Chapter 3

Dissecting Modes of Action of Non-Genotoxic Carcinogens in Primary Mouse Hepatocytes

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Abstract

Under REACH, the European Community Regulation on chemicals, the testing strategy for carcinogenicity is based on *in vitro* and *in vivo* genotoxicity assays. Given that non-genotoxic carcinogens are negative for genotoxicity and chronic bioassays are no longer regularly performed, this class of carcinogens will go undetected. Therefore, test systems detecting non-genotoxic carcinogens, or even better their modes of action, are required. Here, we investigated whether gene expression profiling in primary hepatocytes can be used to distinguish different modes of action of non-genotoxic carcinogens. For this, primary mouse hepatocytes were exposed to 16 non-genotoxic carcinogens with diverse modes of action. Upon profiling, pathway analysis was performed to obtain insight into the biological relevance of the observed changes in gene expression. Subsequently, both a supervised and an unsupervised comparison approach were applied to recognize the modes of action at the transcriptomic level. These analyses resulted in the detection of three out of eight compound classes, *i.e.* peroxisome proliferators, metalloids and skin tumor promotors. In conclusion, gene expression profiles in primary hepatocytes, at least in rodent hepatocytes, appear to be useful to detect some, certainly not all, modes of action of non-genotoxic carcinogens.

Introduction

The current carcinogenicity testing strategy under REACH consists of a tiered approach, focusing on genotoxic endpoints. REACH is the European policy that deals with the Registration, Evaluation, Authorization and Restriction of Chemicals. This regulation requires every chemical with a production volume over 1 tonne per year to be evaluated for human health and environmental risk (Hernandez et al. 2009; Lilienblum et al. 2008; Thomas et al. 2009). The testing strategy for carcinogenicity consists of *in vitro* genotoxicity tests, where a positive result triggers further *in vivo* confirmation (Pfuhler et al. 2007; Thybaud et al. 2007). For substances with positive *in vivo* genotoxicity results, it is decided on a case-by-case basis whether a 2-year carcinogenicity study in rodents is necessary. Criteria to test a substance in the 2-year bioassay include: a production volume greater than 1,000 tonnes per year, a causing concern due to possible mutagenic effects, and evidence of frequent or long-term human (occupational) exposure (Lilienblum et al. 2008). In contrast to genotoxic carcinogens, non-genotoxic carcinogens (NGTXC) induce neoplasia without reacting directly with DNA (Williams 2001; Melnick et al. 1996). Consequently, these substances are negative in (*in vitro/in vivo*) genotoxicity tests. As such, this class of carcinogens goes undetected under REACH (Hernandez et al. 2009; Lilienblum et al. 2008).

Twelve percent (45/371) of the known, probable or possible human carcinogens classified by the International Agency for Research on Cancer (IARC) appear to be non-genotoxic. For one third of these substances, exposure is high enough to expect a significantly increased cancer risk (Hernandez et al. 2009). This underlines the importance of alternative, preferable *in vitro*, test systems predicting non-genotoxic carcinogenic features of an unknown substance. However, the 'class' of NGTXC consists of substances with a wide variety of modes of action, including endocrine modification, immune suppression, tissue-specific toxicity, and inflammatory responses. (Hernandez et al. 2009; Melnick et al. 1996; Hattis et al. 2009; Waters et al. 2010). Due to this diversity, it is not feasible to detect all these substances within one test system (Hernandez et al. 2009). Presumably, a test battery is required to cover all modes of action of the NGTXC.

As a first step towards the development of such an *in vitro* test battery, we tested 16 NGTXC in primary mouse hepatocytes. The selected carcinogens consisted of both human and rodent carcinogens and represent eight different modes of action. We knowingly included rodent carcinogens, since the experiments were performed in a rodent setting. The benefit of using primary mouse hepatocytes as a test system is their biotransformation capacity (Gebhardt et al. 2003; Hewitt et al. 2007; Mathijs et al. 2009). Previous studies have shown that these cells can be used to detect genotoxic carcinogens (Mathijs et al. 2010; van Kesteren et al. 2011). In the present study, some but not all modes of action of NGTXC could be detected, making mouse hepatocytes an attractive component for a test battery.

