

Successful validation of genomic biomarkers for human immunotoxicity in Jurkat T cells *in vitro*

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ABSTRACT: Previously, we identified 25 classifier genes that were able to assess immunotoxicity using human Jurkat T cells. The present study aimed to validate these classifiers. For that purpose, Jurkat cells were exposed for 6 h to subcytotoxic doses of nine immunotoxicants, five non-immunotoxicants and four compounds for which human immunotoxicity has not yet been fully established. RNA was isolated and subjected to Fluidigm quantitative real time (qRT)–PCR analysis. The sensitivity, specificity and accuracy of the screening assay as based on the nine immunotoxicants and five non-immunotoxicants used in this study were 100%, 80% and 93%, respectively, which is better than the performance in our previous study. Only one compound was classified as false positive (benzo-e-pyrene). Of the four potential (non-)immunotoxicants, chlorantraniliprole and Hidrasec were classified immunotoxic and Sunset yellow and imidacloprid as non-immunotoxic. ToxPi analysis of the PCR data provided insight in the molecular pathways that were affected by the compounds. The immunotoxicants 2,3-dichloro-propanol and cypermethrin, although structurally different, affected protein metabolism and cholesterol biosynthesis and transport. In addition, four compounds, i.e. chlorpyrifos, aldicarb, benzo-e-pyrene and anti-CD3, affected genes in cholesterol metabolism and transport, protein metabolism and transcription regulation. qRT–PCR on eight additional genes coding for similar processes as defined in ToxPi analyzes, supported these results. In conclusion, the 25 immunotoxic classifiers performed very well in a screening with new non-immunotoxic and immunotoxic compounds. Therefore, the Jurkat screening assay has great promise to be applied within a tiered approach for animal free testing of human immunotoxicity. Copyright © 2014 John Wiley & Sons, Ltd.

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Introduction

Direct immunotoxicity is defined as the action of a compound on components of the immune system that lead to either immunostimulation or immunosuppression. The assessment of direct immunotoxicity is an important part of the overall toxicological investigation of chemicals (Lankveld *et al.*, 2010). Several authorities including the European Medicines Agency (EMA, 2006) and the US Food and Drug Administration (FDA, 2002) emphasize the need to evaluate immunotoxic effects during the preclinical phase of drug development. Other authorities such as the Organisation for Economic Cooperation and Development (OECD) (Institoris *et al.*, 1998) and the Environmental Protection Agency (EPA, 2013) stress the need for safety assessment of food contaminants and environmental pollutants. Current immunotoxicity screening still focuses on rodent *in vivo* models. Since the implementation of REACH, that requires the evaluation of chemical hazards for chemicals of which the yearly production exceeds 1 tonne, progress has been made towards development of *in vitro* alternatives for immunotoxicity testing (EC, 2006).

In our previous work, using the *in vitro* Jurkat T cell model, we identified and validated 25 biomarker genes representative for different mechanisms underlying direct immunotoxicity (Shao

et al., 2013, 2014) (Table 1). Jurkat T cells were chosen as they are easy to work with, cheap, readily available and of human origin, making extrapolation to human hazard easier than an animal cell model. Jurkat cells have also been applied in assessing the effect of compounds on mitogen-induced proliferation and migration (Fischer *et al.*, 2004; Fernandez-Riejoes *et al.*, 2008).

On the basis of the outcome of the previous study, two important questions were raised. First, how does the screening system perform towards new classes of immunotoxicants that have not been tested yet? This question is relevant because the biomarkers are based on a set of chemicals belonging to a variety of compound classes such as metals, mycotoxins, herbicides,

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Table 1. Overview of 28 primers that were used as classifiers of immunotoxicity. The last three primers (italics) were used as reference genes

Gene Symbol	Description	mRNA accession	Taqman assay
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	NM_005502	Hs01059118_m1
ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	NM_004915	Hs00245154_m1
AK4	adenylate kinase 4	NM_001005353	Hs03405743_g1
ALDH8A1	aldehyde dehydrogenase 8 family, member A1	NM_001193480	Hs00988965_m1
ARRDC3	arrestin domain containing 3	NM_020801	Hs00385845_m1
BZRAP1	benzodiazepine receptor (peripheral) associated protein 1	NM_004758	Hs00270490_m1
CCNG2	cyclin G2	NM_004354	Hs00171119_m1
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	NM_005194	Hs00270923_s1
CHAC1	ChaC, cation transport regulator homolog 1 (<i>E. coli</i>)	NM_001142776	Hs00225520_m1
CRIM1	cysteine rich transmembrane BMP regulator 1 (chordin-like)	NM_016441	Hs00212750_m1
FBXO32	F-box protein 32	NM_001242463	Hs01041408_m1
GPR18	G protein-coupled receptor 18	NM_001098200	Hs00245542_m1
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	NM_001098272	Hs00940429_m1
HSPA1B	heat shock 70 kDa protein 1B	NM_005346	Hs01040501-sH
HSPA5	heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	NM_005347	Hs00946084_g1
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	NM_002165	Hs00357821_g1
ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	NM_002166	Hs00747379_m1
KLF2	Kruppel-like factor 2 (lung)	NM_016270	Hs00360439_g1
KLHL24	kelch-like 24 (<i>Drosophila</i>)	NM_017644	Hs00214210_m1
NQO1	NAD(P)H dehydrogenase, quinone 1	NM_000903	Hs01045995_m1
SLC7A11	solute carrier family 7 (anionic amino acid transporter light chain, xc- system), member 11	NM_014331	Hs00921938_m1
SRXN1	sulfiredoxin 1	NM_080725	Hs00607800_m1
TM6SF1	transmembrane 6 superfamily member 1	NM_001144903	Hs00224823_m1
VLDLR	very low density lipoprotein receptor	NM_001018056	Hs01047538_m1
VPREB1	pre-B lymphocyte 1	NM_007128	Hs00356766_g1
<i>B2M</i>	<i>β2 microglobulin, present on all nucleated cells</i>	<i>NM_004048</i>	<i>H2000984230_m1</i>
<i>GET4</i>	<i>Golgi To ER Traffic Protein 4 Homolog</i>	<i>NM_015949.2</i>	<i>Hs00944514_m1</i>
<i>PIGG</i>	<i>Phosphatidylinositol Glycan Anchor Biosynthesis, Class G</i>	<i>NM_017733.3</i>	<i>Hs01107608_m1</i>

insecticides and fungicides. Therefore, how this biomarker-based system will perform when exposed to a compound from a class that has not been tested should be further investigated. Second, only a limited number of non-immunotoxic compounds has been tested, which resulted in a less satisfactory specificity. How will this screening system perform when more non-immunotoxicants are included? To answer these important issues, new compounds were searched for in literature and reports.

