Chapter 4

A novel toxicogenomics-based approach to categorize (non-)genotoxic carcinogens

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Mirjam M. Schaap
Paul F. K. Wackers
Edwin P. Zwart
Ilse Huijskens
Martijs J. Jonker
Giel Hendriks
Timo M. Breit
Harry van Steeg
Bob van de Water
Mirjam Luijten

Abstract

Alternative methods to detect non-genotoxic carcinogens are urgently needed, as this class of carcinogens goes undetected in the current testing strategy for carcinogenicity under REACH. A complicating factor is that non-genotoxic carcinogens act through several distinctive modes of action, which makes prediction of their carcinogenic property difficult. We have recently demonstrated that gene expression profiling in primary mouse hepatocytes is a useful approach to categorize non-genotoxic carcinogens according to their modes of action. In the current study we improved the methods used for analysis and added mouse embryonic stem cells as a second *in vitro* test system, because of their features complementary to hepatocytes. Our approach involved an unsupervised analysis based on the 30 most significantly up- and down-regulated genes per chemical. Mouse embryonic stem cells and primary mouse hepatocytes were exposed to a selected set of chemicals and subsequently subjected to gene expression profiling. We focused on non-genotoxic carcinogens, but also included genotoxic carcinogens and non-carcinogens to test the robustness of this approach. Application of the optimized comparison approach resulted in improved categorization of non-genotoxic carcinogens. Mouse embryonic stem cells were a useful addition, especially for genotoxic substances, but also for detection of non-genotoxic carcinogens that went undetected by primary hepatocytes. The approach presented here is an important step forward to categorize chemicals, especially those that are carcinogenic.

Introduction

Toxicogenomics, the integration of toxicology, genomics and bioinformatics endeavors to elucidate the response of a cell or tissue to toxicants. It encompasses a number of technical approaches, including transcriptomics, proteomics, and metabolomics (Afshari et al. 2011). To date, transcriptomics is the most widely used approach in research aiming to improve testing strategies for human health risk assessment (Currie 2012; Goetz et al. 2011; Keller et al. 2012). For the evaluation of the carcinogenic potential of a chemical, an inefficient, costly, and animal demanding process is currently used (Lilienblum et al. 2008). Alternative tests are therefore required. The application of transcriptomics approaches to detect carcinogenic features of chemicals has been extensively investigated *in vivo* as well as *in vitro* (Ellinger-Ziegelbauer et al. 2009a; Fielden et al. 2011; Thomas et al. 2009; Tsujimura et al. 2006; Yamada et al. 2012). Overall, these studies have yielded biologically relevant gene signatures, which can be employed to distinguish different chemical classes, *e.g.* (non-) genotoxic carcinogens versus non-carcinogens (Ellinger-Ziegelbauer et al. 2008; Melis et al. 2014; Thomas et al. 2009; Watanabe et al. 2012; Yamada et al. 2012). Besides classification, gene expression profiling has also been demonstrated to be a useful tool to gain insight into the possible mode of action of a (carcinogenic) substance (Fielden et al. 2011; Guyton et al. 2009; Waters et al. 2010).

In vitro systems in which toxicogenomics has been explored for assessment of the carcinogenic features of substances are, just like *in vivo*, mainly of hepatic origin: (primary) hepatocytes or hepatic (cancer-) cell lines (*e.g.* HepG2, MH1C1) (Ellinger-Ziegelbauer et al. 2009b; Magkoufopoulou et al. 2012; Mathijs et al. 2009; Tsujimura et al. 2006). The results of these studies indicated that toxicogenomics-based *in vitro* assays are promising tools to detect mainly genotoxic carcinogens. However, when using an *in vitro* test system the biological meaning and relevance with respect to an *in vivo* exposure should always be part of the hazard (risk) evaluation, as has been demonstrated in several studies (Heise et al. 2012; Schug et al. 2013; van Kesteren et al. 2012).

Carcinogens are, for classification purposes, often subdivided into genotoxic and non-genotoxic chemicals. Non-genotoxic carcinogens (NGTXC) act through several distinct biological pathways, which complicates their detection within

one test system (Hernandez et al. 2009). It is therefore necessary to develop tailored assays for these carcinogens. Using gene expression profiling in primary hepatocytes, we recently demonstrated that some, but certainly not all, non-genotoxic carcinogens can be categorized according to similarity in their proposed modes of action (Schaap et al. 2012). To be able to cover a larger set of chemical 'classes', we investigated whether the introduction of a second cell system, *i.e.* mouse embryonic stem cells (mESC), would improve the performance of the assay. Both primary hepatocytes and stem cells are non-tumor derived, which is an important aspect when detecting responses of substances with carcinogenic potential. Furthermore, the cell systems complement each other in terms of being metabolically competent (PMH), being immortal (mESC) and divide infinitely (mESC). mESC have already shown to be highly sensitive to genotoxic and oxidative stress (Hendriks et al. 2011). Besides the addition of an extra cellular test system we also improved our bioinformatics approach to recognize similarities in expression profiles by using only a limited set of most significantly regulated genes. By testing not only non-genotoxic carcinogens, but also other chemicals like genotoxic carcinogens and (toxic) non-carcinogens we verified the robustness of our approach. The results show that selected gene sets were sufficiently informative to categorize most (non-)genotoxic carcinogens according to similarities in their proposed modes of action.

Materials and methods

Chemicals

Chemicals tested in this study (see Table 1) comprised: 1,1,1-Trichloroethane (TCE), 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), Aroclor 1254 (ARO), Bisphenol A (BPA), Calyculin A (CA), Carbon Tetrachloride (CT), Cisplatin (CSPT), Clofibrate (CF), Cyclosporine A (CSA), Diethyl Maleate (DEM), Disodecyl Phthalate (DIDP), D-Mannitol (D-M), Etoposide (ETP), Heptachlor epoxide (HCE), Lead Acetate (LAC), Menadion (MEN), Mitomycin C (MMC), N-Methyl-N-nitrosourea (MNU), Okadaic Acid (OA), Phenobarbital (PB), Sodium Arsenite (SAR), Tacrolimus (FK506), Tributyltinoxide (TBTO), Wyeth-14,643 (WY), β-Hexachlorocyclohexane (HCH). Details regarding CAS numbers, suppliers and solvents are given in Table 1. Substances were added to the medium as a 200x stock solution, resulting in a 0.5% solvent concentration. The selection of substances was based on

carcinogenicity and genotoxicity reviews using CPDB, IARC, NTP and TOXNET (http://potency.berkeley.edu, http://monographs.iarc.fr, http://ntp-server.niehs.nih.gov and http://toxnet.nlm.nih.gov, respectively) and a study reported by Kirkland et al. (Kirkland et al. 2006).

