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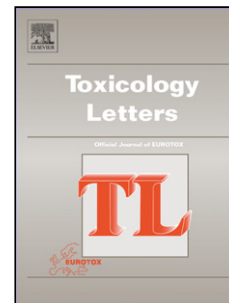
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Gene profiles of a human alveolar epithelial cell line after *in vitro* exposure to respiratory (non-)sensitizing chemicals: identification of discriminating genetic markers and pathway analysis.

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Short title: Transcriptomics-based markers and pathways discriminating respiratory sensitizing from respiratory non-sensitizing chemicals in A549 cells.

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Abbreviations

3D: 3-dimensional

ACR: acrolein

ATCC: American Type Culture Collection

cDNA: complimentary DNA

cRNA: complimentary RNA

Cy: cyanine

DNCB: 1-chloro-2,4-dinitrobenzene

DMSO: dimethylsulfoxide

ERK: extracellular signal-regulated kinase

FDR: false discovery rate

GO: Gene Ontology

GOFFA: Gene Ontology For Functional Analysis

HCpt: ammonium hexachloroplatinate IV

HDI: hexamethylene diisocyanate

IC20: 20% cell growth inhibition

Ig: immunoglobulin

IPA: Ingenuity Pathways Analysis

LFC: logarithmic fold change

MAPK: mitogen-activated protein kinase

MeSA: methyl salicylate

PXR: pregnane X receptor

REACH: Registration, Evaluation and Authorization of CHemicals

RXR: retinoid X receptor

SAM: Significance Analysis of Microarrays

TMA: trimellitic anhydride

Abstract

There are currently no accepted biological prediction models for assessing the potential of a substance to cause respiratory sensitization. New tests should be based on mechanistic understanding and should be preferentially restricted to *in vitro* assays. The major goal of this study was to investigate the alterations in gene expression of human alveolar epithelial (A549) cells after exposure to respiratory sensitizing and non-respiratory sensitizing chemicals, and to identify genes that are able to discriminate between both groups of chemicals. A549 cells were exposed during 6, 10, and 24 hours to the respiratory sensitizers ammonium hexachloroplatinate IV, hexamethylene diisocyanate, and trimellitic anhydride, the irritants acrolein and methyl salicylate, and the skin sensitizer 1-chloro-2,4-dinitrobenzene. Overall changes in gene expression were evaluated using Agilent Whole Human Genome 4x44K oligonucleotide arrays. A Fisher Linear Discriminant Analysis was used to obtain a ranking of genes that reflects their potential to discriminate between respiratory sensitizing and respiratory non-sensitizing chemicals. Among the 20 most discriminating genes, which were categorized into molecular and biological Gene Ontology (GO) terms, *CTLA4* could be associated with asthma and/or respiratory sensitization. When categorizing the top-1000 genes into biological GO terms, 22 genes were associated with immune function. Using a pathway analysis tool to identify possible underlying mechanisms of respiratory sensitization, no known canonical signaling pathway was observed to be activated in the A549 cell line.

1. Introduction

Development of respiratory allergy to low-molecular weight industrial compounds (e.g. ammonium hexachloroplatinate IV (HCpt), hexamethylene diisocyanate (HDI), and trimellitic anhydride (TMA)) can trigger the onset of occupational asthma (Boverhof *et al.*, 2008).

The new European Union rules regarding Registration, Evaluation and Authorization of Chemicals (REACH) require widespread toxicological safety testing of existing and new chemicals. Given the enormity of new and already in-service chemicals that fall under this new rule, obtaining toxicological data on respiratory allergy has unique challenges, not least because there are no universal or accepted test systems available for the identification and characterization of chemicals that have the potential to cause allergic sensitization of the respiratory tract. This is attributable, at least in part, to the fact that there is uncertainty about the exact mechanisms through which respiratory sensitization can be acquired. There remains controversy about the roles played by immunoglobulin (Ig) E antibody-mediated mechanisms, and whether IgE represent a mandatory universal requirement for the induction by chemicals of allergic sensitization of the respiratory tract. Preferentially the use of animals should be kept to a minimum and *in vitro* systems should be included in hazard assessment (Verstraelen *et al.*, 2008a).

