by metabolizing enzymes in the epidermis. Compounds that are metabolized by CYP1A isoforms are known to produce reactive intermediates. CYP1A is present in human epidermis and in HaCaT cells. Successful cell models should have CYP profiles consistent with the expected route of exposure.

For these reasons, CYP1A1 expression was monitored in this study. Compounds such as isoeugenol, 1-phenyl-1,2-propanedione, 2-mercaptobenzothiazol, 2-aminophenol, propyl gallate, metol, and p-benzoquinone produced a concentration-dependent increase in CYP1A1 mRNA. In general, compounds classified as strong or extreme sensitizers showed greater potency toward CYP1A induction than those classified as moderate sensitizers. The CYP induction and molecular structures of these compounds can be seen in Figure 4. For comparison, 2 compounds (phenylacetaldehyde and phenylenediamine) that are classified as moderate potency sensitizers, and have molecular structures similar to those that would be expected to induce CYP1A1, did not increase the expression of this gene in HaCaT cells (Figure 4). This difference can be explained by the fact that phenylacetaldehyde is rapidly metabolized by aldehyde dehydrogenase, whereas phenylenediamine

is N-acetylated. These chemicals are not substrates for CYP metabolism and, therefore, would not be expected to induce CYP1A1 (42–44). These findings demonstrate that the incorporation of structure–activity relationships and CYP induction into the screening paradigm could improve the method.

Because most sensitizers have electrophilic properties and are reactive, the activity of CYP1A1 is important; this enzyme is responsible for the metabolic activation of many chemicals, and the primary metabolites generated are usually highly reactive electrophiles. Menadione and benzo(a)pyrene are examples of CYP1A1 substrates that are activated to electrophilic intermediates and both are classified as sensitizers (45,46). Even the well-tolerated and widely used drug acetaminophen (APAP; paracetamol) has been linked to increased risk for asthma and other allergic diseases (sensitization) in humans (47). Acetaminophen is metabolized by CYP1A1/2, which yields the primary N-acetyl-p-benzoquinone imine (NAPQI). It is the formation of NAPQI in liver and the subsequent induction of oxidative stress that produces toxicity (48). In humans, APAP is metabolized to NAPQI by CYP1A2, CYP3A4, and CYP2E1 (49). Finally, it has been demonstrated that NAPQI directly interacts with Keap1 and activates Nrf2 induction of cytoprotective proteins (40,50).

Monitoring the expression of a single gene controlled by ARE/EpRE is not sufficient to identify the majority of chemical sensitizers in unmodified dermal cell models. In this study, several strong sensitizers such as phthalic anhydride, 2-aminophenol, and propyl gallate had little or no effect on NQO1 expression, but did produce pronounced induction of other Nrf2/ARE/EpRE genes. In addition, 2-aminophenol showed low reactivity in our study in the absence of metabolizing enzymes, but reactivity was increased more than 3-fold in the presence of microsomal enzymes. These data indicate that in normal human skin keratinocytes, NQO1 may not be the most sensitive gene for sensitization, and underscore the need for multiple gene markers and the inclusion of chemical reactivity.

Many metals are known to be sensitizers, but their detection in standard *in vivo* sensitization models can sometimes be difficult. The LLNA was able to correctly identify most metals known to be sensitizers as well as those metals that are not associated with sensitization (8). Therefore, it would be useful if the *in vitro* model could also detect sensitizers, such as metals, that may not act directly through Nrf2. Nickel, cobalt, and chromium are examples of metals that can cause ACD (51–55). These metals also induce a stress response and cytoprotective protein known as metallothionein (MT). Metallothionein is present in skin epidermis and in HaCaT cells (56,57). MT genes

possess multiple copies of the MRE. MT expression is upregulated by zinc and other metals (58). Metals bind to a metal responsive transcription factor (MTF-1) that enables binding the MRE and subsequent upregulation of MTs (59). Interestingly, MT genes also possess ARE in the promoter region and thus can be induced by oxidative stress. Both the loss in cellular GSH and the generation of reactive oxygen species stimulate this pathway (60). Metals can induce MT genes by direct interaction with MTF-1 and indirectly by affecting the redox state of the cell and subsequent activation of the Nrf1/ARE pathway (61–63). Both MT1 and MT2 mRNA were monitored in this study. Only benzyl cinnamate and 1, 2-benzisothiazolin-3-one produced a significant concentration-dependent increase in MT1, whereas MT2 induction was observed only following exposure to 1, 2-dihydroeugenol. For MT1, the 2 compounds induced expression only at concentrations that also produced significant cytotoxicity. These data indicate that expression of MT mRNA is not sensitive to a broad range of chemicals. When the exposure concentrations tested do not cause cytotoxicity, an increase in MT gene expression may indicate the presence of cationic metals.