Isolation, culture and exposure of primary mouse hepatocytes

Primary mouse hepatocytes (PMH) were isolated, maintained *in vitro* and exposed as previously described (Schaap et al. 2012). In short, primary hepatocytes were isolated from male C57BL/6J mice by a two-step liver perfusion and exposed for 24 hours to one of the selected chemicals or the solvent controls (DMSO and PBS). Concentrations were selected based on a cytotoxicity assay, as described in detail by Schaap et al. (Schaap et al. 2012).

Culture and exposure of mouse embryonic stem cells

Wild type mouse ES cells (mESC; B4418 with C57BL/6 genetic background) were kindly provided by Dr. H. de Waard (Erasmus Medical Center, Rotterdam, The Netherlands). Cells were cultured as previously described (Kruse et al. 2007). In short, mESC were cultured on mouse embryonic fibroblast feeder layers. Prior to exposure, cells grown in the absence of mouse embryonic fibroblast feeder layers for two passages were seeded on gelatin-coated plates. Cells were allowed to adhere overnight.

For gene expression profiling, mESC were exposed for 8 hours to one of the selected chemicals or solvent controls (DMSO and PBS). Experiments were performed in triplicate for all chemicals. Concentrations were selected based on the apoptotic response and cytotoxicity measurements. The apoptotic response was determined after 24 hours of exposure using FACS analysis, performed as previously described (Kruse et al. 2007). Cytotoxicity was measured after 24 and 72 hours of exposure. Four hours prior to the end of exposure 5 mg/ml of MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) was added to each well. The medium was removed, and formazan crystals formed were solubilized in DMSO. Absorbance was measured at 570 nm and a reference wavelength at 670 nm. Vehicle-treated cells were used as a solvent control and were taken as a 100% cell viability control. Dose-response calculations were done using PROAST software (www.rivm.nl/proast, (Slob 2002)).

Table 1. Overview of the test chemicals used

Chemicals	Abbreviation	CAS-number	Supplier	Solvent	Concentration		DEGs[a]	
					mESC	*PMH*	*mESC*	*PMH*
Non-genotoxic carcinogens								
1,1,1,-trichloroethane	TCE	71-55-6	Supelco	DMSO	3 mM	1 mM	16	55
1,4-bis[2-(3,5-dichloropyridyloxy)]benzene	TCPOBOP	76150-91-9	Sigma	DMSO	5 µM	10 µM	54	74
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	TCDD	1746-01-6	Accu standard	DMSO	100 nM	10 nM	30	780
Aroclor 1254	ARO	11097-69-1	Supelco	DMSO	25 µM	30 µM	500	955
Calyculin A	CA	101932-71-2	Sigma	DMSO	0.5 nM	1 nM	96	389
Carbon Tetrachloride	CT	56-23-5	Supelco	DMSO	3 mM	300 µM	23	59
Clofibrate	CF	637-07-0	Sigma	DMSO	100 µM	1 mM	1112	496
Cyclosporin A	CSA	59865-13-3	Novartis	DMSO	10 µM	1 µM	1255	156
Heptachlor epoxide	HCE	1024-57-3	Supelco	DMSO	5 µM	30 µM	186	2813
Lead Acetate	LAC	6080-56-4	Sigma-Aldrich	DMSO	10 µM	10 µM	3976	7517
Okadaic Acid	OA	78111-17-8	Sigma	DMSO	4 nM	1 nM	28	362
Phenobarbital	PB	50-06-6	Sigma	DMSO	3 mM	1 mM	464	294
Sodium Arsenite	SAR	7784-46-5	Sigma-Aldrich	PBS	2 µM	3 µM	1744	5334
Tacrolimus	FK506	109581-93-3	Sigma	DMSO	10 µM	30 µM	164	3133
Wyeth-14643	WY	50892-23-4	Sigma	DMSO	250 µM	100 µM	374	2128
β-Hexachlorocyclohexane	HCH	319-85-7	Fluka	DMSO	50 µM	100 µM	20	127
Additional agents								
Bisphenol A	BPA	80-05-7	Aldrich	DMSO	80 µM	10 µM	741	68
Cisplatin	CSPT	15663-27-1	Sigma	DMSO	5 µM	1 µM	1412	573
Diethyl maleate	DEM	141-05-9	Aldrich	DMSO	100 µM	300 µM	2363	764
Diisodecyl phthalate	DIDP	26761-40-0	Fluka	DMSO	2 mM	1 mM	23	55
D-mannitol	D-M	69-65-8	Sigma-Aldrich	DMSO	2 mM	1 mM	37	51
Etoposide	ETP	33419-42-0	Sigma	DMSO	5 µM	30 µM	3665	459
Menadion	MEN	130-37-0	Sigma	PBS	50 µM	3 µM	927	16
Mitomycin C	MMC	50-07-7	Sigma	PBS	0.5 µM	0.3 µM	483	157
N-Methyl-N-nitrosourea	MNU	684-93-5	Sigma	DMSO	500 µM	1 mM	231	152
Tributyltinoxide	TBTO	56-35-9	Aldrich	DMSO	250 nM	0.3 µM	343	72

[a] DEGs = differentially expressed genes, FDR<0.05

RNA isolation, labeling and hybridization

PMH and mESC were collected in 1 ml RNAprotect (QIAgen, Venlo, The Netherlands) and stored at -80˚C prior to RNA isolation. RNA was extracted from the hepatocyte samples using QIAzol and purified using the miRNeasy mini kit and the QIAcube (Qiagen), according to the manufacturer's protocol. For the mESC the RNeasy mini kit was used for RNA isolation. For each substance tested in mESC and PMH, we used, respectively, three and four biological replicates. RNA concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and RNA quality was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). Labeled RNA was prepared using the GeneChip 3" IVT express kit (Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. Hepatocyte samples were hybridized to HT MG-430 PM Array plates (Affymetrix); embryonic stem cell samples to Affymetrix Mouse Genome 430 2.0 GeneChip arrays. Array images were acquired using a GeneChip HT Array Plate Scanner (Affymetrix) and analyzed with Affymetrix HT software suite including expression console software (Affymetrix).