In total we selected nine known immunotoxicants, five known non-immunotoxicants and four compounds for which it was not fully established whether they are immunotoxic or not (Table 2). Known immunotoxicants from new compound classes include the chloropropanol food contaminant 2,3-dichloro-1-propanol (2,3-DCP) (Lu *et al.*, 2013), the biological anti-CD3 (Ishiguro and Xavier 2004; Weetall *et al.*, 2002), the organophosphorus pesticide chlorpyrifos which causes immunotoxicity in T cells and natural killer cells (Dunier *et al.*, 1991; Noworyta-Glowacka *et al.*, 2012), the lectin phytohaemagglutinin (PHA-L) (Mitsuishi *et al.*, 1986) and the poly-brominated flame-retardant tetrabromobisphenol A (TBBPA) (Pullen *et al.*, 2003; Birnbaum and Staskal, 2004). In addition, immunotoxic compounds from classes tested before were included: the insecticides cypermethrin and aldicarb (Jin *et al.*, 2011), and the mycotoxin zearalenone (Abbes *et al.*, 2013). The insecticide aldicarb increased T cell populations in women drinking contaminated

water (Mirkin *et al.*, 1990), however, aldicarb was assessed to be non-immunotoxic in mice (Thomas *et al.*, 1987). Arsenic trioxide was already tested before in the Jurkat screening model and is tested again as a positive control as it showed the largest effects on the biomarker genes (Shao *et al.*, 2013).

As the results of compounds that have been tested as non-immunotoxic are less likely to be published, a publication bias exists for non-immunotoxic compounds. Therefore, searches for non-immunotoxicants also concerned governmental reports such as those from the European Chemicals Agency (ECHA) and the Australian Pesticides and the Veterinary Medicines Authority (APVMA). Known non-immunotoxicants selected in this study were ametoctradin (APVMA), arsenobetaine (Sakurai *et al.*, 2004; Borak and Hosgood, 2007), carbon tetrachloride (CCL4) (Smialowicz *et al.*, 1991), diuron (EC, 2006) and benzo-e-pyrene (White *et al.*, 2012).

A third category contains compounds of which immunotoxicity has not been definitively observed. Four compounds were included: sunset yellow FCF, racecadotril (Hidrasec), chlorantraniliprole (trade name Rynaxypyr) and imidacloprid. Sunset yellow FCF is a food contaminant that was tested immunotoxic in mice (Hashem *et al.*, 2010; Yadav *et al.*, 2013). Sunset yellow FCF reduced the number of monocytes to 50% in mice *in vivo* without affecting other immune cells (Hashem *et al.*, 2010). Hidrasec, which is used as a treatment for diarrhoea, was assessed to be non-immunotoxic in rodents

Table 2. Overview of the compounds, classes and concentrations used. Compounds ($n = 18$) were divided in immunotoxic ($n = 9$, top), non-immunotoxic ($n = 5$, middle) and unknowns ($n = 4$, bottom)

Compound name	Class	Concentration (μM) CV80	Immunotoxicity
2,3-dichloro-1-propanol	Food contaminant, chloropropanol	6000	Yes
Aldicarb	Insecticide	1000	Yes
Anti-CD3	Biological	2.5 ng/ μl	Yes
Arsenic trioxide	Metal	3	Yes
Chlorpyrifos	Organophosphorous pesticide	1000	Yes
Cypermethrin	Insecticide	1000	Yes
Phytohaemagglutinin	Lectin	100 ng/ml	Yes
Tetrabromobisphenol A	Flame retardant	75	Yes
Zearalenone	Fungal mycotoxin	50	Yes
Ametoctradin	Fungicide	25	No
Arsenobetaine	Organoarsenic compound	20	No
Benzo[e]pyrene	Polyaromatic hydrocarbon	250	No
Carbon tetrachloride	Organic compound	1000	No
Diuron	Herbicide	100	No
Chlorantraniliprole	Insecticide	100	?
Imidacloprid	Pesticide/insecticide, endocrine disrupter	1000	?
Hidrasec	Antidiarrheal drug	500	?
Sunset yellow – FCF	Food additive	500	?

(Abbott Healthcare Products Limited, <http://www.medicines.org.uk/emc/print-document?documentId=27109> and www.mims.com.sg/Singapore/drug/info/Ferinject?type=full). Although the information leaflet mentions that up to 1% of the users suffered from a rash and erythema, which are clinical signs of hypersensitivity of the human skin to chemicals. Also chlorantraniliprole was evaluated as non-immunotoxic in rodents (non-peer reviewed publications). However, chlorantraniliprole is suspected to act on the release of calcium from intracellular calcium stores (Lahm *et al.*, 2005) which is an important process in T cell activation. Therefore it was decided to include this drug in the present study as well. Imidacloprid is a neonicotinoid insecticide and has been tested immunotoxic in rats and mice (Badgular *et al.*, 2013; Gawade *et al.*, 2013). Nevertheless, there is little known about the effects of this compound on the human immune system and on human-derived immune cells *in vitro*.