In this study we have extended the work of Natsch and Emter (23) by using the human HaCaT cell line, monitoring direct and indirect chemical reactivity, and measuring changes in the expression of multiple Keap1/Nrf2/ARE/EpRE genes, CYP1A1, and Nrf1/ARE-linked MT genes. In addition, an algorithm for analyzing and processing the vast array of information generated has been developed that provides a single PTI value. This final value correlates well with known LLNA EC₃ values; however, because of the large variation in LLNA data for chemicals in the same potency category, an accurate prediction of the LLNA value is difficult. An additional improvement is the ability of the present method to identify the presence of sensitizing metals. A tiered assay approach will undoubtedly provide the most accurate information. Chemical reactivity, combined with gene expression data, provided more accurate results. Shorter time points revealed that some strong sensitizers have an early response that can be missed at longer time points (oxazolone and glutaraldehyde). It is important to realize that not all of the genes linked to Keap1/Nrf2/ARE/EpRE are upregulated at the same time or to the same extent by any given test chemical.

The method described is a promising *in vitro* method that could replace the need for animal testing. Additional studies are planned in collaboration with various companies and trade groups that will provide further validation of this approach. It will also be important to extend the chemical space by testing compounds outside of the cosmetic and personal care

industries. A limitation to this assay is its apparent inability to separate nonsensitizers from very weak sensitizers. Additional work will focus on improving resolution between these potency categories. Test compounds with low solubility and complex mixtures are difficult to assess in a monolayer cell culture system. In order for *in vitro* sensitization data to be fully incorporated into the regulatory process of safety assessment, it will be necessary for the assay to provide a good estimation of NOELs and no-expected-sensitization-induction levels (NESILs), which are quantitative values used in risk-assessment models (64). Future work with this model will focus on developing a means of estimating these important values.

The method described here accurately placed a broad range of sensitizers into 3 potency categories, extreme/strong, moderate, and weak/nonsensitizers. Overall sensitivity was 81% and specificity was 92%. This work has demonstrated the need for multiple endpoint analysis and the importance of understanding the complex array of signaling pathways involved with the endpoints being monitored. The method presented here can identify skin sensitizers with a level of sensitivity and specificity that is similar to that of the LLNA. Therefore, this method offers a viable *in vitro* alternative to the current animal tests.

Acknowledgments

The authors would like to express their thanks to Drs. Richard Wallin, Joe Carraway, Alan Brown, and Robert Parker for critically reviewing the manuscript. A special thanks to Ms. Muriel Reva for her administrative support in the preparation of this work.

Declaration of interest

This work was funded by Ceetox, Inc. The authors alone are responsible for the content and writing of the paper.

