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Keratinocyte gene expression profiles discriminate sensitizing and irritating compounds[$]

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ABSTRACT

Many chemicals can induce allergic contact dermatitis. Since evaluation of skin sensitizing potential by animal testing is prohibited for cosmetics, and screening of many chemicals is required within REACH, urgent need exists for predictive *in vitro* assays to identify contact allergens. Keratinocytes (KC) are the first cells encountered when chemicals land on the skin. Therefore, KC form an important site of haptenization and their metabolism is likely to be important. Moreover, KC secrete mediators that affect processing and presentation of haptenized proteins by dendritic cells. To develop a KC-based *in vitro* assay to predict sensitizing potential of chemicals, *in vitro* exposure effects of 8 contact sensitizers and 6 irritants on the KC cell line HaCaT were examined by gene profiling. Classifiers predictive of the class sensitizers or irritants were calculated, based on Support Vector Machine (SVM) and Random Forest (RF) algorithms. Classifiers using high-ranking genes were 70% (SVM) and 62% (RF) accurate, based on 3 (SVM) and 2-5 (RF) features. Classifiers using oxidative stress pathway gene sets were 68-73% (SVM) and 69-71% (RF) accurate. Cross-validation showed that the top-3 of most discriminating genes added up to 13 genes, and included oxidative stress gene *HMOX1* irrespective of the chemical left out. Moreover, *HMOX1* was the most significantly regulated gene. GSEA showed upregulation of "Keap-1 dependent" and "oxidative stress" gene lists. In conclusion, KC expression profiling can identify contact sensitizers, providing opportunities for non-animal testing for sensitizing potential. Moreover, our data suggest that contact sensitizers induce the oxidative stress pathway in KC.

INTRODUCTION

A large number of chemicals possess skin sensitizing potential. Repeated contact with such a chemical results in allergic contact dermatitis, the most prevalent form of immunotoxicity found in humans (Kimber et al., 2002). The sensitizing potential of chemicals is currently assessed in experimental animals. However, with the ban on animal testing for cosmetic ingredients according to the 7th Amendment to the European Union Cosmetics Directive and the evaluation of a wide range of chemicals within the European chemical legislative framework REACH, development of non-animal alternatives for the detection of sensitizing potential of chemicals is needed (reviewed by Vandebriel & Van Loveren, 2010). Validated *in vitro* assays for identification of contact allergens are not yet available. The cell types that are involved in the process of skin sensitization should provide an appropriate basis for such predictive screens (Jowsey et al., 2006; Basketter & Maxwell, 2007; Grindon et al., 2007).

Keratinocytes (KC) are the first cells encountered when chemicals when chemicals come into contact with the skin. Therefore, KC form an important site of haptenization and their metabolism is likely to be important. They respond to haptenization with secretion of mediators such as IL-1α, TNF-α, and GM-CSF. These mediators influence processing of haptenized proteins by dendritic cells (DC), migration of these cells to the draining lymph nodes (LN), and presentation of the haptenized proteins to T-cells (reviewed by Vandebriel et al., 2005). Studies by us and others have shown that KC cell lines can indeed be used to detect sensitizing capacity and, moreover, allow some potency determination (van Och et al., 2005)

and distinguish contact from respiratory allergens (Corsini et al., 2009). In order to identify biomarkers/reporter assays for skin sensitization based on responses of KC to contact allergen exposure, this response should be studied in more detail. A promising approach to study comprehensively the molecular mechanisms is gene expression profiling (Baken et al., 2007).

The oxidative stress pathway has been proposed as a crucial pathway induced by contact sensitizers (Natsch, 2009). Kelch-like ECH-associated protein 1 (Keap1) is a sensor protein that contains highly reactive Cys residues. In the absence of electrophiles (haptens) the basic leucine zipper transcription factor nuclear factor–erythroid 2-related factor 2 (Nrf2) is associated with Keap1. Keap1 anchors the Nrf2 in the cytoplasm, thereby targeting it for ubiquitination and proteasomal degradation. Upon electrophile binding Keap1 dissociates from Nrf2, resulting in translocation of Nrf2 to the nucleus, where it forms a heterodimer with small MAFs (Fig. 1). This heterodimer binds to antioxidant response elements (ARE), inducing transcription of genes that contain ARE in their promoter (Itoh et al., 1997; Motohashi et al., 2004; Katsuoka et al., 2005).

Here we describe the genome-wide transcriptional response of the human KC cell line HaCaT to 8 sensitizers and 6 irritants, and the calculation of classifiers based on the Support Vector Machine and Random Forest algorithms. Classifiers used either high-ranking genes or oxidative stress pathway genes. The prediction performance of classifiers was determined by leave-one-chemical-out cross-validation. Gene Set Enrichment Analysis was used to evaluate pathway enrichment.