Data analysis

Data analysis was performed as previously described (Schaap et al. 2012). In short, raw data were subjected to a set of quality control checks, annotated according to de Leeuw et al. (de Leeuw et al. 2008) and expression values were calculated using the robust multi-array average (RMA) algorithm (Affy package, version 1.22.0; (Irizarry et al. 2003). All gene expression data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (accession nos. GSE44088 (PMH) and GSE47345 (mESC)).

Normalized data was statistically analyzed for differential gene expression (Smyth 2004; Wolfinger et al. 2001) and a contrast analysis was applied to compare each exposure with the corresponding vehicle control. For hypothesis testing, a permutation-based Fs test was used (Cui et al. 2005). False discovery rate (FDR) correction was performed globally across all contrasts according to Storey and Tibshirani (Storey and Tibshirani 2003)T. The number of differentially expressed genes (DEGs) was based on a FDR <0.05.

In order to categorize substances based on common mechanisms a comparison approach was applied (see Figure 2). This approach completely depended on the T-statistics, which is the ratio of the effect size and variation of each

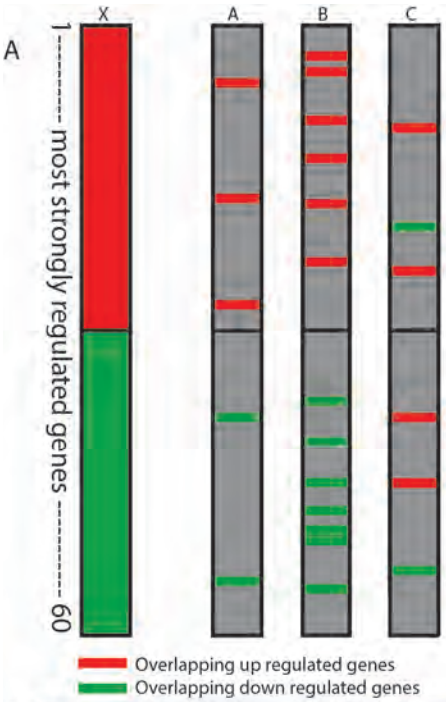
individual gene. For all substances tested in both *in vitro* methods the differential expression values of all genes were ranked using T-statistics, both for up- and down-regulated genes. Subsequently the top 60, *i.e.* the 30 most significantly up regulated and the 30 most down regulated genes, was selected and used as input for the comparison approach. This set of 60 genes of each chemical was compared to the gene sets of the other 25 chemicals. For each combination we determined the number of overlapping genes regulated in the same direction (further referred to as "hits"). We also calculated a score, which is the sum of the absolute T-statistics of all overlapping genes. To state whether the outcome is relevant, cut-off values for the hits and score are required. To start, we arbitrary set the cut-off at 10 hits and a minimal score of 100.

Results

Concentration selection for gene expression studies

The main objective of the present study was to recognize commonalities in gene expression profiles induced by substances having similar modes of action. In total, we tested a set of 26 substances (Table 1), of which 16 were non-genotoxic carcinogens. These 16 chemicals were divided into eight pairs according to their presumptive overlapping modes of action (Table 2). The ten remaining chemicals consisted of four genotoxic agents (GTX), two oxidative stress inducing chemicals (Ox. Dam.) and four non-carcinogenic (NC) but potentially toxic substances (Table 3). These ten chemicals were included to test the specificity and/or robustness of our approach. Each of the 26 substances was tested for its competence to affect transcription in both primary mouse hepatocytes (PMH) and mouse embryonic stem cells (mESC). Since the experiments for PMH and ESC were performed independently and the cell systems differ in terms of origin, cell cycling and metabolic activity, we determined optimal (subtoxic) concentrations for gene expression profiling using two different criteria. For PMH the criterion applied was a relative cell viability of at least 90% using the MTT reduction method. If exposure did not result in a detectable level of cytotoxicity, the ultimate concentration used was 1 mM. Cytotoxicity data are depicted in Supplementary Figure S1. For mESC the selection criteria were a maximum induction of apoptosis of approximately 30%, and/or a 20% decrease in cell viability (Supplementary Table S1, Supplementary Figures S2, S3 and S4). For substances that were found to induce neither apoptosis nor cytotoxicity,

the test concentration was arbitrarily set at 2-3 mM. For both cell types the final concentrations used for gene expression profiling are listed in Table 1.



Example calculation of the score:

	X	A	B	C	Etc.
Gene1	37.3		40.8		
Gene2	14.3		23.9		
Gene3	12.3	5		6.4	
Gene4	10.0		12.6		
Gene5	-7.8	-5.3	-8.1		
Gene6	7.5		9.1		
Gene7	7.2	6.1		5.8	
Gene8	-7.2		-6.3	5.7	
Gene9	-7.1	-7.6	-8.4		
Gene10	6.8			-4.6	
Etc...					

Values represent the T-statistic values. For calculation of the score the absolute T-values are used.

Score + common genes:
X + A: 58; 5 hits (gene 3, 5, 7 and 9)
X + B: 200; 7 hits (gene 1, 2, 4, 5, 6, 8 and 9)
X + C: 32; 2 hits (gene 3, 7)*
* Gene 8 and 10 are not unidirectionally regulated and, as such, not taken into account.