The aim of this study is twofold. The first aim is to validate the 25 biomarkers previously identified by screening new immunotoxic and non-immunotoxic compounds. In our previous study, the sensitivity, specificity and accuracy were 88%, 67% and 85%, respectively (Shao *et al.*, 2014). The low specificity was likely as a result of the fact that only three non-immunotoxic controls were examined. Therefore, in the present work, the performance of the classifier genes is tested using an extended set of non-immunotoxic controls. The second aim is to predict the immunotoxicity of five compounds for which insufficient data are available. Next to the 25 marker genes identified before, we selected eight additional genes that are known to be biomarkers for specific processes including T cell activation, endoplasmic reticulum (ER) stress, cholesterol homeostasis and the cell cycle (Table 4). It was envisaged that the response of these genes in combination with the 25 markers for immunotoxicity will provide insight in to the mechanisms of action of the compounds. For assessing the mRNA expression response of the 33 genes to the 19 compounds in Jurkat cells, we used a Fluidigm high-throughput PCR system, which has proven to be a fast and reliable system to study gene expression (Shao *et al.*, 2014). The set of 25 biomarkers yielded a

sensitivity of 100%, specificity of 80% and accuracy of 93%. This performance was better than that obtained in the previous validation experiment (Shao *et al.*, 2014).

Materials and Methods

Chemicals

All chemicals were ordered from Sigma-Aldrich (Zwijndrecht, The Netherlands), except anti-CD3 and IgG isotype control (Abcam, Cambridge, UK).

Cell Culture

The human T-lymphocyte cell line (Jurkat) was obtained from the American Type Culture Collection (ATCC). Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 100 U ml⁻¹ penicillin and 100 $\mu\text{g ml}^{-1}$ streptomycin (Invitrogen Life Science, Breda, The Netherlands). Cells were cultured at 37 °C with 5% CO₂ in a humidified atmosphere. The medium was refreshed three times a week.

ATPlite Assay

Cell viability was tested using the ATPlite assay (Perkin Elmer, Oosterhout, The Netherlands) which is based on the production of light caused by the reaction of ATP with luciferase and D-luciferin. The emitted light is proportional to the amount of ATP, which is a marker for cell viability. Jurkat cells were plated 20 h before exposure in a 96-well plate (20 000 cells per well). Exposure was done in triplicate in 100 μl medium in 96 well plates for 24 h to increasing concentrations of compounds, or to the vehicle controls. After exposure, the assay was performed according the manufacturer's protocol.

Exposures

Jurkat cells (passage number between 15 and 19) were seeded in six-well plates containing 2.7 ml (750 000 cells per well) per well. After growing the cells for 20 h, exposure was initiated by adding 0.3 ml of medium containing a non-cytotoxic concentration of the compounds or vehicle controls. Subsequently, cells were exposed to the compounds for 6 h. The maximum final dimethyl sulfoxide (DMSO) concentration in the medium was 0.1% (v/v) for all the samples which had no effect on viability. For each compound, exposures were performed on three different days. IgG was used as a control for the anti-CD3 exposures. DMSO was not present in either anti-CD3 or IgG treatments.

RNA Isolation and Quality Control

After exposure, the culture medium was removed after centrifugation of the cell suspension (5 min at 300 *g*, 4 °C). The resulting cell pellet was homogenized in 600 µl lysis buffer (RLT) (Qiagen, Venlo, The Netherlands) supplemented with 10% β-mercaptoethanol and stored at –80 °C until further processing. RNA was isolated with the QIAshredder kit (Qiagen) according to the manufacturer's protocol. Subsequently, RNA was purified using the mRNAeasy kit (Qiagen) including DNase treatment according to the manufacturer's protocol. RNA yield was assessed spectrophotometrically (NanoDrop 2000; Isogen Life Science, De Meern, The Netherlands).

Quantitative Real Time–PCR Verification

Detection of biomarker gene expression changes was performed at ServiceXS (ServiceXS B.V., Leiden, The Netherlands) using the 96.96 BioMark™ Dynamic Array for Real-Time PCR (Fluidigm Corporation, San Francisco, CA, USA), according to the manufacturer's instructions. cDNA samples were synthesized using a miScript Reverse Transcription kit according to the manufacturer's protocol (Qiagen). Before use on the BioMark™ array, the cDNA was first subjected to 14 cycles of Specific Target Amplification (STA) using a 0.2× mixture of all Taqman Gene Expression assays in combination with the Taqman PreAmp Master Mix (Applied Biosystems), followed by five-fold dilution. Water was included as No Template Control (NTC) at the indicated positions. The No Template Controls were also included in the STA reaction, to serve as a true negative control for the entire procedure. After the 5× dilution, thermal cycling and

real-time imaging of the BioMark™ array was done on the BioMark™ instrument, and threshold cycle (Ct) values were extracted using the BioMark™ Real-Time PCR analysis software. Ct values were used to determine compound effects on mRNA expression levels. Pair-wise combinations of all samples were made with each of the assays in duplicate on the array. The empty assay positions were filled with No Assay Controls, in which the 20× assay mix was substituted with water. The default Taqman PCR protocol was used on the BioMark™ instrument with an annealing temperature of 60 °C and a total of 35 cycles of PCR.

Analyzes of qRT–PCR Data

At the end of each PCR cycle, data were collected from the 9216 reaction chambers on each array, and Ct values were extracted using the BioMark™ RT–PCR analysis software version 3.0.2. The quality threshold was set at 0.65 (default value).

The relative mRNA expression levels were calculated for each individual sample by applying the delta-delta-CT method. Three genes were selected as reference genes, based on the low variability of the relative mRNA expression levels quantified by the microarrays performed before (Shao *et al.*, 2013), being beta-2-macroglobulin (B2M, highly abundant), Golgi to ER traffic protein 4 (GET4, lowly abundant) and phosphatidylinositol glycan anchor biosynthesis class G1 (PIGG). Compounds are considered immunotoxic when one or more of the classifier genes are affected by factor ≥ 2 vs. the control in at least two out of three replicates. Clustering of genes was performed using Cluster v3.0 (uncentered correlation; average linkage clustering) developed by Stanford University. Treeview was used for visualization of the clustering analysis.

Functional Interpretation Based on ToxPi Analysis

The 25 classifiers were divided into eight functional classes (Table 3). The effects of each compound on these functional themes were visualized using ToxPi software from the US EPA (Reif *et al.*, 2013). The mRNA levels of the 25 classifier genes of the 16 compounds were used as the input for ToxPi software. ToxPi calculates a ToxPi score for each compound on each functional theme, and ranks the compounds by their overall ToxPi scores. The ToxPi score (between 0 to 1) is calculated by dividing the results of each chemical on the genes that are involved in this functional theme by the maximum results on the same genes from the 16 compounds.