It is also of interest to search for relevant endpoints in the context of respiratory sensitization. New tests should be based on the mode of action of chemicals and should focus on toxicity pathways. For this purpose, transcriptomics, proteomics, and metabolomics are promising tools to better understand the underlying molecular and biological mechanisms through which allergic sensitization to chemicals is induced and regulated.

In the present study, we aimed to find genetic markers that are able to distinguish respiratory sensitizers from respiratory non-sensitizing chemicals using a transcriptomics approach. Alveolar epithelial cells were chosen as a test system to develop a transcriptomics-based *in vitro* assay. A549 cells exhibit many features of alveolar type II cells, which are likely to be involved in immunotoxicity (Verstraelen *et al.*, 2008a). These cells have been used extensively as a model to study alveolar reactivity to various stimuli. Following *in vitro* exposure of A549 cells to the respiratory sensitizer HDI, keratin 18, the 78-kD glucose-regulated protein, trans-1,2-dihydrobenzene-1,2-diol dehydrogenase, and actin were identified as prominent diisocyanate-conjugated proteins through use of a combination of immunocytochemical and mass spectrometric techniques. The data from this study identified proteins as potential carriers for diisocyanates *in vivo*, and hypothesized that HDI conjugation of these proteins may play a role in the pathogenesis of diisocyanate-induced asthma (Wisniewski *et al.*, 2000).

Another study using the A549 cell model showed that glutathione plays an important role in protection against HDI exposure and may help prevent the development of allergic sensitization and asthma (Wisnewski *et al.*, 2005).

Here, A549 cells were exposed to 3 respiratory sensitizers (HCpt, HDI, and TMA) and 3 respiratory non-sensitizers (acrolein (ACR), methyl salicylate (MeSA), 1-chloro-2,4-dinitrobenzene (DNCB)). Gene expression was studied and the top-20 most discriminative genes in this cell model are presented. Furthermore, using Ingenuity Pathways Analysis (IPA) epithelial cell-specific signaling pathways which can contribute to a better understanding of underlying molecular and biological mechanisms of respiratory sensitization were identified based on the resulting gene patterns.

2. Materials and Methods

Cell culture

The alveolar epithelial A549 cell line was obtained from American Type Culture Collection (ATCC number: CCL-185, LGC Promochem, Teddington, UK). A549 cells were cultured in complete cell culture medium containing 89% RPMI 1640 Medium with GlutaMAXTM-1 and 25 mM HEPES (Invitrogen, Paisley, UK), 10% fetal bovine serum (Biochrom AG, Berlin, Germany) and 1% penicillin (5000 U/ml)-streptomycin (5000 µg/ml) (Invitrogen), and were kept in a humidified atmosphere at 37°C and 5% CO₂. Before reaching 90% confluence, cells were subcultured using Trypsin (500 mg/l) – Versene (200 mg/l) (EDTA) solution (Lonza, Verviers, Belgium). Cells were seeded in 6-well plates at 0.5×10^6 cells/ml/well complete cell culture medium for 24 hours before exposure to the test substance (Auwerx, 1991).

Chemical exposure of the cells

A549 were exposed in 6-well plates to the respiratory sensitizers ammonium hexachloroplatinate IV (HCPT, 485 µM), hexamethylenediisocyanate (HDI, 386 µM), and trimellitic anhydride (TMA, 450 µM), the irritants acrolein (ACR, 170 µM) and methyl salicylate (MeSA, 822 µM), and the contact sensitizer 1-chloro-2,4-dinitrobenzene (DNCB, 150 µM) (all from Sigma-Aldrich Chemie GmbH) for 6, 10, and 24 hours. The exposure concentrations yielded 20% cell growth inhibition (IC₂₀) at 24 hours, as determined by neutral red uptake experiments (figure 3), and were chosen based on previous experiments which showed that a weak distortion of the cells could trigger the process of sensitization (Hulet et al., 2005; Jacobs et al., 2006; Hooyberghs et al., 2008). No reduction in cell growth was found for TMA and MeSA and therefore the highest soluble concentration was used for these chemicals. For each chemical and its corresponding solvent, 3 independent experiments were performed. The chemicals HDI, TMA, ACR, MeSA, and DNCB were dissolved in dimethylsulfoxide (DMSO, LabScan Ltd., Dublin, Ireland). The final concentration of DMSO in the culture medium was 0.01% for ACR and 0.05% for the other chemicals. These concentrations of the solvent did not reduce cell growth (data not shown). The respiratory sensitizer HCPT was dissolved in growth medium. Negative control samples were obtained by treating A549 cells from the same experiment with the respective solvent for the same time periods.