Materials and methods

Chemicals

Chemicals tested in this study (see Table 1) consisted of 16 NGTXC: Cyclosporine A (CSA), Tacrolimus (FK506), β-Hexachlorocyclohexane (HCH), Heptachlor epoxide (HCE), Wyeth-14,643 (WY), Clofibrate (CF), 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), Aroclor 1254 (ARO), 1,4-bis[2-(3,5-dichloropyridyloxy)]Benzene (TCPOBOP), Phenobarbital (PB), Carbon Tetrachloride (CT), 1,1,1-Trichloroethane (TCE), Okadaic Acid (OA), Calyculin A (CA), Sodium Arsenite (SAR), Lead Acetate (LAC). For details on CAS number, supplier and solvent see Table 1. The selection of these NGTXC was based on carcinogenicity and genotoxicity reviews by 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) and a study reported by Kirkland et al. (2005).

Table 1. Overview of the 16 non-genotoxic carcinogens tested

Non-genotoxic carcinogen	Abbreviation	CAS-number	Supplier	Solvent [a]	Concentration [b]
1,1,1,-trichloroethane	TCE	71-55-6	Supelco	DMSO	1 mM
1,4-bis[2-(3,5-dichloropyridyloxy)]benzene	TCPOBOP	76150-91-9	Sigma	DMSO	10 µM
2,3,7,8-Tetrachlorodibenzo-p-dioxin	TCDD	1746-01-6	Accu standard	DMSO	10 nM
Aroclor 1254	ARO	11097-69-1	Supelco	DMSO	30 µM
Calyculin A	CA	101932-71-2	Sigma	DMSO	1 nM
Carbon Tetrachloride	CT	56-23-5	Supelco	DMSO	300 µM
Clofibrate	CF	637-07-0	Sigma	DMSO	1 mM
Cyclosporin A	CSA	59865-13-3	Novartis	DMSO	1 µM
Heptachlor epoxide	HCE	1024-57-3	Supelco	DMSO	30 µM
Lead Acetate	LAC	6080-56-4	Sigma-Aldrich	DMSO	10 µM
Okadaic Acid	OA	78111-17-8	Sigma	DMSO	1 nM
Phenobarbital	PB	50-06-6	Sigma	DMSO	1 mM
Sodium Arsenite	SAR	7784-46-5	Sigma-Aldrich	PBS	3 µM
Tacrolimus	FK506	109581-93-3	Sigma	DMSO	30 µM
Wyeth-14643	WY	50892-23-4	Sigma	DMSO	100 µM
β-Hexachlorocyclohexane	HCH	319-85-7	Fluka	DMSO	100 µM

[a] The substances were added to the medium as a 200x stock solution, resulting in a 0.5% solvent concentration.
[b] See also Supplementary Figure S1.

Isolation and culture of primary mouse hepatocytes

Primary mouse hepatocytes were isolated from 8-10 weeks old male C57BL/6J mice by a modified two-step collagenase perfusion technique (collagenase type IV, Sigma-Aldrich, Zwijndrecht, The Netherlands), as described by van Kesteren et al. (2011). To obtain a homogeneous cell suspension containing sufficient cells to test all substances, hepatocyte suspensions with at least 80% viability, determined by trypan blue exclusion, from two mice were mixed in equal proportions before they were seeded to 6-wells plates coated with 1 mg/ml neutralized collagen type I (BD Biosciences, Breda, The Netherlands) at 1.3 x 10^6 cells per well. Unattached hepatocytes were removed by washing and a sandwich configuration was achieved by adding a second layer of neutralized collagen to the cells. After one hour at 37°C, serum-free DMEM (Invitrogen, Bleiswijk, The Netherlands) was added, containing 2% penicillin/streptomycin (Invitrogen), 7 ng/ml glucagon (Sigma-Aldrich), 7.5 µg/ml hydrocortisone (Sigma-Aldrich) and 0.5 U/ml insulin (from bovine pancreas, Sigma-Aldrich). Cells were kept in serum-free medium and the culture medium was changed daily until exposures were performed.