Table 3. Functional themes in which the 25 classifiers are involved

Category	Genes
Regulation of cell cycle and apoptosis	CCNG2, CRIM1, CHAC1, HSPA1B, ID1, ID2
Metabolism and transport of cholesterol	HMGCS1, ABCA1, ABCG1
Cellular signal transduction	BZRAP1, ARRDC3, CRIM1, GPR18, KLHL24, CHAC1
Cellular stress responses	KLF2, CHAC1, HSPA1B, HSPA5, NQO1, SRXN1, AK4, SLC7A11, VLDLR, TM6SF1, ABCA1, ABCG1
Immune regulation	KLF2, VPBEB1, ID1, ID2, CEBPB
Protein metabolism	FBXO32, CRIM1, AK4
Retinoic acid metabolism	ALDH8A1
Regulation of transcription	ID1, ID2

qRT-PCR on Additional Genes for Functional Confirmation

Eight genes were selected for additional qRT-PCR experiments to confirm the functional themes that were used in ToxPi analyzes. These genes were involved in processes such as T cell activation, apoptosis, ER stress, cholesterol homeostasis, immune regulation, cell cycle and regulation of transcription (Table 4). The qRT-PCR procedure was identical to the methods described above.

Hierarchical Clustering

Hierarchical clustering was performed with the programs Cluster (uncentered correlation; average linkage clustering) and Treeview (Eisen *et al.*, 1998). Red and green indicate up- and downregulation vs. average expression of control samples.

Results

ATPlite Assay

The viability of Jurkat cells that were exposed to the different compounds was assessed by means of the ATPlite assay. Compound concentrations that lead to a decrease in viability of 20% or less after 24-h incubation (CV80) were selected for gene expression analysis, as published previously (Schmeits *et al.*, 2013). For some compounds, the viability did not decrease after exposure to the highest concentration not giving rise to precipitation. In that case, this highest concentration was taken for further analysis. An overview of the viability data is presented in Fig. 1. The concentrations that were selected for exposures are listed in Table 2.

Biomarker Characteristics

The qRT-PCR data were subjected to hierarchical clustering analysis (the outcome is visualized in the heat-map in Fig. 2). The results of the qRT-PCR analysis are also shown in Supplementary Fig. S1. All nine known immunotoxicant are classified as immunotoxic based on the 25 gene markers. The sensitivity is therefore 100%. For the known non-immunotoxicants, four out of five were correctly classified as non-immunotoxic. Benzo-e-pyrene downregulated one gene (HMGCS1) and is consequently classified as false positive. The specificity of this set is therefore 80%. The overall accuracy is 93%. The performance

characteristics of the present analysis are also shown in Supplementary Table S1.

Compounds with Unknown Immunotoxic Properties

This study included four compounds for which the immunotoxic properties have not been precisely described. The reason for including these compounds was to verify whether the expression profiles of these 25 marker genes can provide more insight into the modes of action and point towards possible immunotoxic properties of these compounds. Chlorantraniliprole upregulates two genes (GPR18 and SLC7A11) and is classified as immunotoxic. Hidrasec upregulates seven genes and downregulates one and is also classified as immunotoxic. Imidacloprid and Sunset yellow do not affect any of the genes and are therefore considered not immunotoxic.

ToxPi Analyzes

We categorized the 25 candidate classifier genes into eight functional themes, based on the major GO processes and molecular functions that these genes are involved in (Table 3). The ToxPi tool was then used to gain insight into the modes of action of the compounds. Results of this analysis are shown in pie graphs in Fig. 3. A first observation is that all compounds affected protein metabolism to an almost similar degree. Based on similarities in ToxPi results, the compounds could be classified into three groups. The first group was formed by zearalenone, Hidrasec and As2O3 which all affected genes in cellular signal transduction and cellular stress responses (panel A). Next to these similarities, zearalenone affects immune regulation and Hidrasec affects retinoic acid metabolism which are processes that are hardly affected by the other two compounds.

Two compounds with almost a similar pie graph, but no similarities in structure are 2,3-DCP and cypermethrin (panel B). Both compounds affect protein metabolism and cholesterol metabolism and transport. In addition, cypermethrin affects immune regulation and transcription regulation that are not or to a lesser extent regulated by 2,3-DCP. ToxPi analysis of four compounds, chlorpyrifos, benzo-e-pyrene, aldicarb and anti-CD3, resulted in similar pie graphs (panel C). These four compounds all affect cholesterol metabolism and transport, protein metabolism and transcription regulation, and to a lesser extent retinoic acid metabolism and immune regulation.

Table 4. Overview of eight gene markers for investigating possible modes of action

Gene Symbol	Description	Process	mRNA accession	Taqman assay
CD69	Cluster of Differentiation 69	T cell activation	NM_001781	hs00934033_m1
DDIT3	DNA damage inducible transcript 3	DNA damage, apoptosis	NM_001195053	Hs00358796_g1
DDIT4	DNA damage inducible transcript 4	Immune regulation	NM_019058	Hs01111686_g1
HERPUD1	Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1	ER stress / Unfolded protein response	NM_001010989	Hs01124269_m1
LGALS1	Galectin-1	Apoptosis, cell cycle	NM_002305	Hs00355202_m1
SGK1	Serine/threonine-protein kinase 1	Regulation of transcription	NM_001143676	Hs00985033_g1
SQLE	squalene epoxidase	Cholesterol homeostasis	NM_003129	Hs01123768_m1
TRIB3	Tribbles homolog 3	Apoptosis, ER stress	NM_021158	hs01082394_m1