RNA isolation

After the appropriate exposure times, the supernatants of the cells was removed and the cells were lysed in the plates with RNA lysis buffer, consisting of 50% (v/v) buffer saturated phenol (Invitrogen), 50% (v/v) RLT buffer (Qiagen, Hilden, Germany), and 0.25 mg/ml 8-hydroxyquinolin (Merck, Brussels, Belgium). Total RNA was isolated using mini RNeasy RNA isolation kits, according to the manufacturer's specifications (Qiagen). The RNA concentration was determined using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, USA) and quality was visually inspected for absence of degradation using agarose gel electrophoresis. RNA was stored in RNase-free water (Qiagen) at -80°C.

Microarray hybridization

For hybridization onto Agilent Whole Human Genome 4x44K 60mer oligonucleotide arrays (G4112F, Agilent Technologies, Palo Alto, CA), 1 µg of total RNA was used in the Agilent LIRAK PLUS, two-colour Low RNA input Linear Amplification method, according to the manufacturer's instructions. Briefly, first the total RNA was reverse transcribed into complimentary DNA (cDNA) using T7-promotor primer and MMLV reverse transcriptase. The cDNA was transcribed into complimentary RNA (cRNA), during which it was fluorescently labelled by incorporation of cyanine (Cy)5-CTP (exposed samples) or Cy3-CTP (negative control samples). After purification, using the RNeasy mini kit (Qiagen), cRNA yield and Cy incorporation efficiency (specific activity) into the cRNA were determined using a NanoDrop Spectrophotometer (NanoDrop Technologies). cRNAs showing a yield >825 ng and a specific activity of 8-20 pmol/µg cRNA were selected for further processing. Equal amounts of the exposed and negative control sample were competitively hybridized onto Agilent Whole 4x44K Human oligonucleotide arrays in a hybridization oven at 60°C for 17 hours. Slides were washed according to the manufacturer's instructions with washing buffers and finally dipped in Stabilization and Drying Solution (Agilent Technologies) to protect them from environmental ozone. The arrays were scanned on an Agilent scanner (G2565BA) and further processed using Agilent Feature Extraction Software (version 9.5.1). The software automatically finds and places microarray grids, rejects outlier pixels, accurately determines feature intensities and ratios, flags outlier pixels, and calculates statistical confidences. For the two-color microarrays, gProcessedSignal values from Agilent's Feature Extraction software were used as input into experimental analyses and includes additional preprocessing to adjust

for possible dye bias within a microarray. Data used in the two-color analyses was either the red and green ProcessedSignal or LogRatio values. Dye normalization includes both linear scaling and Lowess normalization to a rank invariant set of microarray features. Further details on the data processing steps used to generate the Agilent two-color output can be found in the Agilent protocol GE2-v5_95_Feb07.

The resulting data set contains the expression values of each spotted gene in 3 independent biological experiments per exposure condition (6, 10, 24 hours).

Statistical analysis

Significance Analysis of Microarrays (SAM) (one-class) was used, with an estimated false discovery rate (FDR) ≤ 0.05 , to find genes with a significant difference in expression between control and exposed cells for each chemical and time point from 3 independent biological experiments (Tusher *et al.*, 2001).

To obtain a ranked gene list, each gene on the Agilent array was assessed for its potential to discriminate between respiratory sensitizers and respiratory non-sensitizers by the following procedure. To use the information of all time points, the logarithmic fold changes at the 3 time points were first combined into a weighted average (feature value): per gene a Fisher LDA was applied to determine 3 weights that maximize the separation in feature space between exposures to sensitizers and non-sensitizers. From these feature data the significance of the separation was extracted by a t-test and the genes were ranked according to it. Not all genes react at the same time. Therefore, the method of utilizing the data from all time points seems meaningful to rank all the genes in a similar way, regardless of their kinetics.