B

Chemical	ARO		Chemical	HCE		Chemical	TCDD	
#genes (input)	60		#genes (input)	60		#genes (input)	60	
1st match	HCE		1st match	ARO		1st match	ARO	
Hits	22		Hits	22		Hits	13	
Symbol	**ENTREZ ID**	**T-stats**	**Symbol**	**ENTREZ ID**	**T-stats**	**Symbol**	**ENTREZ ID**	**T-stats**
Cyp1a1	13076	37.29	Hmgcs2	15360	-10.59	Cyp1a1	13076	40.62
Cyp1a2	13077	14.29	Cyp2c55	72082	10.57	Cyp1b1	13078	30.69
Cyfip2	76884	12.28	Hmgcr	15357	10.30	Cyfip2	76884	16.39
Cyp2c55	72082	9.99	Ces6	102022	9.52	NA_1435459	NA	15.68
Fgf21	56636	-7.80	NA_1460232	NA	-9.33	Cyp1a2	13077	15.11
Cyp2c29	13095	7.48	Hyal1	15586	-9.09	Fmo2	55990	14.57
Tiparp	99929	7.19	5430435G22Rik	226421	8.81	Tiparp	99929	14.51
Nqo1	18104	7.18	AA986860	212439	-8.77	Ereg	13874	13.64
Hmgcs2	15360	-7.10	Nnt	18115	-8.77	Sytl2	83671	13.35
NA_1424390	NA	6.79	NA_1437003	NA	8.76	Insl6	27356	12.92
Tns1	21961	-6.77	Tnfaip3	21929	-8.69	Hsd17b6	27400	11.29
Cpt1a	12894	-6.71	Hsd3b3	15494	-8.63	Nqo1	18104	10.87
Slc16a2	20502	-6.36	Sntb2	20650	8.60	Spon1	233744	10.13
Hyal1	15586	-6.22	Cyp26a1	13082	8.56	Dclk3	245038	10.01
Sntb2	20650	6.19	Ptges	64292	8.55	Reln	19699	9.55
Ghrl	14645	-5.98	NA_1455770	NA	-8.42	Xdh	22436	9.46
Ces6	102022	5.98	Klf12	16597	-8.42	NA_1428323	NA	-8.29
Exosc8	69639	5.94	100042616	100042616	-8.41	AU018778	234564	7.94
NA_1416998	NA	5.79	Pla2g12a	66350	8.40	Lrp4	228357	7.92
NA_1435691	NA	-5.75	Cpt1a	12894	-8.36	Dfna5h	54722	7.87

Figure 2. Outline comparison approach. A) Per chemical (X in this figure) the most significantly regulated genes (top 30 up and top 30 down ranked according to T-statistics) were compared to those of the other chemicals tested (given as A, B, C, etc.). For each combination of chemicals, the number of common genes (=hit) was determined (provided the direction of regulation was the same), and the sum of T-statistics (=score) was calculated. These two parameters were used to find the best match for the chemical of interest. B) Example of PMH data, showing only 20 of the most significantly regulated genes. Blue indicates the hits for ARO and HCE; yellow indicates the hits for ARO and TCDD. Genes overlapping between ARO, HCE and TCDD are marked in green. Complete gene sets are listed in Supplementary Table S2.

Table 2. Non-genotoxic carcinogens paired according to known modes of action.

Feature used for grouping of NGTXC	Pairs of substances tested[a]		Characteristics[b]	References
Arylhydrocarbon receptor agonist	TCDD	ARO	Ligand activated TF, regulates target genes encoding for metabolizing enzymes of Phase I and Phase II	(Aly and Domenech 2009; Sato et al. 2008; Silkworth et al. 2008)
Constitutive androstane receptor agonist	TCPOBOP	PB	Regulates the transcription of target genes involved in drug metabolism and bilirubin clearance	(Lempiainen et al. 2011)
Halogenated hydrocarbon	CT	TCE	Cause lipid peroxidation (Tse et al. 1990), CCL_3* radical, metabolite of CT, reacts with amino acids, nucleotides and fatty acids, proteins, nucleic acids and lipids, or abstracts hydrogen to form chloroform	(Weber et al. 2003)
Immunosuppressant	CSA	FK506	Induces endoplasmatic reticulum stress, hepatotoxic, induce cholestasis	(Hamawy 2003; Kitamura 2010; Matsuda and Koyasu 2000)
Ligand independent estrogen receptor activator	HCH	HCE	Organochlorine pesticides, activates ER through a signaling pathway involving the membrane-bound RTK c-ErbB2 and p44/42 MAP kinase	(Hatakeyama et al. 2002; Tessier and Matsumura 2001)
Metalloid	SAR	LAC	Induce oxidative stress, ROS production, interaction with DNA repair processes	(Beyersmann and Hartwig 2008)
Peroxisome proliferator	WY	CF	Activate TF PPARα, regulate target genes encoding for peroxisomal beta-oxidizing enzymes, fatty acid transporters, cyp4A family and thioesterases	(Fidaleo 2009; Peters et al. 2005)
Skin tumor promotor	CA	OA	Inhibitors of protein serine/threonine phosphatases	(Fujiki and Suganuma 2009)

[a] For the full names of the substances, see Table 1

[b]The described characteristics are an indication of the known modes of action from literature. Abbreviations; ER estrogen receptor, PPARα peroxisome proliferator-activated receptor alpha, ROS reactive oxygen species, RTK receptor tyrosine kinase, TF transcription factor

Table 3. Features of additional (geno)toxic chemicals tested

Chemicals[a]	Abbreviations	Features	References
Cisplatin	CSPT	GTX carcinogen, clastogen, induces mainly intrastrand DNA crosslinks	(Roos and Kaina 2013)
Etoposide	ETP	GTX carcinogen, inhibits topoisomerase II in the cell that unravels DNA, causing the strands to break and leading to cell death	(Attia et al. 2003)
Mitomycin C	MMC	GTX carcinogen, clastogen, crosslink DNA with high efficiency and absolute specificity for the sequence CpG	(Tomasz 1995; Weng et al. 2010)
N-Methyl-N-nitrosourea	MNU	GTX carcinogen, mutagen, alkylating agent, exhibits its toxicity by transferring its methyl group to nucleobases in nucleic acids	(Tsubura et al. 2011)
Diethyl maleate	DEM	Ox. Dam. Intracellular glutathione-depleting agent, increases oxidative stress	(Yamauchi et al. 2011)
Menadion	MEN	Ox. Dam. Causes oxidative stress through increased oxidation of NADH and NADPH and through conjugation of glutathione	(Criddle et al. 2006)
Bisphenol A	BPA	NC Endocrine disruptor which closely mimics the structure and function of estradiol and has the ability to bind to and to the estrogen receptor	(Rubin 2011)
Diisodecyl phthalate	DIDP	NC Plasticizer, inducing peroxisome proliferation effects in the liver	(Saravanabhavan and Murray 2012)
D-mannitol	D-M	NC Osmotic diuretic that is metabolically inert in humans	(Saha and Racine 2011)
Tributyltinoxide	TBTO	NC Biocide, environmental pollutant, immunomodulating chemical	(Osman and van Loveren 2012)

Microarray analyses

Gene expression profiles generated upon exposure to one of the 26 chemicals (as listed in Table 1) were first analyzed using ANOVA. The number of differentially expressed genes (DEGs, FDR<0.05) varied substantially, ranging in PMH from 16 (MEN) up to 7,517 (LAC) and from 16 (TCE) up to 3,976 (LAC) in mESC (Table 1). Generally, exposure to non-genotoxic carcinogens did elicit a stronger response (in terms of DEGs) in PMH than in mESC, while the opposite was observed for the genotoxic and oxidative stress inducing substances.