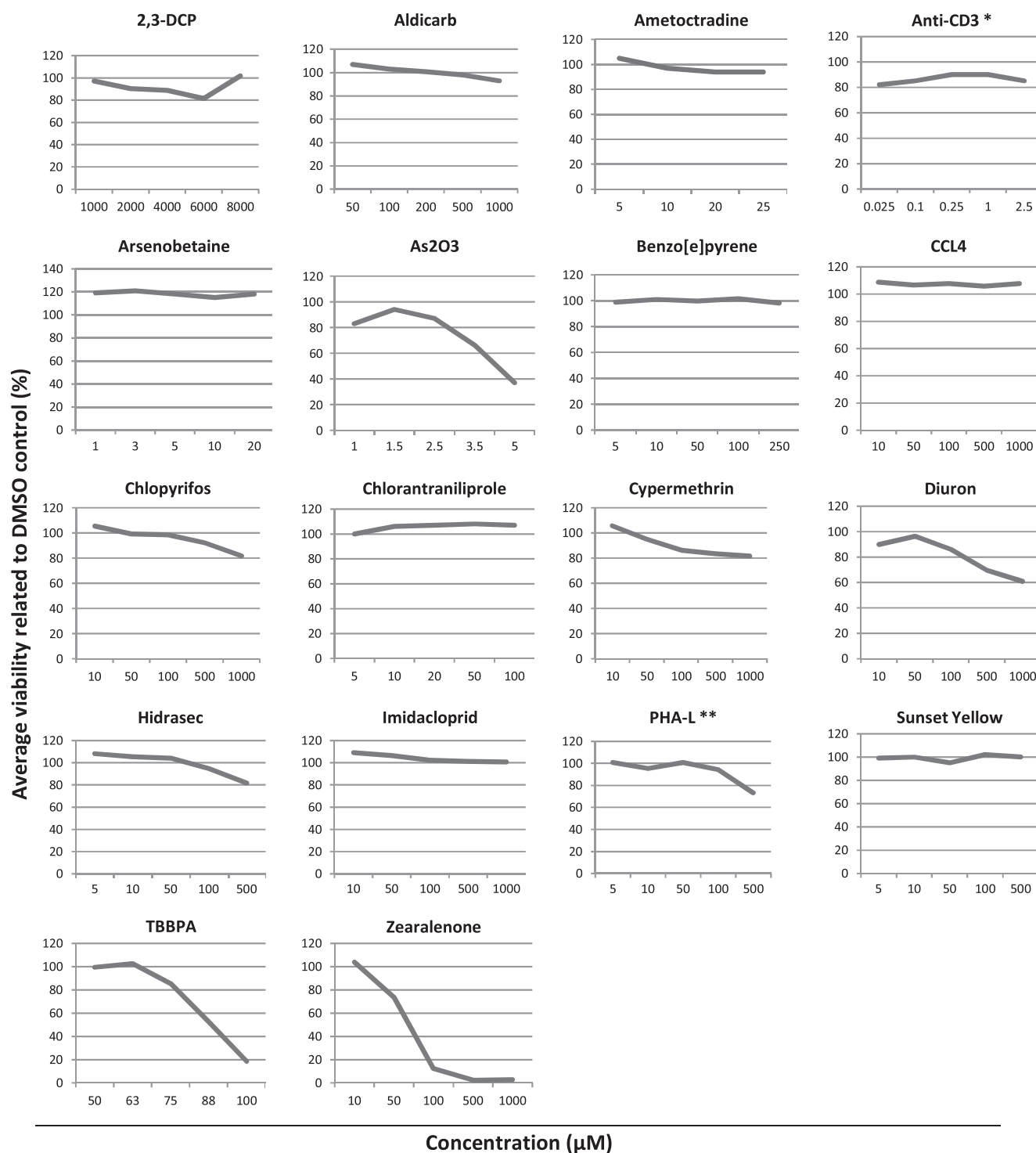


Figure 1. Average viability of compounds as measured by ATPlite assay. Results are based on at least three biological replicates of Jurkat cells and viability is corrected for solvent control dimethyl sulfoxide (DMSO). Average viability is shown on the Y-axis as percentage to DMSO, compound concentrations are present on the X-axis in μM . Exceptions *Anti CD3 was dosed in $\text{ng } \mu\text{L}^{-1}$ and was corrected for the IgG control. ** PHA-L was dosed in $\text{ng } \text{mL}^{-1}$.

Effects on Mode of Action (MOA) Signature Genes

In order to validate the MOAs identified with the present study, we also performed qRT-PCR for all compounds on eight additional genes with known functions. An overview of the primers for these genes is given in Table 4. Seven compounds resulted in significantly altered expression of at least one of the eight

genes. The results of the PCR experiments for these seven compounds are visualized as bar graphs in Fig. 4. PHA-L upregulated the early T cell activation marker CD69 (Ziegler *et al.*, 1994), which corresponds to the mechanism of action of T cell activation. CD69 was also upregulated by TBBPA which indicates that this compound induces T cell activation as well. TBBPA also induced expression of HERPUD1, a known marker for an induced

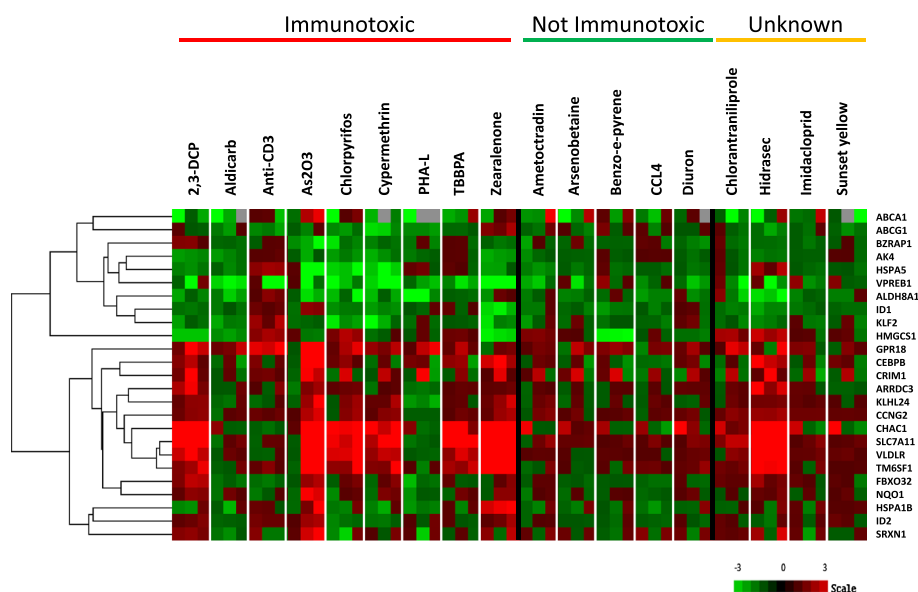


Figure 2. Average expression of $n = 25$ classifier genes in human Jurkat T cells exposed to 18 different compounds. Values represent the average \pm SD of technical duplicates. Each compound is tested in three independent biological replicates. The order of the genes was determined by hierarchical clustering. Red indicates that the particular gene is upregulated, green indicates a downregulation and grey represents missing values. Colour scale represents 2log ratios vs. vehicle control.