Cytotoxicity analysis

For all 16 substances a cytotoxicity analysis was performed. Forty-six hours after isolation, hepatocytes were exposed for 24 hours to varying concentrations of the substances dissolved in DMSO or PBS (Supplementary Material, Table S1). Final DMSO or PBS concentrations in culture medium were 0.5 % (v/v) in all exposure studies, including the vehicle controls. Cytotoxicity was tested after an additional 48 hours with normal serum-free culture medium, using the MTT reduction method as described by Mosmann (1983), with modifications. In short, cultures were incubated for one hour with 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Invitrogen). The medium was removed and the formazan crystals formed were solubilized in DMSO. Absorbance was measured in triplicate 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).

Gene expression profiling

Forty-six hours after isolation, hepatocytes were exposed to one of the NGTXC. Concentrations were selected based on the cytotoxicity assay (Supplementary Table S1). After 24 hours of exposure, cells were collected in 1 ml RNAprotect (QIAgen, Venlo, The Netherlands) and stored at -80˚C for RNA isolation. RNA was extracted using QIAzol and purified using the miRNeasy mini kit and the QIAcube (Qiagen), according to the manufacturer's protocol. For each substance tested, four biological replicates were used. 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).

Microarrays and labelling of RNA

The HT MG-430 PM Array plate (Affymetrix Inc., Santa Clara, CA, USA) represents 39,000 well-characterized transcripts on a single array. In short, biotins labeled cRNA samples were prepared as described in the Affymetrix GeneChip HT 3" IVT Express Technical Manual (Affymetrix) using 200 ng of purified total RNA as template for the reaction. For this the GeneChip 3" IVT express kit (Affymetrix) was used. The 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

The raw data were subjected to a set of quality control checks. The quality check revealed significant hybridization and experimental blocking effects. After passing array quality control, the arrays were annotated according to 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) available from the Bioconductor project (http://www.bioconductor.org) for the R statistical language (http://cran.r-project.org). The normalized data was statistically analyzed for differential gene expression using a mixed linear model with coefficients for block (random) and each experimental group (fixed) (Smyth 2004; Wolfinger et al. 2001). 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 (2003). Only the annotated genes were used for further analysis. The gene expression results have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (accession no. GSE35058). Per substance, the differentially expressed genes (DEG) were further analyzed for overrepresentation in MetaCore from GeneGo Inc. to identify significant biological processes with a FDR < 0.05 (http://www.genego.com/metacore.php). If the number of DEG was less then 1,000, a top 1,000 of genes (based on the *P*-value) was analyzed.

To further investigate if two NGTXC with expected similar mode of action resulted in similar gene expression responses, we used two comparison analyses. The first analysis was a supervised comparison; we presumed a priori that two substances with a similar mode of action should yield the same responses, and we aimed to find the genes that showed consistently a higher log fold change in response to the substances with a similar mode of action. This was quantified by applying Fisher's ratio (denoted as *C*) on the log fold changes (calculated with respect to the solvent control). The genes were ranked according to their *C* value. We also calculated a significance value for *C* by comparing it to a distribution of 5000 *C* values calculated from random sets of substances without a similar mode of action.

The second analysis was an unsupervised comparison; we presumed a priori that substances inducing similar gene expression responses have a similar mode of action, intended to find for each NGTXC another substance with the most similar response profile. For this a Gene Set Enrichment Analysis (GSEA) was used (Lamb et al. 2006; Subramanian et al. 2005). We basically tested whether the upregulated genes found in response to one substance (*x*) were also upregulated in response to any other substance, and we did the same for the downregulated genes. The similarity of substance *x* with substance *y* ($S_{x\rightarrow y}$) was quantified using the enrichment score of the upregulated genes (ES_{up}) and the enrichment score of the downregulated genes (ES_{down}) as:

$$S_{x\rightarrow y} = \begin{cases} ES_{up} - ES_{down} \text{ if } ES_{up} > ES_{down} \\ ES_{down} - ES_{up} \text{ if } ES_{down} > ES_{up} \end{cases}$$

The substance yielding the highest value for *S* was the substance with the highest similarity to *x*. Note that $S_{x\rightarrow y} \neq S_{y\rightarrow x}$. The leading edge subsets were used for interpretation using MetaCore from GeneGo Inc. in those cases where

$ES_{up} > 0$ or $ES_{down} < 0$. This analysis was executed using the differentially expressed genes (see above), and also using the top 200 most significantly upregulated and top 200 most significantly downregulated genes.