We performed Principle Component Analysis (PCA) using the complete dataset, for both the PMH and mESC exposure studies (Figure 1). The metalloids LAC and SAR induced the strongest response in PMH. Additionally, two clusters could be identified: one consisting of five non-genotoxic carcinogens (ARO, CF, FK506,

HCE and WY) and another consisting of the remaining substances (Figure 1A). In the PCA plot of the mESC (Figure 1B) all non-genotoxic carcinogens, except for LAC and SAR, and all (toxic) non-carcinogens clustered together. LAC and SAR as well as the genotoxic carcinogens (CSPT, ETP, MMC and MNU) and oxidative stress inducing agents DEM and MEN induced a rather pronounced response compared to the other compounds.

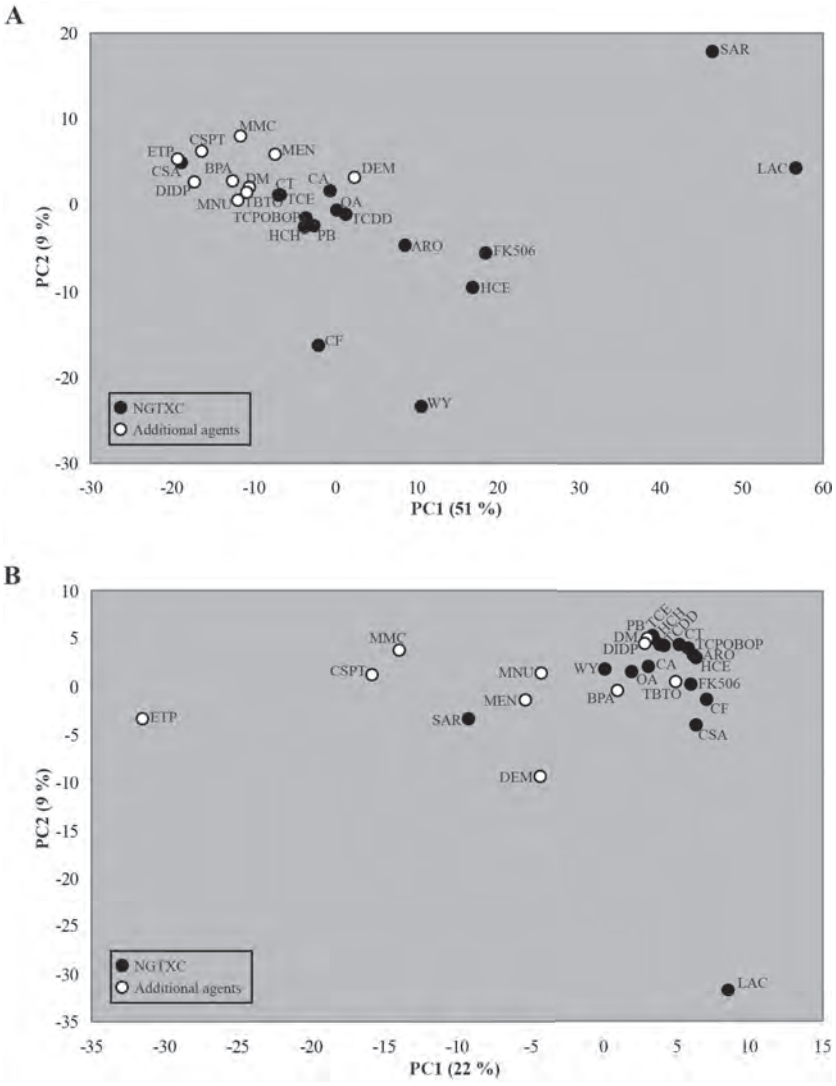


Figure 1. Principle component analysis, based on all genes. A) Primary mouse hepatocytes. B) Embryonic stem cells. Closed circles indicate the non-genotoxic carcinogens, open circles indicate the additional test chemicals. For explanation of the abbreviations, see Table 1.

Comparison approach

We hypothesize that chemicals with a comparable mode of action will induce, at least in part, overlapping expression patterns. To identify these commonalities we compared the expression profiles of all 26 substances tested for each *in vitro* system separately. The comparison approach used is outlined in Figure 2. The top 60 most significantly regulated genes (*i.e.* the 30 most up- and the 30 most down-regulated) were selected and ranked according to T-statistics (see Methods section). This set of 60 genes for each chemical was compared to the gene sets for the other 25 chemicals. For each combination we determined the number of overlapping genes regulated in the same direction (further referred to as "hits"). We also calculated a score, which is the sum of the absolute T-statistics of all overlapping genes (see example in Figure 2A). The score together with the number of hits for a given chemical was taken as a measure for the degree of similarity between two substances. We based this approach on T-statistics since this parameter comprises both the significance as well as the direction of regulation (up or down) of each gene. The top 60 gene sets for the 26 chemicals are listed in Supplementary Tables S2 (PMH) and S3 (mESC). The results obtained for the comparison approach are summarized in Table 4 (PMH) and Table 5 (mESC). For each chemical the three best matching chemicals (referred to as 'match 1', 'match 2' and 'match 3') are indicated in Tables 4 and 5. Besides using a top 60 we also tested this approach using higher numbers of transcripts, *i.e.* a top 100 and top 200. This did not alter or improve the outcome of the analysis (data not shown). To distinguish between noise and most likely valuable results, cut-off values were arbitrarily set at 10 hits and a minimum score of 100. The results that did not reach these values are marked grey in Tables 4 and 5.

Dissecting modes of action in hepatocytes

Application of the comparison approach in hepatocytes showed that the majority of the NGTXCs gave a combination with a chemical as proposed in literature to have a comparable mode of action (Table 2 and 4). The combinations found, meeting the cut-off criteria (≥ 10 hits and a minimum score of 100), are WY + CF, LAC + SAR, TCDD + ARO, HCH + HCE and OA + CA (Table 4). Although at first sight a promising result, we observed a large variation in the scores (from 101.2 up to 859.4) between these five pairs of NGTXC, indicating that more in depth analysis are needed. The scores of 859.4 (WY and CF) and 621.0 (SAR and

LAC) clearly indicate a large overlap in their biological activities (see Table 2 and Supplementary Table S2 for detailed gene lists). In addition we found for ARO an overlap with two different chemicals, *i.e.* HCE (318; 22 hits) and TCDD (277; 13 hits). As both are respectable matches (in terms of score and hits) we further studied the corresponding gene sets of these three chemicals (Figure 2B). ARO and TCDD, both primarily Aryl hydrocarbon receptor (AhR) agonists, had 13 genes in common (see Figure 2B, yellow), including the expected AhR-mediated target genes *Cyp1a1* and *Cyp1a2*. In addition, we observed a clear overlap of 22 genes between ARO and HCE (Figure 2B, blue). The three chemicals (ARO, TCDD, and HCE), however, had only three genes in common, suggesting that perhaps two different (related?) responses are involved.