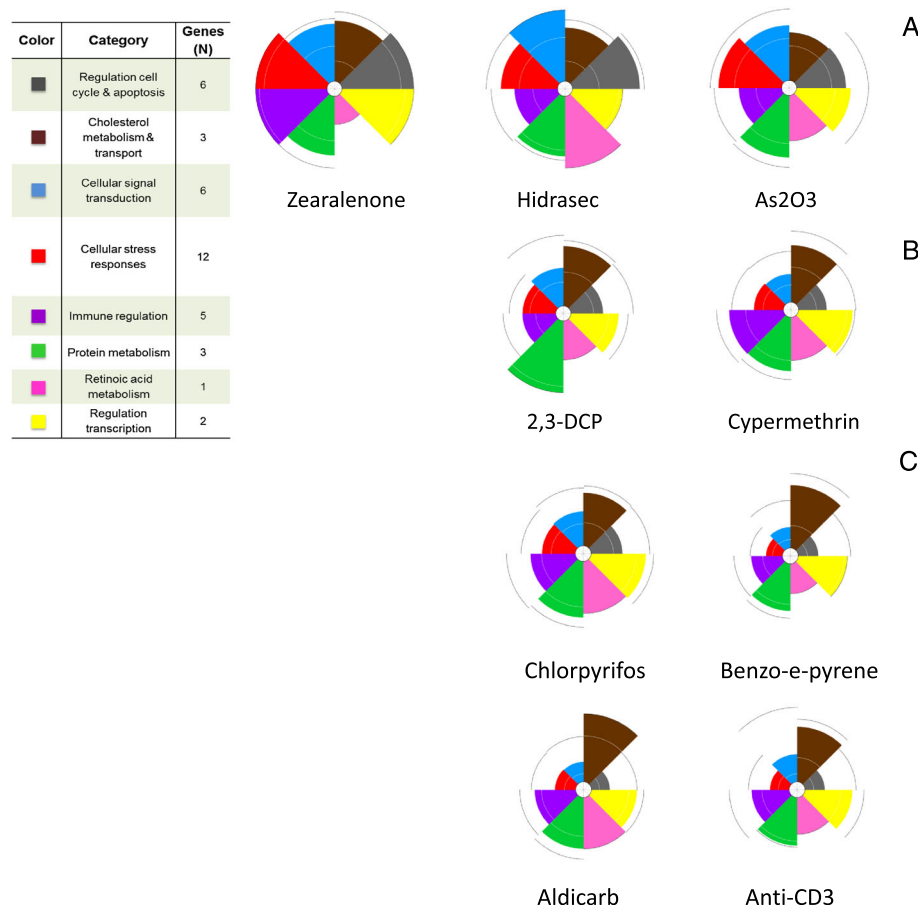


Figure 3. Visualization of the effects of example compounds on eight functional themes. The pie graphs visualize the effects of each compound on the functional themes as listed in the table (left side of Fig. 3A). Each pie represents one compound. Each piece of the pie represents one single functional theme. The eight functional themes were weighted equally, so the graphic widths of all slices are equal. The size of each piece (the distance to the centre, between 0 to 1) shows the ToxPi score that was calculated by the ToxPi software. Pie graphs are shown for the following compounds: (A) Zearalenone, Hidrasec and As2O3; (B) 2,3-DCP and cypermethrin; and (C) Chlorpyrifos, benzo-e-pyrene, aldicarb and anti-CD3.