Results

Concentration selection for gene expression studies

To determine a concentration of each NGTXC for gene expression profiling, we used selection criteria based on cytotoxicity. A concentration range study was performed using the MTT reduction method. Gene expression profiles were determined in cells with a viability of at least 90% (see Supplementary Material, Table S1). If no cytotoxicity was induced, the maximum concentration used was 1 mM. Due to limited solubility, the concentration of TCDD was based on literature data (Flaveny et al. 2010). Plots of the cytotoxicity tests are depicted in Supplementary Fig. S1, A-P, ultimate chosen concentrations per substance are depicted in Table 1.

Gene expression analysis – general impression

To obtain a general impression of the transcriptomics data, differences in gene expression between the substances and corresponding vehicle controls were calculated with a FDR<0.05. This analysis resulted in a set of differentially expressed genes (DEGs) that varied highly in number, ranging from 40 (TCE) up to 5,693 (LAC) genes (see Table 2). A principle component analysis (PCA) based on all genes revealed essentially three clusters of NGTXC (Figure 1 and Table 2). The largest cluster (cluster A) consisted of ten substances inducing low to intermediate changes in gene expression. The gene expression responses of the other two clusters were more distinct. These clusters included a collection of four carcinogens with different modes of action (cluster B) and a group containing the two metalloids (cluster C).

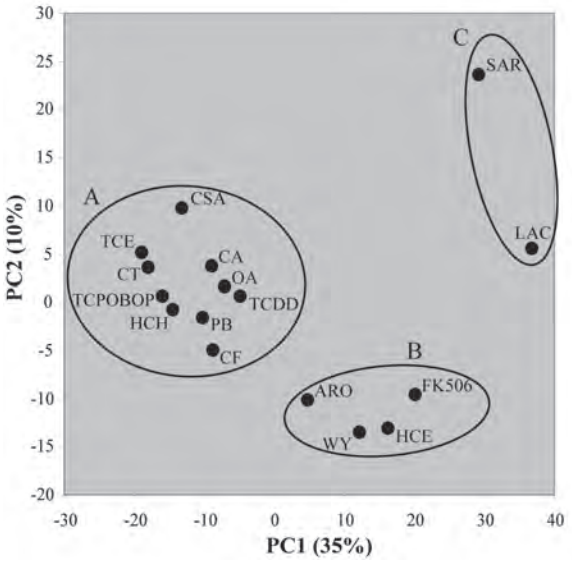

Figure 1. Principle component analysis. Based on all genes, the 16 NGTXC can be divided into three clusters. A = low number of DEGs, B = mid, and C = high number of DEGs. For explanation of the abbreviations see Table 1.

Table 2. Results of the gene expression analysis

NGTXC[a]	Mode of action	DEGs[b]	PCA[c]	Response[d]
ARO	Arylhydrocarbon receptor agonist	762	B	1
TCDD	Arylhydrocarbon receptor agonist	567	A	1
CA	Skin tumor promoter	281	A	3
OA	Skin tumor promoter	282	A	3
CF	Peroxisome proliferator	308	A	1
WY	Peroxisome proliferator	1611	B	1
CSA	Immunosuppressant	124	A	1
FK506	Immunosuppressant	2404	B	2
CT	Halogenated hydrocarbon	56	A	3
TCE	Halogenated hydrocarbon	40	A	3
HCE	Ligand-independent estrogen receptor activator	2088	B	2
HCH	Ligand-independent estrogen receptor activator	115	A	3
LAC	Metalloid	5693	C	2
SAR	Metalloid	4130	C	2
PB	Constitutive androstane receptor agonist	238	A	3
TCPOBOP	Constitutive androstane receptor agonist	65	A	3

[a] For the full names of the substances, see Table 1.
[b] DEGs = differentially expressed genes, FDR<0.05.
[c] Based on the PCA plot (Fig. 1) samples were divided into three clusters. A = low number of DEGs, B = mid, and C = high number of DEGs.
[d] The response column indicates whether a substance, in terms of pathways, induced (1) a specific response, (2) a non-specific response, or (3) no response.

Pathway analyses

For each carcinogen pathway analysis was performed using GeneGo Pathways in Metacore. A detailed list of the top 50 of the pathways involved is shown in Supplementary Table S2. Based on this analysis and literature data, the 16 NGTXC could be divided into three different sets (marked 1, 2 and 3 in Table 2), to be discussed in more detail in the next sections. Set 1 comprised carcinogens, of which the regulated pathways fit the literature data on these substances (see Table 2). Substances belonging to the second set showed a more general response not directly related to their published modes of action. The results of the remaining NGTXC (set 3) did not show any significantly and/or expected regulated pathways.