Gene Ontology For Functional Analysis (GOFFA) is the primary biological interpretation tool using Gene Ontology (GO) in ArrayTrack (<http://www.fda.gov/nctr/science/centers/toxicoinformatics/ArrayTrack/>). GOFFA first ranks GO terms in the order of prevalence for a list of selected genes and then allows the user to interactively select GO terms according to their significance and specific biological complexity within the hierarchical structure. In this study, a gene list generated by Fisher LDA and cut at a top ranking of 1000 genes were uploaded into the GOFFA library as input data.

Pathway analysis

The IPA application (Ingenuity Systems, Mountain View, CA) was used to find functionally related genes that correspond to canonical signaling pathways. The GenBank Accession

Numbers for the top-1000 differentially expressed genes were entered in the IPA database as input. A detailed description on the statistics behind the canonical pathways can be found at www.ingenuity.com.

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3. Results and discussion

Differential gene expression changes in A549 cells

SAM was used to identify differentially expressed genes of each chemical relative to its vehicle control (figure 1A and B). The highest number of modulated genes was observed after exposure to HCpT (figure 1A) and MeSA (figure 1B) in the group of respiratory sensitizers and respiratory non-sensitizers, respectively. The response of A549 cells exposed to respiratory (non-)sensitizers differed in timing. An early (6 and 10 hours) transcriptional response was observed after exposing A549 cells to the skin sensitizer DNCB. A later (24 hours) response was observed after exposure to all the respiratory sensitizers. A549 cells exposed to the irritants ACR and MeSA showed the largest differential gene expression, respectively after 6 and 24 hours and 10 and 24 hours.

The intersections of the different areas contain the number of common genes that were differentially expressed by the corresponding agents. Overlap in common genes was observed between the different time points for each group separately, but also between the group of respiratory sensitizers and respiratory non-sensitizers (data not shown). The shared differentially expressed genes with associated gene information for all 3 respiratory sensitizers were e.g. *HSPA6*, *KITLG*, *LDHA*, *SLC16A4*, *SOCS3*, and *TLR2*. Of these examples, the genes *KITLG* (Verstraelen et al., 2008b), *SOCS3* (Gao and Ward, 2007), and *TLR2* (Fuchs and Braun, 2008) are genes already described in the context of respiratory sensitization. The highest number of shared genes was observed between HCpT and HDI at all time points measured, suggesting that these chemicals may induce similar molecular and biological responses in A549 cells despite their different chemical structures.

Fisher LDA was used to identify selective markers that can discriminate respiratory sensitizers from non-sensitizers and a skin sensitizer (*supplementary data, table SI*). A ranking of the top-20 genes in the A549 model is provided in table I. For 7 out of 20 genes no associated gene information was found. After BLAST searches, the transcript *AK123107* showed 99% homology with human chromosome 17 genomic contig, alternate/reference assembly. BLAST searches did not reveal any homologues for the other 6 transcripts. A visual presentation of the discriminative power of the top-20 genes is shown in figure 2. In the next paragraph, GO terms are assigned to these 20 most discriminating markers and the top-1000 genes are considered for further functional analysis.

GO classification and enrichment analysis of the most selective markers

The GOFFA tool was used to categorize the 20 selected candidate discriminating genes into molecular and biological process GO terms (table I). The top-20 list included genes involved in different biological processes, being immune response (*CTLA4*), regulation of transcription (*ZNF195*, *ZNF228*, and *SUPT6H*), chromatin remodeling (*SUPT6H*), transport (*ERGIC1*), nucleosome assembly (*H3F3B*), gluconeogenesis and lipid biosynthesis (*PC*), protein folding and fertilization (*CLGN*), and proteolysis (*SCPEP1*). Some genes were members of more than one biological process and for 11 genes (*NM_144580*, *AK123107*, *EIA_r60_a22*, *THC2586764*, *AT_ssh_PC_3*, *BF083139*, *A_23_P64962*, *DCP_22_7*, *DCP_22_9*, *AF130050*, and *EIA_r60_3*) no assigned biological GO terms were found. The possible role of cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) in the process of respiratory sensitization was looked after in literature. CTLA4 was found to be associated with T cells. Airway inflammation after inhaled allergen exposure requires the recruitment, activation, and differentiation of antigen-specific T cells into T helper 2 effector cells. These processes are regulated not only by antigen engagement of the T cell receptor, but also by specific accessory molecules on the surface of the T-cell, such as CD28 and the homologous molecule CTLA4 (Burr *et al.*, 2001). CTLA4 binds to B7 ligands with a 16-fold greater affinity than does CD28 (Linsley *et al.*, 1991). Both molecules have opposing functions. CD28 has been shown to promote T cell activity with costimulatory signals, whereas CTLA4 may function to downregulate T cells activation (Walunas *et al.*, 1994; Krummel and Allison, 1995; Tivol *et al.*, 1995; Waterhouse *et al.*, 1995). CTLA4 is expressed at low levels on resting T cells and is highly induced after T cell activation (Walunas *et al.*, 1994; Leach *et al.*, 1996; Perkins *et al.*, 1996; Walunas *et al.*, 1996). In our study, CTLA4 gene expression in A549 cells was differentially expressed after exposure to respiratory sensitizing and non-sensitizing chemicals in alveolar epithelial cells, but no literature information was found to confirm this finding in this cell model. In line with our findings, several genomic regions have been identified that might contain genes contributing to the development of asthma and atopy. These include chromosome 2q33, for which evidence for linkage with variation in total serum IgE levels in a Dutch asthma population was observed. Both, *CTLA4* and *CD28*, map within this region (Howard *et al.*, 2002).