MATERIALS AND METHODS

Cell culture

The human keratinocyte cell line HaCaT (Boukamp et al., 1988) was purchased from Cell Lines Service (Eppelheim, Germany). Cells were grown in culture flasks to 80% confluency in Dulbecco's modified Eagle's medium supplemented with 1% non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Gibco, Breda, the Netherlands), and 10% heat-inactivated Fetal Calf Serum (PAA, Linz, Austria) (complete medium) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were then trypsinized (0.05% Trypsin with EDTA 4Na; Gibco), washed in complete medium, and resuspended in fresh complete medium to a concentration of $3.33*10^5$ cells/ml. The cell suspension was seeded into 12-wells plates (1.5 ml per well; Greiner, Alphen aan den Rijn, the Netherlands). The cells were allowed to adhere and form a monolayer during 24 h, after which the wells were washed with PBS and exposed to the different chemicals in complete medium.

Chemical exposure

Table 1 shows the 8 sensitizers and 6 irritants used in this study. All chemicals were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands), except for Bandrowski's base (MP Biochemicals, Illkirch, France) and SDS (BDH Biochemicals, Poole, United Kingdom). All chemicals were dissolved in absolute ethanol, except $NiSO_4$, which was dissolved in distilled water due to its low solubility in ethanol. The final concentration of ethanol was 1%, and that of distilled water also 1%.

Chemical concentrations resulting in 80% cell viability after 24 h of exposure (CV80) were used (Table 1). They were determined for each chemical by colorimetric measurement of WST-1 cleavage. To this end, HaCaT cells were seeded in 12-well plates ($5*10^5$ cells/1.5 ml) and incubated with a concentration range of the chemicals or solvent control in triplicate at 37°C in a humidified atmosphere of 5% CO_2 in air. After 21 h of exposure, 100 µl/well WST-1 (Roche, Woerden, the Netherlands) was added and 3 h later WST-1 cleavage was quantified at 450 nm using a microplate reader (FLUOstar Galaxy and software v4.30-0; BMG Labtechnologies, Offenburg, Germany). After blank correction, the mean optical densities of the replicates were compared to the mean of the controls to calculate relative viability (data not shown). Viability of unexposed, distilled water-, and ethanol-exposed cells were similar, and (only) ethanol was included as a control in the microarray analysis. For each chemical, the cells were exposed to CV80 during both 4 h and 8 h to obtain samples for microarray analysis. The results from both time points were used in the subsequent analysis. Four independent replicate experiments were performed.

RNA isolation

At the end of the chemical exposure period medium was removed, cells were washed with PBS, and 600 µl RLT lysis buffer (Qiagen, Westburg, the Netherlands) was added per well. Cells were resuspended and lysates were stored at -80°C until further analysis. After homogenizing the lysates using QIAshredder columns, total RNA was isolated by using RNeasy mini kits in combination with an RNase-free

DNase Set (all from Qiagen) according to the manufacturer's instructions. RNA quantity was assessed spectrophotometrically (Nanodrop Technologies, Wilmington, DE) and integrity was determined by automated gel electrophoresis (Bioanalyzer 2000; Agilent technologies, Amstelveen, the Netherlands). From the four RNA replicates, based on concentration and RIN value three were chosen for subsequent generation of cRNA. So, for each chemical and the ethanol control, three 4 h-samples and three 8 h-samples were obtained. RNA samples were stored at -80°C.

Microarray analysis

Gene expression profiling was performed using Affymetrix U133 Plus 2.0 Arrays (Santa Clara, CA). For each sample, cDNA synthesis from 5 µg of total RNA and subsequent synthesis of biotin labeled cRNA was performed using GeneChip One-Cycle Target Labeling and Control Reagents including the One-Cycle cDNA Synthesis Kit, Poly-A RNA control kit, Sample Cleanup Module, IVT labeling kit, and Hybridization Control Kit (all from Affymetrix) according to the manufacturer's protocol. The yield of cRNA was measured spectrophotometrically (Nanodrop) and the integrity was determined using the Bioanalyzer before and after fragmentation of 15 µg of the labeled cRNA. Fragmented samples were hybridized to the arrays for 16 h at 45 °C (GeneChip Hybridization Oven 640; Affymetrix), after which the chips were washed and stained using the GeneChip Hybridization, Wash and Stain kit and the GeneChip Fluidics Station 450 (both Affymetrix). Chips were then scanned using the Affymetrix GeneChip Scanner 3000 7G and raw data were extracted using the

GeneChip Operating Software (Affymetrix). The scaling factor, background, percentage present spots, and housekeeping controls were checked for each chip.

Data analysis

Madmax

Quality control of microarray data was performed using BioConductor packages (including simpleaffy and affyplm), through the R pipeline which can be accessed via Genepattern (de Groot et al., 2008).

Pre-processing of gene expression data

Genepattern (Reich et al., 2006) was used for pre-processing the data. They were normalized using Robust Multichip Average (RMA), employing the ExpressionFileCreator module. RMA is a common algorithm for converting raw Affymetrix data to gene expression values. In addition, the MBNI Custom CDF, which contains updated probe set definitions for Entrez GeneIDs was applied: http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/11.0.1/entrezg.asp. After normalization, for each probe set, log-ratios were created by log (10) transformation, followed by correcting the value for each sample by the average of the time-matched ethanol control. These log-ratios were converted to fold induction.