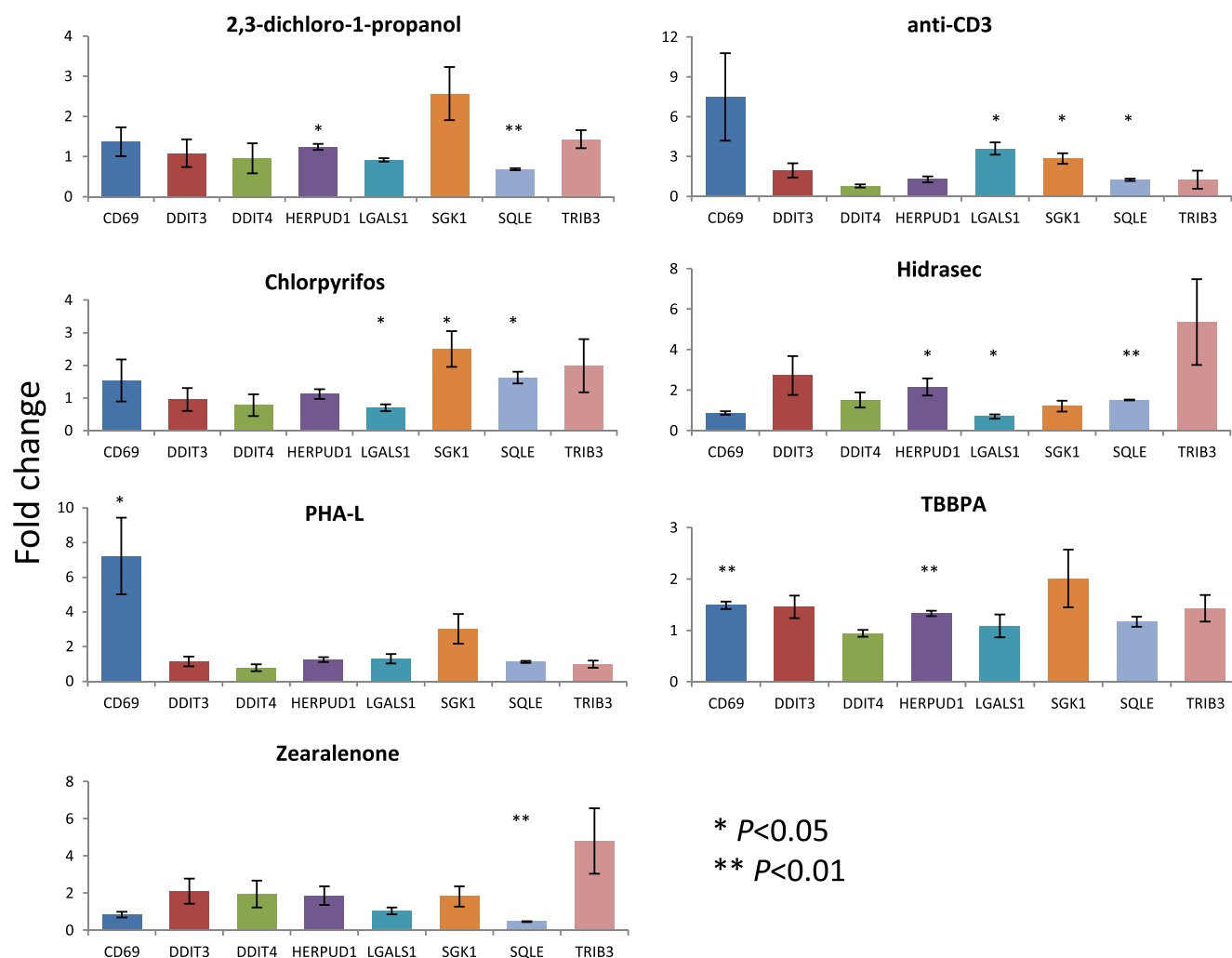


Figure 4. Average expression of genes indicating modes of action. Performance of eight qRT-PCR genes that are involved in T cell activation and proliferation (CD69 and LGALS1), DNA damage and apoptosis (DDIT3, DDIT4 and TRIB3), ER stress induction (HERPUD1), cellular stress response (SGK1) and sterol biosynthesis (SQLE). Data are based on three independent biological replicates. * $P < 0.05$ and ** $P < 0.01$ compared with the control (Student's *t*-test). Y-axis: Fold change as corrected by DMSO and reference genes.

unfolded protein response that may lead to ER stress (Hori *et al.*, 2004). Other compounds that increased the expression of HERPUD1 and are thus likely to induce ER stress are Hidrasec and 2,3-DCP. 2,3-DCP downregulated SQLE, which is involved in cholesterol metabolism and transport (Bonne *et al.*, 2002). This decrease of cholesterol metabolism supports the biological interpretation based on the 25 classifier genes for 2,3-DCP in Fig. 3A. SQLE was also downregulated by anti-CD3 and zearalenone, and upregulated by chlorpyrifos and hidrasec which is in agreement with the results shown in Fig. 3A, B. SGK1 codes for a protein that is involved in cell growth, survival, migration and the cellular stress response (Amato *et al.*, 2009). SGK1 was upregulated by anti-CD3 and chlorpyrifos. Hidrasec downregulated LGALS1, a gene that is involved in the cell cycle and apoptosis, which also supports the outcome of ToxPi analysis. LGALS1 was also downregulated by chlorpyrifos and upregulated by anti-CD3, however, regulation of cell cycle and apoptosis was not obvious from the pie graphs for these compounds (Fig. 3).

Of the compounds that were immunotoxic based on the classifiers, As2O3, aldicarb and cypermethrin did not affect any of the additional genes. Furthermore, benzo-e-pyrene, the false

positive as based on the 25 identifiers had also no effect on these additional genes. Moreover, the compounds selected as non-immunotoxic did not change the expression of any of the eight additional genes.

Discussion

The identification and validation of the 25 classifier genes was performed previously on a training set of 36 immunotoxic and 8 non-immunotoxic compounds, and an external verification set of 17 immunotoxic and 3 non-immunotoxic compounds (Shao *et al.*, 2014). The classifiers were chosen in such a way that they covered a broad range of molecular mechanisms of action.

The present study was designed for two purposes, being (1) further validation of the 25 classifiers with additional immunotoxicants and non-immunotoxic control compounds, and (2) application of the classifiers to screen a group of compounds for which human immunotoxicity has not yet been fully established. For the first purpose, we exposed Jurkat cells to nine known immunotoxicants and five known non-immunotoxicants. These compounds included new compound classes that have

not tested before, such as the polybrominated flame retardant TBBPA and the biological anti-CD3. The four compounds selected for the second purpose were the neonicotinoid imidacloprid, the insecticide chlorantraniliprole, the anti-diarrheal drug hidrasec and the food additive Sunset yellow FCF. In this second round of validation, the 25 classifiers generated a higher sensitivity (100% vs. 88%), specificity (80% vs. 67 %) and overall accuracy (93% vs. 85 %) than obtained in our previous validation (Shao *et al.*, 2014). When the results of our present study were combined with those of Shao *et al.* (2014), the sensitivity, specificity and accuracy would be 91%, 71% and 85%, respectively. In addition, we show that the 25 classifiers correctly predicted the immunotoxicity of compounds from classes not tested before in the Jurkat assay including TBBPA, anti CD3, chlorpyrifos, PHA-L and 2,3-DCP.

This current exercise resulted in only one false positive (benzo-e-pyrene). Benzo-e-pyrene is a structural analogue of benzo-a-pyrene which is a known immunotoxicant (Boorman *et al.*, 1982). Although benzo-e-pyrene is used as a non-immunosuppressive control for polycyclic aromatic hydrocarbon (PAH) exposure (Davila *et al.*, 1999), some studies indicate slight immunotoxic effects of benzo-e-pyrene (Krieger *et al.*, 1995; Davila *et al.*, 1996). The only gene that was regulated by benzo-e-pyrene was HMGCS1, which is regulated in cholesterol metabolism and transport (Horton *et al.*, 2002).