Of the remaining NGTXC the skin tumor promotors OA and CA induced comparable gene sets (see Table 4). The corresponding score was however fairly low, *i.e.* only 101, but based on 10 common genes. These genes, however, could not be related to processes or pathways triggered through inhibition of protein serine/threonine phosphatases, the suggested mechanism for these chemicals. Next, TCE and BPA, CSA and CT, and the non-carcinogens TBTO and DIDP formed mutual combinations. The relevance of these findings is questionable, since both scores and hits were clearly below the arbitrarily set cut-off values. This may suggest that these combinations are coincidental, and therefore not significant. For the remaining test chemicals, the highest score obtained was the one for the genotoxic carcinogens CSPT and MMC (327; 26 hits, Table 4). The gene set for ETP, also a direct DNA damaging agent, was quite similar to that of CSPT and MMC. One of the common genes for these three chemicals is *Ccng1*, which is a p53-responsive gene and can be linked to a DNA-damage response (Vazquez et al. 2008). For the genotoxic carcinogen MNU we did not find any significant overlap with any of the other three genotoxic substances. DEM gave changes in gene expression that were most comparable to those found for LAC and SAR. This is not an unexpected finding as each of these chemicals is known to induce oxidative stress (Beyersmann and Hartwig 2008; Yamauchi et al. 2011). This was supported by the finding that *Blvrb*, a gene which is involved in neutralizing reactive oxygen species ROS (Baranano et al. 2002), was part of the common gene sets. MEN however, which we expected to give a response at least in part similar to that of DEM (Table 3), did not show any similarity to any of the 25 substances tested.

The remaining four substances BPA, DIDP, DM and TBTO did not reach our cut-off values and showed only a weak response with no or low numbers of hits (marked grey in Table 4). These chemicals were included in the study to test the robustness of our approach. Although they all clustered together with the NGTXC in the PCA plot (Figure 1), they did not interfere with recognizing overlap in effects of exposure.

Table 4. Results comparison approach in hepatocytes.

Chemical	Match 1	Score 1	Hits 1	Match 2	Score 2	Hits 2	Match 3	Score 3	Hits 3
WY	CF	859	38	HCE	89	6	ARO	63	5
CF	WY	859	38	TCDD	56	4	HCE	50	4
LAC	SAR	621	20	DEM	277	10	TCDD	89	4
SAR	LAC	621	20	DEM	260	11	HCE	87	4
CSPT	MMC	327	26	ETP	202	15	CA	31	3
MMC	CSPT	327	26	ETP	178	15	DEM	19	2
ARO	HCE	318	22	TCDD	277	13	HCH	219	20
HCE	ARO	318	22	HCH	252	19	PB	178	13
DEM	LAC	277	10	SAR	260	11	ARO	50	3
TCDD	ARO	277	13	LAC	89	4	CF	56	4
HCH	HCE	252	19	PB	226	22	ARO	219	20
PB	HCH	226	22	ARO	211	19	TCPOBOP	179	17
ETP	CSPT	202	15	MMC	178	15	HCH	101	7
TCPOBOP	HCH	194	19	PB	179	17	HCE	112	8
FK506	HCE	112	7	ARO	111	8	SAR	67	3
OA	CA	101	10	TCE	50	6	TBTO	49	6
CA	OA	101	10	CF	46	5	LAC	43	2
TCE	BPA	74	10	TBTO	65	9	DM	51	7
BPA	TCE	74	10	TBTO	53	7	DM	40	5
TBTO	DIDP	71	10	TCE	65	9	CSA	62	8
DIDP	TBTO	71	10	TCE	44	6	CSA	39	5
CSA	CT	70	7	TBTO	62	8	DIDP	39	5
CT	CSA	70	7	OA	34	4	DM	30	4
DM	TCE	51	7	OA	48	6	BPA	40	5
MNU	WY	45	3	BPA	38	5	CA	36	4
MEN	OA	7	2	CT	6	1			

Chemicals are ranked according to score. The number of hits indicates the number of genes present in both gene sets. Grey marked cells did not met the cut-off values (hits ≥ 10 and score ≥ 100). A blank cell indicates no hits.

Comparison approach in embryonic stem cells

The mESC gene expression data were analyzed analogously to the PMH-derived data. In contrast to PMH, genotoxic carcinogens were clearly recognized

in mESC (see Table 3 and 5). As in PMH the three genotoxicants CSPT, MMC and ETP induced quite comparable changes in terms of expressed genes (see Supplemental Table S3), but the gene sets found in mESC were different from those found in PMH. In mESC, the most significantly affected genes were *Aurka*, *Kif23* and *Plk1*, whose down regulation suggests inhibition of cell growth and a pro-apoptotic response. This is supported by the finding of up-regulation of *Apaf1*, which has been reported to induce apoptosis (Bahassi el 2011; Reubold and Eschenburg 2012). Although, the abovementioned genes (except for *Apaf1*) were not present in the gene set of the fourth genotoxic carcinogen MNU, the gene set of this mutagen was similar to those of the other genotoxicants (ETP, CSPT and MMC). These findings implicate that in proliferating cells these genotoxic carcinogens are easily categorized as a cluster of chemicals giving a similar response. Oxidative stressor DEM induced a gene set that was most comparable to SAR and *vice versa*, but also MEN appeared to affect genes similar to DEM and SAR. One of the common genes, *Pgrmc1*, promotes cell death upon oxidative damage accumulation, which confirms the assumption that these substances have a common mode of action (Hand and Craven 2003).