Class prediction

Within the R statistical software environment, the Support Vector Machines (SVM; Rifkin et al., 2003) and Random Forest (RF; Breiman, 2001) algorithms were used for class prediction. While SVM is based on hyperplane separation, RF is based on a prediction network. Since it was unknown beforehand which of them would perform better on the current data set, both algorithms were employed. Moreover, using classifiers based on different algorithms allowed verification of the biological robustness across these classifiers. For classification of the complete set of chemicals, SVM was applied using a linear kernel on non-scaled data, while for classification of the strong and weak chemicals separately (see Table 4), SVM was applied using a radial kernel on scaled data.

The data (log-ratios) were split into a training and a test set. The training set data comprised the data for all-but-one chemical, and the test set the data for the remaining chemical. This type of cross-validation, denoted leave-one-chemical-out cross-validation, keeps replicate samples together. For the training set features (probe sets) were selected by applying a student's t-test using the two classes (sensitizers and irritants), where different numbers of top-ranking features were tested for use in the classifier. Alternatively, probe sets used in the classifier were based on pre-selected pathways that have been described to be involved in sensitization by low molecular weight chemicals (i.e. oxidative stress, MAPK, and PTEN). Genes involved in these pathways were taken from Gene Ontology, BioCarta, and WikiPathways (not shown). The prediction accuracy for the test set samples

was determined. The percentage of correct classifications was calculated as the average of all 14 predictions.

The most predictive set of genes of every prediction was combined to a biomarker set. The recursive support vector machine (R-SVM) algorithm (Zhang et al., 2006) was used to the select, within the given gene set, the smallest set of biomarkers without losing prediction accuracy.

Gene set enrichment analysis

In Gene Set Enrichment Analysis (GSEA) *a priory* sets of genes are tested in their relative position in sorted lists of gene log-ratios. Sorting is done within the GSEA software (http://www.broadinstitute.org/gsea/) based on the difference in log-ratios of samples between phenotypes (here: sensitizers and irritants). Gene sets of which the genes are located more towards the top or the bottom of the ordered list can be considered up- or down-regulated, respectively, in one phenotype compared to the other one.

In the current study, GSEA was performed to assess whether indeed the oxidative stress, MAPK, and PTEN pathways were significantly regulated. In this way the *a priory* choice of the pathways as features for classification was verified.

RESULTS

Cell viability

Cells were seeded in 12-well plates and exposed to a concentration range of each of the chemicals indicated in Table 1. After 24 h, viability was assessed by measuring WST-1 cleavage. Based on these data, the concentrations that resulted in 80% viability after 24 h of exposure (CV80) were chosen, and used in the microarray experiments (Table 1).

Data analysis

Class prediction

SVM and RF were used to calculate classifiers. When the classifiers were based on high-ranking genes in a t-test the optimal number of features was 3 when SVM was used, and 2-5 when using RF, and the class prediction accuracy decreased with an increasing number of probe sets (see Fig. 2). Using SVM and RF the accuracy of class prediction was 70% and 62%, respectively.

Since the most significant differently expressed gene between sensitizers and irritants, *HMOX1*, is Nrf2-dependent, we chose two gene sets, one compiled from literature as being Nrf2-dependent and one listed as being significantly upregulated by Keap1 gene knockdown in the HaCaT cell line (MacLeod et al., 2009). The gene sets are listed in Tables 2A and 2B, respectively. When the prediction was based on the literature-compiled oxidative stress pathway gene set, its accuracy was 73% and 69% (SVM and RF, respectively) and when the prediction was based on the MacLeod gene set, its accuracy was 68% and 71% (SVM and RF, respectively).

In an attempt to evaluate the prediction accuracy of the oxidative stress pathway, a comparison was made to (the prediction accuracy of) other pathways implicated in sensitization by low molecular weight sensitizers, being the MAPK and PTEN pathways. The MAPK pathway has been implicated in the response of DC to contact allergens (Trompezinski et al., 2008), while the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) pathway was revealed in the response of a bronchial epithelial cell line to respiratory sensitizers (Verstraelen et al., 2009). Their respective accuracies were only 32% and 30% (SVM), and 41 and 43% (RF), so well below the accuracies based on high-ranking genes or using the oxidative stress pathway gene sets.

We assumed that certain chemical-specific characteristics such as being a sensitizer or irritant, or its CV80 value, might influence the contribution to the prediction model. To assess this contribution, the number of genes that was significantly ($P < 0.001$) different between sensitizers and irritants was established for each left-out chemical, the rationale being that a lower number of genes would implicate a higher contribution of the left-out chemical. The chemicals were ranked by number of genes (Table 3A). The 6 highest-ranking chemicals, corresponding to the lowest numbers of genes and thus contributing most to the prediction model, were 3 sensitizers and 3 irritants. In the *in vitro* exposure experiments, these chemicals showed the lowest CV80 values. This suggests that chemicals with a low CV80 value contribute most to the prediction model and that this holds for both sensitizers and irritants. After omitting the data on the metal salt $NiSO_4$, being the only inorganic sensitizer tested, a significant ($P = 0.002$) correlation (r = 0.938; 2-

sided Pearson correlation; SPSS, Chicago, IL) between LLNA EC3 values (converted to mM) and the exposure concentrations *in vitro* was found (Fig. 3).