References

1. Aeby P, Sieber T, Beck H, Gerberick GF, Goebel C. Skin sensitization to p-phenylenediamine: the diverging roles of oxidation and N-acetylation for dendritic cell activation and the immune response. *J Invest Dermatol* 2009; 129:99-109.
2. Kimber I, Hilton J, Dearman RJ, Gerberick GF, Ryan CA, Basketter DA, et al. An international evaluation of the murine local lymph node assay and comparison of modified procedures. *Toxicology* 1995; 103: 63-73.
3. Gerberick GF, Vassallo JD, Bailey RE, Chaney JG, Morrall SW, Lepoittevin JP. Development of a peptide reactivity assay for screening contact allergens. *Toxicol Sci* 2004; 81: 332-343.
4. Gerberick GF, Ryan CA, Dearman RJ, Kimber I. Local lymph node assay (LLNA) for detection of sensitization capacity of chemicals. *Methods* 2007; 41(1):54-60.
5. Ryan CA, Gildea LA, Hulet BC, Dearman RJ, Kimber I, Gerberick GF. Gene expression changes in peripheral blood-derived dendritic cells following exposure to a contact allergen. *Toxicol Lett* 2004; 150:301-316.
6. Sailstad DM. Allergic Contact Hypersensitivity: Mechanisms and Methods: in *Alternative Toxicological Methods*, 2003; pp 193-205 (Ed. H Salem and S Katz), CRC Press, New York.
7. Basketter DA, Dearman RJ, Kimber I, Gerberick GF, Ryan CA. The impact of LLNA group size on the identification and potency classification of skin sensitizers: a review of published data. *Cutan Ocul Toxicol* 2009; 28:19-22.
8. Basketter DA, Lea LJ, Cooper KJ, Ryan CA, Gerberick GF, Dearman RJ, Kimber I. Identification of metal allergens in the local lymph node assay. *Am J Contact Dermat* 1999; 10:207-212.
9. Landsteiner K, Jacobs J. Studies on the sensitization of animals with simple chemical compounds. II. *J Exp Med* 1936; 64: 625-639.
10. Dupuis G, Benezra C. (1982) Allergic contact dermatitis to simple chemicals: A molecular approach. Marcel Dekker, New York & Basel.
11. Lepoittevin JP, Basketter DA, Goossens A, Karlberg AT. (1988) Allergic Contact Dermatitis: The Molecular Basis, Springer Berlin.
12. Mutschler J, Giménez-Arnau E, Foertsch L, Gerberick GF, Lepoittevin JP. Mechanistic assessment of peptide reactivity assay to predict skin allergens with Kathon CG isothiazolinones. *Toxicol In Vitro* 2009; 23:439-446.
13. Wang W, Jaiswal AK. Nuclear factor Nrf2 and antioxidant response element regulate NRH:quinone oxidoreductase 2 (NQO2) gene expression and antioxidant induction. *Free Radic Biol Med* 2006; 40:1119-1130.
14. Burczynski ME, Lin HK, Penning TM. Isoform-specific induction of a human aldo-keto reductase by polycyclic aromatic hydrocarbons (PAHs), electrophiles, and oxidative stress: implications for the alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogenase. *Cancer Res* 1999; 59: 607-614.
15. Ma Q, Lu AYH. CYP1A Induction and Human Risk Assessment: An Evolving Tale of In Vitro and in vivo studies. *Drug Metab Disp.* 2007; 35: 1009-1016.
16. Nebert DW, Petersen DD, Puga A. Human AH locus polymorphism and cancer: inducibility of CYP1A1 and other genes by combustion products and dioxin. *Pharmacogenetics* 1991; 1(2):68-78.
17. Whitlock JP Jr. Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-p-dioxin action. *Annu Rev Pharmacol Toxicol* 1990; 30:251-277.
18. Whitlock JP Jr. Induction of cytochrome P4501A1. *Annu Rev Pharmacol Toxicol* 1999; 39:103-125.
19. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, et al. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci U S A* 2002; 99:11908-11913.
20. Kang KW, Lee SJ, Kim SG. Molecular mechanism of Nrf2 activation by oxidative stress. *Antioxid Redox Signal* 2005; 7:1664-1673.
21. Wakabayashi N, Dinkova-Kostova AT, Holtzclaw WD, Kang MI, Kobayashi A, Yamamoto M, et al. Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor

- modified by inducers. *Proc Natl Acad Sci U S A* 2004; 101: 2040–2045.
22. Noguchi N. Role of oxidative stress in adaptive responses in special reference to atherogenesis. *J Clin Biochem Nutr* 2008; 43(3):131–8.
23. Natsch A, Emter R. Skin sensitizers induce antioxidant response element dependent genes: application to the *in vitro* testing of the sensitization potential of chemicals. *Toxicol Sci* 2008; 102:110–119.
24. Hooyberghs J, Schoeters E, Lambrechts N, Nelissen I, Witters H, Schoeters G, et al. A cell-based *in vitro* alternative to identify skin sensitizers by gene expression. *Toxicol Appl Pharmacol* 2008; 231:103–111.
25. Trompezinski S, Migdal C, Tailhardat M, Le Varlet B, Courtellemont P, Haftek M, et al. Characterization of early events involved in human dendritic cell maturation induced by sensitizers: cross talk between MAPK signaling pathways. *Toxicol Appl Pharmacol* 2008; 230:397–406.
26. Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 1980; 106: 207–212.
27. Chien CW, Chiang MC, Ho IC, Lee TC. Association of chromosomal alterations with arsenite-induced tumorigenicity of human HaCaT keratinocytes in nude mice. *Env Hlth Perspect* 2004; 112: 1704–1710.
28. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65(1-2):55–63.
29. Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 2004; 64: 5245–5250.
30. Vandesompele J, Preter KD, Pattyn F, Poppe B, Van Roy N, de Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* 2002; 3: 34.1–34.11.
31. Altman DG, Bland JE. Statistics notes: diagnostic tests 1: sensitivity and specificity. *Br Med J* 1994; 308:1552.
32. Zhou, XH, Obuchowski NA, McClish DM. *Statistical Methods in Diagnostic Medicine*; New York: John Wiley and Sons. 2002.
33. Dvorak et al. 2006.
34. Janmohamed A, Dolphin CT, Phillips IR, Shephard EA. Quantification and cellular localization of expression in human skin of genes encoding flavin-containing monooxygenases and cytochromes P450. *Biochem Pharmacol* 2001; 62: 777–786.
35. Wondrak GT, Cabello CM, Villeneuve NF, Zhang S, Ley S, Zheng YL, Zhang DD. Cinnamoyl-based Nrf2-activators targeting human skin cell photo-oxidative stress. *Free Rad Biol Med* 2008; 45: 385–395.
36. Devling TWP, Lindsay CD, McLellan LI, McMahon M, Hayes JD. Utility of siRNA against keap1 as a strategy to stimulate a cancer chemopreventive phenotype. *PNAS* 2005; 102: 7280–7285.
37. Eilstein JE, Giménez-Arnau E, Duché D, Cavusoglu N, Hussler G, Rousset F, et al. Sensitization to p-amino aromatic compounds: study of the covalent binding of 2,5-dimethyl-p-benzoquinonediimine to a model peptide by electrospray ionization tandem mass spectrometry. *Bioorg Med Chem* 2008; 16:5482–5489.
38. Kato H, Okamoto M, Yamashita K, Nakamura Y, Fukumori Y, Nakai K, et al. Peptide-binding assessment using mass spectrometry as a new screening method for skin sensitization. *J Toxicol Sci* 2003; 28(1):19–24.
39. Natsch A, Gfeller H, Rothaupt M, Ellis G. Utility and limitations of a peptide reactivity assay to predict fragrance allergens *in vitro*. *Toxicol In Vitro* 2007; 21:1220–1226.
40. Aleksunes LM, Manautou JM. Emerging role of Nrf2 in protecting against hepatic and gastrointestinal disease. *Toxicol Pathol* 2007; 35:459–473.
41. Cross SE, Magnusson BM, Winckle G, Anissimov Y, Roberts MS. Determination of the effect of lipophilicity on the *in vitro* permeability and tissue reservoir characteristics of topically applied solutes in human skin layers. *J Invest Dermatol* 2003; 120:759–764.
42. Panoutsopoulos GI, Kouretas D, Gounaris EG, Beedham C. Enzymatic oxidation of 2-phenylethylamine to phenylacetic acid and 2-phenylethanol with special reference to the metabolism of its intermediate phenylacetaldehyde. *Basic Clin Pharmacol Toxicol* 2004; 95:273–279.
43. Panoutsopoulos GI, Kouretas D, Gounaris EG, Beedham C. Metabolism of 2-phenylethylamine and phenylacetaldehyde by precision-cut guinea pig fresh liver slices. *Eur J Drug Metab Pharmacokinet* 2004; 29:111–118.
44. Hu T, Bailey RE, Morrall SW, Aardema MJ, Stanley LA, Skare JA. Dermal penetration and metabolism of p-aminophenol and p-phenylenediamine: application of the EpiDerm human reconstructed epidermis model. *Toxicol Lett* 2009; 188:119–129.
45. Schulz KH, Garbe I, Hausen BM, Simatupang MH. The sensitizing capacity of naturally occurring quinones. Experimental studies in guinea pigs. I. Naphthoquinones and related compounds. *Arch Dermatol Res* 1977; 258(1):41–52.
46. Casale GP, Cheng Z, Cavalieri EL, Singhal M. Profiles of cytokine mRNAs in the skin and lymph nodes of SENCAR mice treated epicutaneously with dibenzo[a,h]pyrene or dimethylbenz[a]anthracene reveal a direct correlation between carcinogen-induced contact hypersensitivity and epidermal hyperplasia. *Mol Carcinog* 2000; 27(2):125–140.
47. Davey G, Berhane Y, Duncan P, Aref-Adib G, Britton J, Venn A. Use of acetaminophen and the risk of self-reported allergic symptoms and skin sensitization in Butajira, Ethiopia. *J Allergy Clin Immunol* 2005; 116:863–868.
48. Zhao C, Shichi H. Prevention of acetaminophen-induced cataract by a combination of diallyl disulfide and N-acetylcysteine. *J Ocul Pharmacol Ther* 1998; 14:345–355.
49. Sarich T, Kalhorn T, Magee S, al-Sayegh F, Adams S, Slattery J, et al. The effect of omeprazole pretreatment on acetaminophen metabolism in rapid and slow metabolizers of S-mephenytoin. *Clin Pharmacol Ther* 1997; 62(1):21–28.
50. Copple IM, Goldring CE, Jenkins RE, Chia AJ, Randie LE, Hayes JD, et al. The hepatotoxic metabolite of acetaminophen directly activates the Keap1-Nrf2 cell defense system. *Hepatology* 2008; 48:1292–1301.
51. Day GA, Virji MA, Stefaniak AB. Characterization of exposures among cemented tungsten carbide workers. Part II: assessment of surface contamination and skin exposures to cobalt, chromium and nickel. *J Expo Sci Environ Epidemiol* 2009; 19:423–434.
52. Reichenberger M, Ebke M, Patiri C. Nickel sensitization in women and its relevance to occupation [German]. *Berufsdermatosen* 1976; 24(4):91–99.
53. Majumder S, Ghoshal K, Summners D, Bai S, Datta J, Jacob ST. Chromium (VI) down-regulates heavy metal-induced metallothionein gene transcription by modifying transactivation potential of the key transcription factor, metal-responsive transcription factor 1. *J Biol Chem* 2003; 278:26216–26226.
54. Vital AL, Goncalo M, Cruz MT, Figueiredo A, Duarte CB, Celeste Lopes M. The sensitizers nickel sulfate and 2,4-dinitrofluorobenzene increase CD40 and IL-12 receptor