No information was found on the possible association between the other 19 genes and asthma and/or respiratory sensitization.

Besides the 20 most selective markers, we identified discriminative gene markers that were specifically involved in immune function. Therefore, the top-1000 most discriminative genes

were analyzed for GO classification and enrichment using GOFPA. Of these, 382 genes had GO information for functional analysis (molecular, biological, and cellular). Statistical analysis identified 837 enriched GO terms for biological processes. Most of these genes are involved in signal transduction, cell organization and biogenesis, metabolism, regulation of biological and physiological processes, biosynthesis, localization, and transport. Table II provides an overview of the immune-related genes. A number of these genes information is available for their role in airway inflammation, asthma, or respiratory sensitization, namely *CTLA4* (Hellings *et al.*, 2002), *SPP1* (Pardo *et al.*, 2005; Tanino *et al.*, 2006), *CCL4* (Li *et al.*, 2004; Bisset and Schmid-Grendelmeier, 2005), *CCL26* (Abonyo *et al.*, 2006; Paplinska *et al.*, 2007), and *TLR2* (Chu *et al.*, 2005; Fuchs and Braun, 2008). These data are based on single-cell experiments using A549 cells and this model is too simple to explain regulation of the immune system. (In-)direct contact with other cell types and the cell environment is of uttermost importance in this context. This may be the reason why only a small minority of the genes were related to the immune system. Furthermore, cellular processes other than immune might be induced by chemical exposure which are part of an earlier response and may be more abundant than the immune effects.

Canonical signaling pathway analysis

To identify well-characterized canonical signaling pathways that can play a role in the respiratory sensitization process, IPA was used to analyze possible overlap of 57 available signaling pathways with our network of 382 most discriminative gene markers with an assigned biological function. Only 9 signaling pathways were associated with significant p-values of less than 0.05. These significant signaling cascades are shown in table III together with their associated p-value and molecules. These resulting pathways were protein ubiquitination pathway, estrogen receptor signaling, pregnane X receptor (PXR)/ retinoid X receptor (RXR) activation, glucocorticoid receptor signaling, amyloid processing, axonal guidance signaling, coagulation system, extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling, and p53 signaling. Of these pathways, the estrogen receptor signaling, protein ubiquitination pathway, amyloid processing, and axonal guidance signaling are less relevant in the context of respiratory sensitization and will not be used in further analysis. To find out if the remainder 5 signaling cascades are possibly specific for the respiratory sensitization process, different Fisher LDA approaches were performed and the complete set of gene expression data and the ranking of the 7 pathways was compared with the results of the initial set-up in which 3 respiratory sensitizers (HCPT, HDI, and TMA)