The finding that the optimal number of probe sets is 3 (SVM), implicates that for classification it is sufficient to measure the top-3 of most discriminating genes per chemical (Table 3B). This means that measuring the 13 genes *CCDC43*, *COL16A1*, *HMOX1*, *HOXD10*, *HSPA1A*, *HSPA1B*, *IL6R*, *OSGIN1*, *SPSB1*, *SNX16*, *UGDH*, *USP28*, and *ZFAND5* is sufficient for classification. Notably, *HMOX1* is in this list for every chemical left out, again suggesting the importance of the oxidative stress pathway. Besides that, the top-3 was partly different for each chemical left out. Using recursive SVM feature selection, we found that all 13 genes were required for classification.

Since sensitizers and irritants that are "strong" *in vitro* contributed most to the prediction model (described above), next to the classification accuracy for all chemicals the accuracy was also determined for "strong" *in vitro* sensitizers and irritants only, and for "weak" *in vitro* sensitizers and irritants only (Table 4). In this way, remarkably, "strong" *in vitro* sensitizers and "weak" *in vitro* sensitizers (defined here as having a CV80 lower or higher than 0.5 mM, respectively) were classified with 85% and 73% accuracy, respectively, based on the top-3 for each classification.

Gene Set Enrichment Analysis

After a False Discovery Rate (FDR) correction of 5%, GSEA showed significant upregulation of the "Keap1 dependent" gene list (Table 2B) in sensitizers vs.

irritants, sensitizers vs. ethanol controls, and irritants vs. ethanol controls. In addition, this analysis showed significant upregulation of the "oxidative stress consensus" (Table 2A), MAPK, and PTEN gene lists in sensitizers vs. irritants, sensitizers vs. ethanol controls, but not irritants vs. ethanol controls.

It may be suggested that, possibly, pro-haptens and haptens can be discriminated based on gene expression profiling. GSEA showed, however, that after FDR correction (5%, and even 25%), no processes were significantly regulated between the prohaptens CA, Eug, and Isoeug, and the haptens BB, DNCB, HCA, and TETD.

DISCUSSION

Here we have shown that gene expression profiling after *in vitro* exposure of the KC cell line HaCaT to 8 sensitizers and 6 irritants resulted in a classification model based on the SVM algorithm, that was 70% accurate in predicting chemicals as sensitizers or irritants using 3 features. A classification model based on the RF algorithm, was 62% accurate using 2-5 features. This means that using the SVM-based model 70% accuracy is obtained by measuring the top-3 of most discriminating genes per chemical, adding up to 13 genes. *HMOX1* was among the top-3 of most discriminating genes irrespective of the chemical left out.

HMOX1 is well known for its dependency on Nrf2, a critical component of the oxidative stress pathway. This finding prompted us to perform predictions based on oxidative stress gene sets. One gene set was compiled by us from literature (19 genes, Table 2A) and another gene set comprised the genes that were significantly upregulated by Keap1 gene knockdown in HaCaT cells (MacLeod et al., 2009), Keap1 being the sensor molecule in the oxidative stress pathway (18 genes, Table 2B). Using SVM the prediction accuracy was similar (around 70%) compared to the original prediction (i.e. without using pathway information), while using RF the accuracy increased from 62% to 70%. Remarkably, when the number of probe sets used in the pathway-based prediction models (18 or 19) would have been used in the original prediction models the prediction accuracy would have been only 50%, as can be seen in Fig. 2. This may suggest that the models using pathway information are more robust.

Since "strong" *in vitro* sensitizers and irritants contributed most to the prediction model, the classification accuracy was also determined for "strong" *in vitro* sensitizers and irritants only, and for "weak" *in vitro* sensitizers and irritants only (Table 4). Grouping these chemicals based only on CV80 values resulted in classification of "strong" *in vitro* and "weak" *in vitro* sensitizers with 85% and 73% accuracy, respectively. It may be suggested that strong and weak sensitizers both induce the oxidative stress pathway, while additional pathways (of unknown nature) are differentially induced. A computer model for sensitization underlines this notion, since the relative contributions of individual pathways were likely to vary with sensitizing potency (Maxwell & Mackay, 2008). Next, while the irritants SDS and isopropanol have similar CV80 values, SDS is accurately predicted using the panel of "weak" in vitro sensitizers and irritants, while isopropanol is accurately predicted using the other panel. This may suggest that for irritants a CV80-threshold may not be as straightforward as for sensitizers. Testing additional chemicals is required to settle this issue. In any case, the prediction accuracies for sensitizers are promising.

The observed correlation between EC3 and CV80 values cannot be explained in a straightforward manner, since the underlying mechanisms of sensitization (hapten binding) and cytotoxicity *in vitro* are likely to be very different. Nonetheless, a weak albeit significant association exists between contact sensitization potency and skin irritancy (Auton et al., 1995; Basketter et al., 2007).

We exposed the cells at chemical concentrations that induced 80% viability, suggesting that the cells experience oxidative stress that is not necessarily related to

sensitizing activity. This "background" induction of the oxidative stress response may mask weak sensitizing activity. Therefore, in future follow-up experiments we will also expose cells to chemical concentrations that do not induce cell death.