Genes differentially expressed upon NGTXC exposure revealed similarities between CSA + FK506, and ARO + CF. Both these combinations were not found in the PMH data set. The first two chemicals (CSA and FK506), are immune suppressors, and most of their common genes are involved in endoplasmatic reticulum stress, which is a well-known effect of CSA and FK506 exposure (Kitamura 2010; Oh-Hashi et al. 2010). The combination of ARO and CF was, based on their described modes of action (Table 2), not expected. However, ARO and CF had 29 hits with a convincing score of 510 (Table 5). The common genes for these chemicals (see Supplemental Table S3) cannot be related to an unambiguous response. For instance, *Apoe*, *Fabp3* and *Abcg1* are involved in lipoprotein metabolism and transport, while *Hbegf* and *Lamp1* trigger proliferation and play a role in tumor cell metastasis (Jensen et al. 2013; Miyamoto et al. 2006). Uncovering the mechanisms triggered through ARO and CF exposure requires a more in depth analysis. This was, however, beyond the scope of this study. Other similarities the mESC gene expression profiles were found between LAC and TBTO, and BPA and WY. These combinations were also unexpected. The gene sets induced by BPA and WY had 11 genes in common, of which three genes (*Nsdhl*, *Fdft1* and *Ldlr*) are known to be involved in cholesterol metabolism. Gene sets induced by LAC and TBTO had an overlap of 10 genes.

These genes, however, could not be linked to any biological process. For the remaining chemicals, the similarity in gene expression response upon exposure was fairly low or even absent (marked grey in Table 5).

Table 5. Results comparison approach in embryonic stem cells.

Chemical	Match 1	Score 1	Hits 1	Match 2	Score 2	Hits 2	Match 3	Score 3	Hits 3
CSPT	MMC	593	32	ETP	547	19	MNU	260	15
MMC	CSPT	593	32	ETP	461	17	MNU	221	15
ETP	CSPT	547	19	MMC	461	17	MNU	383	15
ARO	CF	510	29	HCE	171	12	FK506	111	8
CF	ARO	510	29	FK506	166	11	HCE	111	6
DEM	SAR	450	16	MEN	407	16	LAC	242	8
SAR	DEM	450	16	MEN	313	14	ETP	82	3
MEN	DEM	407	16	SAR	313	14	MNU	102	6
MNU	ETP	383	15	CSPT	260	15	MMC	221	15
FK506	CSA	329	13	CF	166	11	ARO	111	8
CSA	FK506	329	13	CF	91	6	LAC	78	3
LAC	TBTO	251	10	DEM	242	8	BPA	164	6
TBTO	LAC	251	10	HCE	69	6	BPA	68	5
BPA	WY	175	11	LAC	164	6	PB	145	9
WY	BPA	175	11	LAC	130	6	DEM	77	4
HCE	ARO	171	12	CF	111	6	BPA	72	4
PB	BPA	145	9	WY	57	5	CSA	46	2
TCPOBOP	CF	66	5	ARO	65	6	CSA	25	2
CA	WY	54	5	ETP	49	2	CSPT	42	3
HCH	HCE	44	3	ARO	36	4	BPA	28	2
TCDD	CA	32	4	ARO	18	2	TCPOBOP	16	2
TCE	HCH	22	3	TCPOBOP	14	2	OA	13	2
OA	CA	15	2	TCPOBOP	15	2	DM	14	2
DM	CA	15	2	OA	14	2	PB	9	1
CT	CA	14	2	OA	13	2	CF	10	1
DIDP	PB	10	1	DM	9	1	ARO	8	1

Chemicals are ranked according to score. The number of hits indicates the number of genes present in both gene sets. Grey marked cells did not met the cut-off values (hits ≥ 10 and score ≥ 100).

Discussion

Current carcinogenicity test strategies are based on *in vitro* and *in vivo* genotoxicity tests and, as such, non-genotoxic carcinogens may go undetected (Hernandez et al. 2009). The diversity of modes of action of NGTXC complicates reliable detection of this 'class' of chemicals. Therefore, we and others explored

the possibility to recognize features of these substances using transcriptomics approaches (Fielden et al. 2011; Schaap et al. 2012; Waters et al. 2010). In a previous study we demonstrated the usefulness of a toxicogenomics-based approach to categorize NGTXC according to their overlap in gene expression profiles using primary mouse hepatocytes (Schaap et al. 2012). One of the two aims of the present study was to improve this approach, which was initially based on an unsupervised clustering approach using Gene Set Enrichment Analysis (Subramanian et al. 2005). As a first step to simplify this method, we now focused on the 60 (30 up and 30 down) most significantly regulated genes per chemical to search for common genes and possibly also pathways. For this, we employed T-statistics, because it provides information on the direction as well as the significance of regulation of expressed genes. Furthermore, we broadened the focus of interest by extending the list of chemicals tested. In addition to NGTXC, we now also included other chemical 'classes'. The second aim was to investigate the added value of an extra *in vitro* cell system, *i.e.* mouse embryonic stem cells (mESC).

Application of our modified approach resulted in the recognition of the majority of expected non-genotoxic modes of action (as described in Table 2). Using primary hepatocytes we detected five mutual NGTXC combinations, *i.e.* WY + CF, LAC + SAR, TCDD + ARO, HCH + HCE, and OA + CA. Addition of mESC as an extra *in vitro* test system revealed another NGTXC pair, *i.e.* CSA + FK506. So, six out of the eight NGTXC combinations tested were uncovered. The chemical pairs CT + TCE and PB + TCPOBOP still went undetected. Looking at the number of DEGs (Table 1) we tend to conclude that a lack of response in both cellular systems (*i.e.* PMH and mESC) is the reason why these chemicals were missed.

Besides the to our knowledge expected combinations, we also found other combinations with convincingly high scores and a large number of overlapping genes. Apparently, some chemicals act via more than one (known) mechanism. In PMH, for example, we found an overlap of 22 genes between the gene sets induced by ARO and HCE. ARO was selected as AhR agonist (Table 2), but it is known that ARO in hepatocytes also binds to other nuclear receptors like the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) (Aly and Domenech 2009; Silkworth et al. 2008). Kojimo and co-workers have shown that organochlorine pesticides HCE and HCH also act as PXR agonists (Kojima et al. 2011). PXR binding is therefore a very plausible explanation for the observed match between ARO and HCE. The second best match for ARO

was TCDD. Comparison of the top genes affected upon ARO and TCDD exposure clearly revealed signaling pathways induced through the AhR. So, our modified approach appears to be also useful for recognizing substances that act through related mechanisms. This is in our view a very important feature with substantial added value for (human health) risk assessment.