were analysed against 3 respiratory non-sensitizers (ACR, MeSA, and DNCB). Table IV represents an overview of the ranking of the 5 selected significant pathways after different Fisher LDA data. The second chemical combination was analysed to identify the canonical pathway ranking after comparison between 3 respiratory sensitizers (HCpt, HDI, TMA) and 2 irritants (ACR, MeSA). In this analysis, DNCB was left out to make sure that this chemical, which is a (skin) sensitizer, did not skew the observed signaling pathways when it is included at the side of non-sensitizers. The third approach represents a comparison between the skin sensitizer DNCB and the irritants ACR and MeSA to evaluate if the observed signaling pathways in the initial set-up were specific for respiratory sensitization and not for contact sensitization. In the last Fisher LDA, 3 respiratory sensitizers (HCpt, HDI, TMA) were distinguished from the skin sensitizer DNCB. The obtained gene lists from all 4 Fisher LDA approaches were imported separately in IPA and the position of the top-10 canonical signaling cascades of the initial set-up (3 sensitizers versus 3 non-sensitizers) were calculated for 3 additional analyses (table IV).

Generally, comparing the ranking positions of the signaling pathways, it can be concluded that no known canonical pathway is specific for the respiratory sensitization process. However, the PXR/RXR activation pathway is highly ranked in all 4 Fisher LDA approaches, and is therefore of possible importance in the global (respiratory and skin) sensitization process.

4. Conclusion

In the present study using the A549 alveolar epithelial cell model, a ranking of genes was obtained that reflects their potential to discriminate between respiratory sensitizing (HCpT, HDI, and TMA) and respiratory non-sensitizing (ACR, MeSA, and DNCB) chemicals. The top-20 discriminating genes were categorized into molecular and biological GO terms. Of these, *CTLA4* was associated with the immune response. When categorizing the top-1000 genes into biological GO terms, 22 genes were associated with immune function. In this study, none of the canonical signaling pathways identified here in alveolar epithelial cells is activated specifically in the context of respiratory sensitization. Further experiments with an expanded panel of chemicals are needed before A549 cell line can be used as an *in vitro* model to screen chemical compounds for their respiratory sensitizing potency.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure captions

- Figure 1 Venn diagrams depicting differences and commonalities in differentially expressed genes (indicated by numbers) by A549 cells exposed to respiratory sensitizers HDI, HCpt, and TMA (A) and respiratory non-sensitizers ACR, MeSA, and DNCB (B) for 6, 10, and 24 hours. SAM one-class analysis was used for each chemical relative to its vehicle control.
- Figure 2 Ranking of top-20 selective markers in the A549 model that can distinguish respiratory sensitizers from respiratory non-sensitizers. The horizontal axis represents the feature values per biological experiment per compound that are calculated using Fisher LDA for each gene. A square box represents a biological experiment that was exposed to a respiratory sensitizer and an asterisk represents a biological experiment that was exposed to an irritant or skin sensitizer. The vertical axis displays the top-20 selective markers with their corresponding FDA p-value as depicted in table I.
- Figure 3 Neutral red uptake data of A549 cells exposed to HCpt (485 μ M or 215 μ g/ml), HDI (386 μ M or 65 μ g/ml), ACR (170 μ M or 10 μ g/ml), and DNCB (150 μ M or 30 μ g/ml) for 24 hours. The exposure concentrations yielded 20% cell growth inhibition (IC₂₀) at 24 hours.

Table I
Overview of top-20 selective marker genes.

Rank	Gene Symbol	Gene Name	GenBank Accession Number	p-value	Molecular Function	Biological Process
1	C1orf85	Chromosome 1 open reading frame 85	NM_144580	2.04E-06	-	-
2	-	CDNA clone IMAGE:5303499	AK123107	2.22E-06	-	-
3	ZNF195	Zinc finger protein 195	NM_007152	7.68E-06	GO:0008270: zinc ion binding	GO:0045449: regulation of transcription, DNA-dependent
4	-	-	E1A_r60_a22	1.39E-05	-	-
5	-	-	THC2586764	1.42E-05	-	-
6	ERGIC1	Endoplasmic reticulum-golgi intermediate compartment (ERGIC) 1	NM_001031711	1.69E-05	-	GO:0006888: ER to Golgi vesicle-mediated transport
7	SUPT6H	Suppressor of Ty 6 homolog (S. cerevisiae)	NM_003170	1.98E-05	GO:0003700: transcription factor activity GO:0016788: hydrolase activity, acting on ester bonds GO:0003723: RNA binding	GO:0045449: regulation of transcription, DNA-dependent GO:0006338: chromatin remodeling
8	CTLA4	Cytotoxic T-lymphocyte-associated protein 4	NM_005214	2.11E-05	-	GO:0006955: immune response
9	ZNF228	Zinc finger protein 228	NM_152354	2.47E-05	GO:0008270: zinc ion binding	GO:0045449: regulation of transcription, DNA-dependent
10	-	-	AT_ssH_PC_3	2.47E-05	-	-
11	TXLNA	Taxilin alpha	BF083139	2.52E-05	-	-
12	CLGN	Calmegin	NM_004362	2.59E-05	GO:0051082: unfolded protein binding GO:0005509: calcium ion binding	GO:0007338: fertilization (sensu Metazoa) GO:0006457: protein folding
13	-	-	A_23_P64962	2.66E-05	-	-
14	H3F3B	H3 histone, family 3B (H3.3B)	NM_005324	2.86E-05	-	GO:0006334: nucleosome assembly
15	PC	Pyruvate carboxylase	NM_001040716	2.87E-05	GO:0004736: pyruvate carboxylase activity GO:0009374: biotin binding GO:0030145: manganese ion binding	GO:0006094: gluconeogenesis GO:0008610: lipid biosynthesis