Although Ni^{++} differs from DNCB in the activation of the MAPK signaling pathways p38 MAPK, ERK, and JNK, at least in DC (Aiba et al., 2003; Ade et al., 2007; Trompezinski et al., 2008), both chemicals induced the oxidative stress pathway in HaCaT cells. This latter finding may be explained by the fact that both chemicals induced p38 MAPK (Aiba et al., 2003). Several studies have shown that the p38 MAPK inhibitor SB203580 inhibited Nrf2 translocation, although, unfortunately, none of these studies investigated sensitizers.

Our finding that the oxidative stress pathway is a major pathway induced by contact sensitizers in the KC cell line HaCaT is in agreement with reports by others for $CD34^{+}$ DC, MoDC, and the THP-1 monocyte cell line (Bruchhausen et al., 2003; Ryan et al., 2004; Ade et al., 2009). The major role of this pathway after contact sensitizer exposure has recently been reviewed (Natsch, 2009). As a corollary, an assay to identify sensitizers has been developed that uses an ARE reporter construct (Natsch and Emter, 2008). Because it makes use of the ARE, it is a pathway-specific reporter assay and not a gene-specific reporter assay, and may therefore be regarded as similar to the KC assay at the pathway level, but not (necessarily) at the level of (a) specific gene(s). After testing 102 chemicals, the accuracy of the reporter assay was found to be 83%. From a statistical point of view, it is too early to compare the classification performance of the reporter assay based on 102 chemicals with the KC database based on 14 chemicals, with (only) 9 chemicals

overlapping. This head-to-head comparison awaits more chemicals to be tested in the KC assay.

A widely used method to detect sensitizing capacity *in vitro* is by measuring DC maturation (reviewed by Casati et al., 2005; Ryan et al., 2007). DC have also been used in combination with microarray analysis, allowing identification of not only strong allergens, but also moderate and weak ones (Cluzel-Tailhardat et al., 2007; Schoeters et al, 2007), showing that weak sensitizer potency is not an obstacle for successful expression analysis. Based on 13 differentially expressed genes contact sensitizers could be discriminated from irritants (Hooyberghs et al., 2008). A drawback of Hooyberghs' model is that it requires $CD34^+$ progenitor-derived DC and thus cord blood. Replacement of $CD34^+$ DC by the THP-1 cell line resulted, unfortunately, in a poorer prediction (Lambrechts et al., 2009), leaving sufficient room for other avenues to detect sensitizing capacity using gene profiling in conjunction with human cell lines.

There is no overlap between the 13 genes identified by Hooyberghs et al. (2008) and the 13 genes identified in the current study. While in the KC cell line HaCaT, *HMOX1* is in the top-3 of most discriminating genes irrespective of which chemical is left out, this gene was not observed as being discriminative between sensitizers and non-sensitizers in dendritic cells (Hooyberghs et al., 2008) and the THP-1 cell line (Lambrechts et al., 2009). Others, however, did find *HMOX1* to be upregulated by sensitizers in both dendritic cells and the THP1 cell line (Ade et al., 2009). This may suggest that overlap (or a lack thereof) between lists of genes is not straightforward.

The assay described in the present study is not yet sufficiently developed for predictive testing of single chemicals. We aim to test a larger number of chemicals in a follow-up study, in order to determine better the assay's capability to identify moderate and weak antigens, which is a critical characteristic for its utility. Next, extending the panel of chemicals tested is required for a head-to-head comparison of sensitivity and specificity with other assays that measure sensitizing capacity. Moreover, this should clarify whether the assay may surpass an accuracy of 70%, since this percentage would not seem sufficient for the assay to enter formal validation. Whether increasing the accuracy by grouping chemicals based on CV80 values, is more generally valid should also be revealed. If the results of our follow-up studies are favorable, the assay can be taken to pre-validation and validation.

R-SVM showed that all 13 genes were required for classification. So, the KC assay cannot be readily used in conjunction with PCR in the present development phase.

In conclusion, gene expression profiling after *in vitro* exposure of the KC cell line HaCaT resulted in a classification model that was 70% accurate in distinguishing sensitizers from irritants. The oxidative stress pathway gene *HMOX1* was most significantly different between sensitizers and irritants. *A priori* lists of oxidative stress related genes gave similar or higher prediction accuracies. The top-3 of most discriminating genes for each chemical left out added up to 13 genes, and included *HMOX1* irrespective of the chemical left out. GSEA showed upregulation of "Keap-1 dependent" and "oxidative stress" gene lists. Remarkably, classification accuracy improved when the chemicals were grouped based on their CV80.

SUPPLEMENTARY DATA

Supplementary data (microarray data, ^{10}log ratio to vehicle) is attached to this manuscript (Vandebriel_Supplementary_Data.xls).

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Table 1. Sensitizers and irritants for which transcriptional effects in the HaCaT cell line were studied. LLNA EC3 values were taken from the NICEATM-ICCVAM website at http://iccvam.niehs.nih.gov/methods/immunotox/llna-panelDocs.htm .