Next to the NGTXC, the direct-acting genotoxic agents CSPT, MMC, ETP and MNU (Attia et al. 2003; Roos and Kaina 2013; Weng et al. 2010) were correctly categorized using mESC. In PMH the response to these genotoxicants was less pronounced but still CSPT, MMC, and ETP were rather easily identified as chemicals having comparable effects of exposure. Furthermore, another group of chemicals, comprising DEM, MEN, but also the non-genotoxic carcinogens LAC and SAR induced a similar gene expression response. These four chemicals have in common that they all induce oxidative stress (Aragon et al. 2006; Beyersmann and Hartwig 2008; Hendriks et al. 2012; Yamauchi et al. 2011). In both cell lines we detected three out of these four substances as chemicals with a common gene expression pattern: LAC, SAR and DEM were recognized in PMH, whereas DEM, MEN and SAR were detected in mESC. This finding demonstrates the added value of using multiple *in vitro* systems, and in particular the complementary value of PMH and mESC for these substances.

So far, our approach seems to be promising in detecting common expression profiles of (carcinogenic) chemicals. However, some points need further attention. An important issue is the need to clearly define what a reliable response is. What are the criteria to decide whether a substance indeed induces a particular expression pattern? We applied a minimum of 10 hits and a score of at least 100 as cut-off values. For the NGTXC, these criteria appeared to yield variation in the appropriateness of the results. For instance, we observed in PMH a robust change in gene expression upon exposure to the peroxisome proliferators WY and CF, resulting in a relatively simple detection of their common mode of action. In contrast, the response upon exposure to the skin tumor promotors OA and CA was far less pronounced. In the overlap between the respective gene sets of these chemicals, we were not able to recognize specific biological processes, *e.g.* effects on cell cycle, cell proliferation and remodeling of the cytoskeleton by inhibition of protein serine/threonine phosphatases. This may be due to inappropriate exposure times, cell specific effects, or the fact that these biological processes are mainly recognized at the protein level (McConnell and Wadzinski 2009).

Another crucial question is how we can ensure the detection of chemicals with an overall low response, *e.g.* chemicals such as the skin tumor promotors CA and OA, or the halogenated hydrocarbons TCE and CT. In the present study we tested all substances at only a single concentration based on cytotoxicity levels. Testing multiple concentrations and/or multiple time points will most likely improve the detection of chemicals with a similar mode of action, and may even enable recognition of different modes of action per chemical. This certainly is of added value when characterizing unknown and new chemicals. Next to this, more *in vitro* testing systems and/or other "omics" approaches are required to cover a wider range of NGTXC. For example, (phospho-) proteomics would surely improve the detection of chemicals like the skin tumor promotors, since it has been shown that these NGTXC act as inhibitors of protein serine/threonine phosphatases (Pan et al. 2008; Pines et al. 2011). As additional *in vitro* system one could consider to use rat primary hepatocytes. These cells have been demonstrated to retain high mRNA levels of CAR and other xenobiotic receptors, as well as Phase I and Phase II genes (Baudoin et al. 2014). This may improve the detection of CAR activators, which were missed using PMH and mESC.

We demonstrated the usefulness of our comparison approach and pointed out how further modifications, like testing multiple concentrations per chemical and implementation of additional *in vitro* systems, may further strengthen the method. Next, it is important to consider how this approach would fit into current test strategies for safety assessments of chemicals (Figure 3). For genotoxic substances, reliable assays such as the Ames test and the *in vitro* micronucleus test are available (Kirkland et al. 2005; Pfuhler et al. 2007). We therefore propose that our toxicogenomics-based approach will be used as additional step in the second tier of the current test strategy for cancer risk assessment. Thus, for chemicals that give a negative result in the standard battery of *in vitro* genotoxicity tests or that are demonstrated to be non-genotoxic *in vivo* (Figure 3). All information available at that point in the test strategy will determine whether further testing is necessary. We consider a detailed description of a complete test strategy for cancer risk assessment beyond the scope of this manuscript, and will therefore present our views and ideas in a separate publication (Luijten et al. *in preparation*). Following gene expression profiling in various *in vitro* test systems (at least including hepatocytes and stem cells),

a weight-of-evidence approach will be required for appropriate interpretation of the data. This implies that we will need to define decision criteria that will take into account the matches within one test system as well as the results across the various cell systems. Such an approach complies with the current shift in focus in regulatory risk assessment from hazard identification to hazard characterization, in which information on modes of action will get a more crucial role.

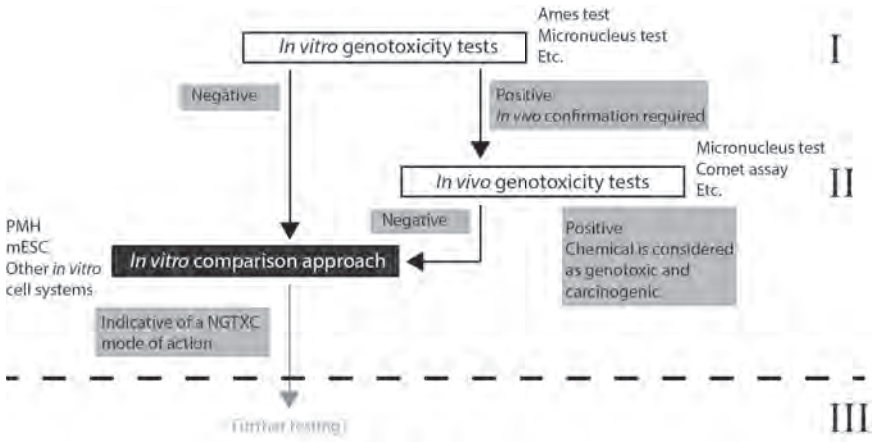


Figure 3. Flowchart of the first tiers of the current test strategy for cancer risk assessment, visualizing the suggested position of the *in vitro* comparison approach.

In conclusion, we showed in the present study that only a limited set of significantly regulated genes is sufficiently informative to categorize chemicals according to their mode of action. We demonstrated that our toxicogenomics-based comparison approach, using hepatocytes as well as embryonic stem cells, is useful to recognize expression patterns of non-genotoxic as well as genotoxic and oxidative stress inducing (carcinogenic) substances. The concept of the approach however is not limited to carcinogens only, but applicable to environmental and pharmaceutical chemicals in general. Given the need for alternative test systems and the fact that mode of action is gaining importance in human health risk assessment, we are confident that this approach will become a useful and feasible tool for future safety evaluation of chemicals.

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Supplemental information

Supplementary data is available at the journal's website.
http://link.springer.com/article/10.1007%2Fs00204-014-1368-6

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