					GO:0005524: ATP binding	
16	-	-	DCP_22_7	3.43E-05	-	-
17	SCPEP1	Serine carboxypeptidase 1	NM_021626	3.56E-05	GO:0004185: serine carboxypeptidase activity	GO:0006508: proteolysis
18	-	-	DCP_22_9	3.92E-05	-	-
19	SEC14L1	SEC14-like 1 (<i>S. cerevisiae</i>)	AF130050	4.17E-05	-	-
20	-	-	E1A_r60_3	4.20E-05	-	-

Top-20 selective marker genes in A549 cells exposed for 6, 10, and 24 hours to the respiratory sensitizers HCpt, HDI, and TMA and respiratory non-sensitizers ACR, MeSA, and DNCB. Fisher LDA and a t-test were used to obtain a ranking of genes reflecting their potential to discriminate between respiratory sensitizers and non-sensitizers. Functional classification with regard to molecular and biological processes using GO terms are provided.

Table II
Genes associated with immune response.

Gene Symbol	Gene Name
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)
STAB1	Stabilin 1
F2	Coagulation factor II (thrombin)
RELA	V-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)
IRAK2	Interleukin-1 receptor-associated kinase 2
CCL4	Chemokine (C-C motif) ligand 4
AOC3	Amine oxidase, copper containing 3 (vascular adhesion protein 1)
TNFSF14	Tumor necrosis factor (ligand) superfamily, member 14
SYK	Spleen tyrosine kinase
SPON2	Spondin 2, extracellular matrix protein
LSP1	Lymphocyte-specific protein 1
NDST1	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1
AIRE	Autoimmune regulator
B2M	Beta-2-microglobulin
TNFRSF13C	Tumor necrosis factor receptor superfamily, member 13C
CCL26	Chemokine (C-C motif) ligand 26
DMBT1	Deleted in malignant brain tumors 1
TLR2	Toll-like receptor 2
TCF7	Transcription factor 7 (T-cell specific, HMG-box)
ALCAM	Activated leukocyte cell adhesion molecule
CTSW	Cathepsin W

Genes associated with immune response using GOFA biological term classification of the top-1000 differentially expressed genes in A549 cells exposed for 6, 10, and 24 hours to a panel of respiratory (non-)sensitizers.

Table II
Top-10 canonical signaling pathways.

Canonical signaling pathway	p-value	Molecules
Protein ubiquitination pathway	1.00E-02	B2M, HSPA8, USP45, HSP90AA1, FBXW7, USP40, UBE2S, NEDD4, UBE3A, ANAPC1
Estrogen receptor signaling	1.10E-02	POLR2L (includes EG:5441), POLR2A, CREBBP, TBP, H3F3B, PCK1, GTF2A1
PXR/RXR activation	2.45E-02	RELA, ABCC2, NR1I3, PRKAR2A, PRKAG2
Glucocorticoid receptor signaling	2.95E-02	HSPA8, RELA, POLR2L (includes EG:5441), POLR2A, CREBBP, PIK3R5, TBP, HSP90AA1, PCK1, SERPINE1, GTF2A1
Amyloid processing	3.09E-02	PRKAR2A, PRKAG2, CDK5R1, PSEN1
Axonal guidance signaling	3.55E-02	NGEF, PAK2, NRP2, PRKAR2A, PIK3R5, MYL6B, NTN1, ADAM12, PFN4, PRKAG2, UNC5D, EPHB3, BMP7, ARPC1A, PDGFD, NRP1
Coagulation system	4.57E-02	SERPINE1, F2, SERPIND1
ERK/MAPK signaling	4.90E-02	PAK2, PLCG2, PLA2G5, PIK3R5, PRKAR2A, PRKAG2, H3F3B, ELK3
p53 signaling	4.90E-02	PIK3R5, BAX, CTNNB1, HIPK2, SERPINE2