	Abbre-viation	CAS no	Conc. (µM)	LLNA EC3 (%)
Sensitizers				
Bandrowski's base	BB	20048-27-5	17	0.04
2,4-Dinitrochlorobenzene	DNCB	97-00-7	20	0.05
Tetraethylthiuramdisulfide	TETD	97-77-8	62	1.4
Alpha-hexylcinnamaldehyde	HCA	101-86-0	140	11
Isoeugenol	IsoEug	97-54-1	540	1.2
Eugenol	Eug	97-53-0	1100	13
Cinnamyl alcohol	CA	104-54-1	2500	21
Nickel sulfate	$NiSO_4$	10101-97-0	4200	2.5
Irritants				
Lactic acid	LA	50-21-5	40	N/A
Hexane	Hex	110-54-3	45	N/A
Sodium dodecyl sulfate	SDS	151-21-3	240	N/A
Isopropanol	Iso	67-63-0	247	N/A
Methyl salicylate	MS	119-36-8	2800	N/A
Benzoic acid	BAc	65-85-0	4250	N/A

Table 2A. "Oxidative stress consensus" gene list. This gene list has been compiled from http://www.biocarta.com/pathfiles/h_ARENRF2PATHWAY.asp, http://www.wikipathways.org/index.php/Pathway:WP3, and http://www.wikipathways.org/index.php/Pathway:WP408. In the latter pathway, only the "Activation" part was used. From this gene list, GPX1 (GeneID 2876), GSTA2 (GeneID 2939), and GSTT2 (GeneID 2953) were not on the array. GeneID's are taken from the NCBI Entrez Gene website at http://www.ncbi.nlm.nih.gov/gene/.

GeneID	Symbol	Gene name
847	CAT	catalase
2729	GCLC	glutamate-cysteine ligase, catalytic subunit
2730	GCLM	glutamate-cysteine ligase, modifier subunit
2878	GPX3	glutathione peroxidase 3 (plasma)
2936	GSR	glutathione reductase
3162	HMOX1	heme oxygenase 1
23764	MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog f
4097	MAFG	v-maf musculoaponeurotic fibrosarcoma oncogene homolog g
7975	MAFK	v-maf musculoaponeurotic fibrosarcoma oncogene homolog k
4257	MGST1	microsomal glutathione s-transferase 1
4501	MT1X	metallothionein 1x
1728	NQO1	nad(p)h dehydrogenase, quinone 1
6647	SOD1	superoxide dismutase 1, soluble
6648	SOD2	superoxide dismutase 2, mitochondrial
6649	SOD3	superoxide dismutase 3, extracellular
25828	TXN2	thioredoxin 2
7296	TXNRD1	thioredoxin reductase 1
10587	TXNRD2	thioredoxin reductase 2
54578	UGT1A6	udp glucuronosyltransferase 1 family, polypeptide a6

Table 2B. "Keap1 dependent" gene list. Genes affected by Keap1 gene knockdown (MacLeod et al., 2009) are listed. All genes from this list were on the array. GeneID's are taken from the NCBI Entrez Gene website at http://www.ncbi.nlm.nih.gov/gene/.

GeneID	symbol	Gene name
231	AKR1B1	aldo-keto reductase family 1, member b1
57016	AKR1B10	aldo-keto reductase family 1, member b10
1645	AKR1C1	aldo-keto reductase family 1, member c1
8644	AKR1C3	aldo-keto reductase family 1, member c3
2235	FECH	ferrochelatase
2512	FTL	ferritin, light polypeptide
2539	G6PD	glucose-6-phosphate dehydrogenase
2729	GCLC	glutamate-cysteine ligase, catalytic subunit
2730	GCLM	glutamate-cysteine ligase, modifier subunit
2936	GSR	glutathione reductase
3162	HMOX1	heme oxygenase 1
4097	MAFG	v-maf musculoaponeurotic fibrosarcoma oncogene homolog g
4199	ME1	malic enzyme 1, nadp(+)-dependent, cytosolic
1728	NQO1	nad(p)h dehydrogenase, quinone 1
5226	PGD	phosphogluconate dehydrogenase
22949	PTGR1	prostaglandin reductase 1
140809	SRXN1	sulfiredoxin 1 homolog
7296	TXNRD1	thioredoxin reductase 1

Table 3A. Ranking of chemicals by number of genes significantly different between sensitizers and irritants. In the leave-one-out cross-validation process, depending on the chemical left out a certain number of genes was significantly different between sensitizers and irritants. S, sensitizer; I, irritant. Conc. (μM), *in vitro* exposure concentration (see Table 1).

Chemical left out	Number of genes	S/I	Conc. (μM)
TETD	33	S	62
LA	80	I	40
Iso	85	I	247
DNCB	87	S	20
Hex	96	I	45
BB	104	S	17
IsoEug	149	S	540
HCA	163	S	140
Eug	185	S	1100
$NiSO_4$	192	S	4200
CA	250	S	2500
SDS	280	I	240
MS	331	I	2800
BAc	490	I	4250

Table 3B. **Top-3 of most discriminating genes for each chemical left out.** In the leave-one-compound-out cross-validation process, depending on the chemical left out a certain number of genes was significantly different between sensitizers and irritants (see Table 3A). For each chemical left out, the top-3 of most discriminating genes is shown. For each gene, the fold induction is indicated. *HMOX1* is in the top-3 irrespective of which chemical is left out.