- expression in a fetal skin dendritic cell line. *Biosci Rep* 2004; 24:191-202.
55. Jankićević J, Vesić S, Vukićević J, Gajić M, Adamić M, Pavlović MD. Contact sensitivity in patients with venous leg ulcers in Serbia: comparison with contact dermatitis patients and relationship to ulcer duration. *Contact Dermatitis* 2008; 58(1):32-36.
 56. Ablett E, Whiteman DC, Boyle GM, Green AC, Parsons PG. Induction of metallothionein in human skin by routine exposure to sunlight: evidence for a systemic response and enhanced induction at certain body sites. *J Invest Dermatol* 2003; 120:318-324.
 57. Cortese-Krott MM, Munchow M, Pirev E, Hessner F, Bozkurt A, Uciechowski P, et al. Silver ions induce oxidative stress and intracellular zinc release in human skin fibroblasts. *Free Radic Biol Med*. 2009; 47:1570-1577.
 58. McKim JM Jr, Liu J, Liu YP, Klaassen CD. Distribution of cadmium chloride and cadmium-metlothionein to liver parenchymal, Kupffer, and endothelial cells: their relative ability to express metallothionein. *Toxicol Appl Pharmacol* 1992; 112:324-330.
 59. Andrews GK. Cellular zinc sensors: MTF-1 regulation of gene expression. *Biometals* 2001; 14:223-237.
 60. Nakano H, Ikenaga S, Aizu T, Kaneko T, Matsuzaki Y, Tsuchida S, et al. Human metallothionein gene expression is upregulated by beta-thujaplicin: possible involvement of protein kinase C and reactive oxygen species. *Biol Pharm Bull* 2006; 29(1):55-59.
 61. Ohtsuiji M, Katsuoka F, Kobayashi A, Aburatani H, Hayes JD, Yamamoto M. Nrf1 and Nrf2 play distinct roles in activation of antioxidant response element-dependent genes. *J Biol Chem* 2008; 283:33554-33562.
 62. Leung L, Kwong M, Hou S, Lee C, Chan JY. Deficiency of the Nrf1 and Nrf2 transcription factors results in early embryonic lethality and severe oxidative stress. *J Biol Chem* 2003; 278:48021-48029.
 63. Chen LM, Kwong M, Lu R, Ginzing D, Lee C, Leung L, et al. Nrf1 is critical for redox balance and survival of liver cells during development. *Mol Cell Biol* 2003; 23:4673-4686.
 64. Api AM, Basketter DA, Cadby PA, Cano MF, Ellis G, Gerberick GF, et al. Dermal sensitization quantitative risk assessment (QRA) for fragrance ingredients. *Regul Toxicol Pharmacol* 2008; 52(1):3-23.
 65. Conney AH. Induction of drug-metabolizing enzymes: a path to the discovery of multiple cytochromes P450. *Annu Rev Pharmacol Toxicol* 2003; 43: 1-30.