Top-10 canonical signaling pathways with their associated p-value, and molecules affected by respiratory sensitizing versus respiratory non-sensitizing chemicals in a human A549 model. A top-1000 differentially expressed gene list obtained by Fisher LDA was used as IPA input data.

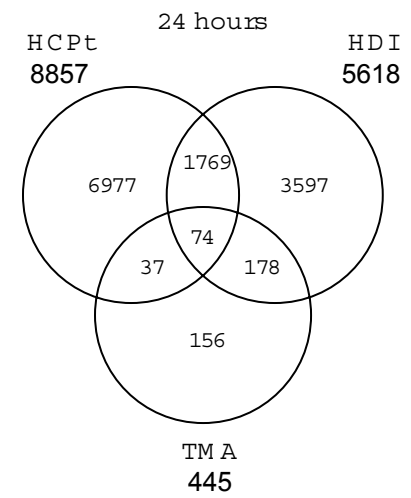
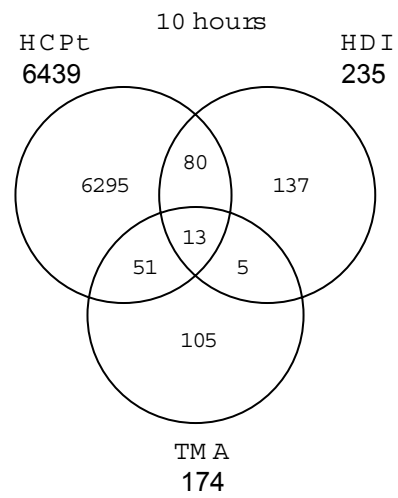
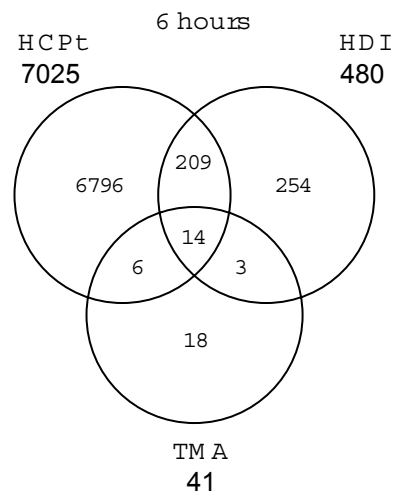
Table IV
Ranking canonical signaling pathways.

Canonical cell signaling pathway	Different chemical combinations			
	*	**	***	****
	HCpt, HDI, TMA ↔ ACR, MeSA, DNCB	HCpt, HDI, TMA ↔ ACR, MeSA	DNCB ↔ ACR, MeSA	HCpt, HDI, TMA ↔ DNCB
PXR/RXR activation	3	5	8	2
Glucocorticoid receptor signaling	4	28	17	24
Amyloid processing	5	3	73	41
Axonal guidance signaling	6	1	21	46
Coagulation system	7	22	40	1
ERK/MAPK signaling	8	8	10	18
p53 signaling	9	9	16	19

Ranking positions of 7 selected canonical signaling pathways are given for a comparison between 4 different test chemical combinations using Fisher LDA:

- * 3 respiratory sensitizers (HCpt, HDI, and TMA) versus 3 respiratory non-sensitizers (ACR, MeSA, and DNCB)
- ** 3 respiratory sensitizers (HCpt, HDI, and TMA) versus 2 irritants (ACR and MeSA)
- *** 1 skin sensitizer (DNCB) versus 2 irritants (ACR and MeSA)
- **** 3 respiratory sensitizers (HCpt, HDI, and TMA) versus 1 skin sensitizer (DNCB)

A



B