Chemical left out	Gene	Fold-induction	Gene	Fold-induction	Gene	Fold-induction
TETD	*SPSB1*	2,9	*HMOX1*	77,1	*HSPA1B*	2,6
LA	*HMOX1*	1,8	*HSPA1B*	1,1	*HSPA1A*	1,1
Iso	*HOXD10*	0,8	*HSPA1B*	1,0	*HMOX1*	1,0
DNCB	*SPSB1*	1,4	*HMOX1*	34,9	*IL6R*	1,2
Hex	*HSPA1B*	1,0	*HMOX1*	1,2	*UGDH*	1,1
BB	*HSPA1B*	1,1	*HMOX1*	5,1	*USP28*	1,0
IsoEug	*HMOX1*	1,3	*OSGIN1*	1,0	*ZFAND5*	1,0
HCA	*HSPA1B*	1,2	*HMOX1*	9,9	*SPSB1*	1,3
Eug	*HMOX1*	3,3	*HSPA1B*	1,1	*COL16A1*	1,1
Ni	*HSPA1B*	1,0	*HMOX1*	3,8	*SPSB1*	1,2
CA	*HMOX1*	1,6	*ZFAND5*	0,9	*UGDH*	0,9
SDS	*ZFAND5*	1,1	*HMOX1*	1,3	*SNX16*	1,4
MS	*HMOX1*	1,0	*ZFAND5*	1,0	*SPSB1*	1,0
BAc	*CCDC43*	1,1	*HMOX1*	1,1	*HSPA1B*	0,9

Table 4. **Number of correctly classified replicate samples for each chemical.** In the classification model, using all chemicals 70% was correctly classified (59/84), using the four strongest sensitizers and irritants 85% was correctly classified (41/48), and using the four weakest sensitizers and irritants 73% was correctly classified (35/48). The number of correctly classified replicate samples is shown (number of replicate samples per chemical = 6).

Chemical	Correct All	Correct Strong	Correct Weak
BB	4	6	
DNCB	6	6	
TETD	6	6	
HCA	5	5	
IsoEug	1		5
Eug	2		5
CA	1		5
NiSO4	2		4
LA	4	5	
HEX	5	6	
SDS	6	1	6
PROP	5	6	0
MS	6		5
BAC	6		5

Figure 1. ARE Nrf2 pathway.

http://www.biocarta.com/pathfiles/h_ARENRF2PATHWAY.asp

Figure 2. Class prediction accuracy plotted against the number of probe sets. Classifiers were calculated using SVM and RF, based on high-ranking probe sets in a t-test.

Figure 3. Sensitizer EC3 values (mM) plotted against the CV80 values (mM). After omitting the data on $NiSO_4$ a significant (P = 0.002) correlation (r = 0.938) between EC3 and CV80 values is seen. For clarity, data are plotted on log scales.

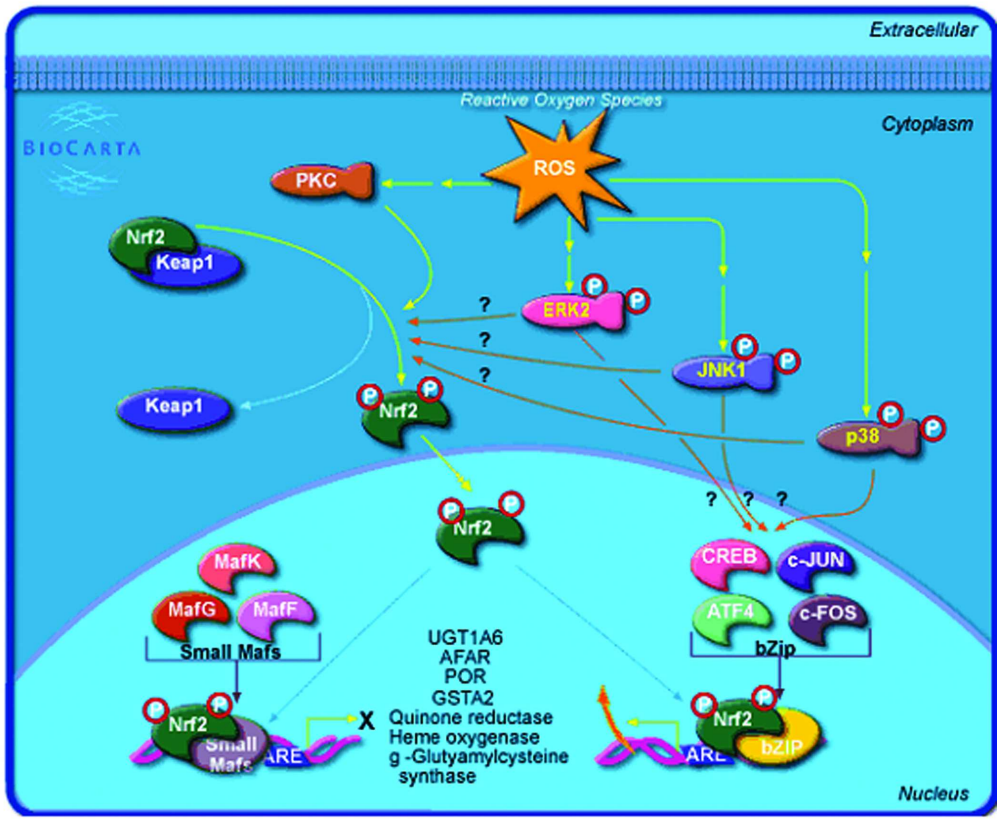

Figure 1. ARE Nrf2 pathway.
177x150mm (300 x 300 DPI)