We investigated possible mode of actions of the test compounds using ToxPi analysis based on the expression of the 25 classifier genes. In addition, we performed PCR on eight additional genes, in order to support the putative MOAs we identified with the 25 classifiers. None of the non-immunotoxic compounds in the present study affected the expression of the eight additional genes tested. We determined the fold changes in expression of these eight additional genes regarding the 31 compounds of the previous study (Shao *et al.*, 2014). Also in this study the non-immunotoxic compounds did not alter the expression of any of the eight additional genes (results not shown).

TBBPA is known to induce oxidative stress followed by DNA damage (Choi *et al.*, 2011) and stimulate immune cells (Koike *et al.*, 2013). In our study, TBBPA induced expression of the T cell activation marker CD69, which corresponds to the results of Koike *et al.* (2013), but also upregulates ER stress/UPR gene HERPUD1 (Hori *et al.*, 2004), which is a new finding (Fig. 4). In addition, TBBPA upregulated the pro-apoptosis marker CHAC1 (Mungro *et al.*, 2009) almost four times. Furthermore, TBBPA is known to induce cell death (in Sertoli cells) by affecting calcium homeostasis (Ogunbayo *et al.*, 2008). Disruption of calcium homeostasis in T cells may lead to ER stress (Gwack *et al.*, 2007; Katika *et al.*, 2012a, 2012b). Cells that do not recover from ER stress will undergo apoptotic events.

Induction of ER stress is not limited to immune cells; however, immune cells are more sensitive to ER stress inducing compounds such as deoxynivalenol and tributyltin oxide. Previously we demonstrated that T cells are more sensitive because of the fact that ER stress leads to a leakage of calcium ions from the ER, leading to a calcium signal that in turn generates a T cell activation response, which is a measure for immunotoxicity (Katika *et al.*, 2012a, 2012b; Schmeits *et al.*, 2014). Although ER stress may occur in other cells as well (at higher concentrations than in immune cells), in those cells this will not lead to a T cell activation response.

Currently, the mechanism by which the mycotoxin zearalenone exerts its immunotoxicity is not clear. We show that zearalenone affects genes in cellular stress responses,

cholesterol metabolism/transport, immune regulation, cellular signal transduction, cell cycle and apoptosis and transcription. In addition, zearalenone downregulated one of the eight additional genes tested, SQLE, which is involved in cholesterol metabolism. The fact that some of the immunotoxic compounds affect genes involved in cholesterol metabolism might very well reflect the cellular response to disruption of cholesterol homeostasis.

Based on our results obtained with the 25 classifier genes, 2,3-DCP mainly affects cholesterol metabolism and transport and protein metabolism. PCR analysis of additional genes, such as HERPUD1 and SQLE, supported these findings. A previous study indicated that 2,3-DCP inhibits T cell activation and downregulates activation of transduction pathways *in vitro* (mouse T cells purified from splenocytes) and mice *in vivo* (Lu *et al.*, 2013). However, 2,3-DCP did not significantly affect expression of the T cell activation marker CD69 in the present study. This is most likely caused by the fact that Jurkat cells generally were cultured without stimulus (PHA/LPS). An inhibition of T cell activation is therefore hard to detect.

In the present study, chlorpyrifos induces cellular stress responses and cholesterol biosynthesis in Jurkat T cells. Previous studies found that chlorpyrifos induced oxidative stress (Gultekin *et al.*, 2001; Ambali *et al.*, 2007). Also, chlorpyrifos induces hyperlipidaemia, a state in which cholesterol synthesis is elevated in rats (Acker and Nogueira, 2012) which is in line with the results obtained in our study.

Of the four compounds that might be immunotoxic, chlorantraniliprole and Hidrasec were classified as immunotoxic, and the other two (imidacloprid and Sunset yellow) as non-immunotoxic, as based on the genetic markers used in this Jurkat study. Imidacloprid has been tested immunotoxic in rats and mice *in vivo* (Badgular *et al.*, 2013; Gawade *et al.*, 2013). No data were available on human immune cells. Imidacloprid does not affect any of the 25 classifiers or eight additional genes tested in this study. Sunset yellow is known to affect monocytes but no other blood cells in mice *in vivo* (Hashem *et al.*, 2010). Apparently, Sunset yellow is also not toxic for human Jurkat T cells. This result points to the limitation of the current assay that compounds targeting types of immune cells other than T cells might be not detected. Both chlorantraniliprole and Hidrasec were assessed non-immunotoxic in rodent immunotoxicity studies. The discrepancy between the outcome of the animal studies and the current Jurkat study with these compounds might be as a result of inter-species differences. It is generally recognized that the predictability of rodent studies for human toxicity is rather low (Shanks *et al.*, 2009). For this reason, the US National Research Council, and other organisations, developed a future vision in which animal models are being replaced by human *in vitro* models Schmidt (2009). Chlorantraniliprole is suspected to act on release of calcium from intracellular calcium stores (Lahm *et al.*, 2005) that is known to lead to T cell activation in T cells (Gwack *et al.*, 2007; Katika *et al.*, 2012a, 2012b). Chlorantraniliprole mainly affects cellular stress and signal response pathways in our present experiments. One of the genes upregulated by chlorantraniliprole is SLC7A11 (alias XCT). SLC7A11 represents the rate limiting step of glutathione synthesis, and overexpression of SLC7A11 reduces the need for calcium for early cell cycle progression in the early cell cycle (Lastro *et al.*, 2008).

In conclusion, we have proven the good prediction performance of the Jurkat T cell screening system based on 25 classifiers when challenged by new classes of immunotoxicants. The

sensitivity, specificity and accuracy were even slightly higher in the present study as compared with the previous study. In addition to its use for predicting direct immunotoxicity, this set of genes can be exploited to give indications for the modes of action of compounds. This system however may not detect compounds that exert immunotoxic action on other immune cells than T cells, which likely is the case for Sunset yellow. Therefore, the Jurkat T cell line is a valuable model for immunotoxicity screening not on itself but as part of an integrated testing strategy.

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Conflict of Interest

The authors did not report any conflict of interest.

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