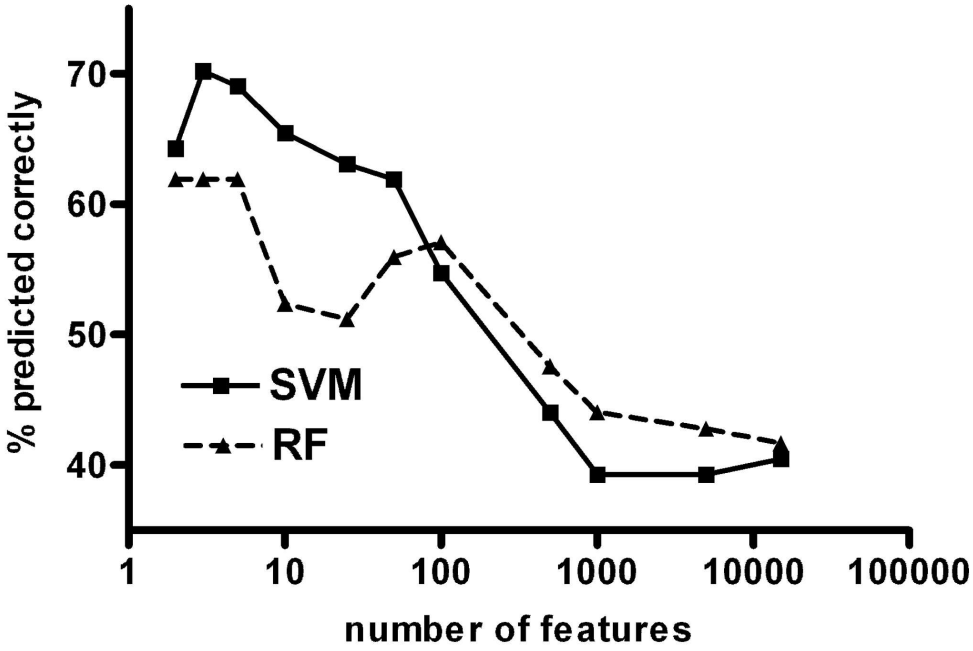

Figure 2. Class prediction accuracy plotted against the number of probe sets. Classifiers were calculated using SVM and RF, based on high-ranking probe sets in a t-test.
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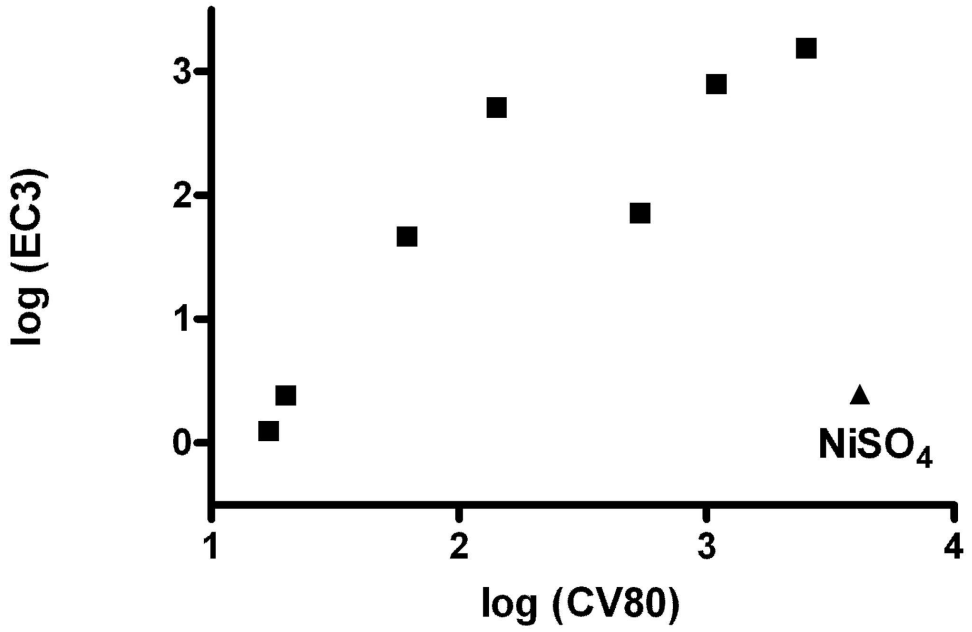

Figure 3. Sensitizer EC3 values (mM) plotted against the CV80 values (mM). After omitting the data on NiSO4 a significant (P = 0.002) correlation (r = 0.938) between EC3 and CV80 values is seen. For clarity, data are plotted on log scales.
88x59mm (600 x 600 DPI)