The NGTXC of set 1 that induced known specific responses were CSA, CF, WY, ARO and TCDD. CSA is a well-known immunosuppressant and a human carcinogen (Hamawy and Knechtle 2003; Matsuda and Koyasu 2000). In our study, the main observed effect of CSA was upregulation of *OSM, IL1R1* and *IL1rap*, receptors triggering pathways involved in immune responses. Interestingly, CSA also changed expression of genes involved in cellular genotoxic stress responses, i.e. downregulation of *ATM, ATR* and *DNA-PK* (Yang et al. 2003).

WY, a rodent carcinogen (possibly not human), is often used as model compound for studying peroxisome proliferation, whereas CF is a lipid lowering peroxisome proliferator used for controlling high cholesterol and triacylglyceride levels in blood. Peroxisome proliferators bind to and activate the peroxisome proliferators-activated receptor-α (PPARα) regulating target genes encoding for peroxisomal beta-oxidizing enzymes, fatty acid transporters, lipoprotein lipase, cytochrome P450 4A family and thioesterases (Fidaleo et al. 2009; Peters et al. 2005). Although these primary processes are well described, the precise carcinogenic mechanism of PPARα agonists is not yet fully understood. WY and CT showed a clear upregulation of genes belonging to the cytochrome P450 4A family (*cyp4a10, cyp4a14, cyp4a31*) and genes encoding thioesterases (*Acot1,2,3 and 5*). At the pathway level, the major effect observed was suppression of blood coagulation and induction of peroxisomal and mitochondrial pathways involved in lipid metabolism and fatty acid beta-oxidation. These effects have also been observed in rat *in vivo* studies and in studies employing primary rat hepatocytes (Hirode et al. 2009; Tamura et al. 2006).

Two other NGTXC that induced a specific response were the Aryl hydrocarbon Receptor (AhR) agonists ARO and TCDD. ARO is a mixture of polychlorinated biphenyls (PCBs), some of which are structurally related to the potent toxicant TCDD (Aly and Domenech 2009). These two substances have been shown previously to alter gene expression related to cholesterol biosynthesis, lipogenesis, and glucose metabolism through the AhR-mediated pathway (Mendoza-Figueroa et al. 1992; Sato et al. 2008). In our study, both substances did, as expected, decrease processes involved in lipid metabolism. In addition, TCDD induced an apoptotic response, which has also been frequently observed in both *in vivo* and *in vitro* test systems (Chopra and Schrenk 2011). ARO and TCDD strongly induced the expression of cytochrome P450 genes *Cyp1a1* and *Cyp1a2*. These genes are well-known targets of the aryl hydrocarbon receptor (Schwarz and Appel 2005; Whitlock 1993). As with the peroxisome proliferators, the molecular mechanism underlying the tumor promoting effect of AhR agonists is still poorly understood (Mandal et al. 2005).

The second set of NGTXC (see response 2 in Table 2), consisting of FK506, HCE, LAC and SAR, caused an effect that could not be related to a specific mechanism. These substances have in common that their exposure resulted in a strong response at the gene expression level (> 500 differentially expressed genes, Table 2). The major affected pathways (see Supplementary Table S2) were related to the regulation of lipid metabolism via the farnesoid X receptor (FXR) and the liver X receptor (LXR). Since FXR and LXR are important regulators of cholesterol and fatty acids, regulation of pathways via these receptors is indicative of a cholestatic and thus a hepatotoxic effect (Wagner et al. 2009). All four substances negatively regulated *Slc10a1* and *Abcb11*. These genes are important for bile acid uptake and export, and are both known to be triggered through the FXR pathway (Cheng et al. 2007).
Upon LAC exposure a complicated set of pathways, sometimes opposite to each other (e.g. cell growth and apoptosis) was found. Regulated pathways included mainly processes like stimulation of cell growth and proliferation. This proliferative effect of LAC has been reported in the IARC monographs on LAC (IARC 2006). In contrast, exposure to LAC also resulted in an upregulation of *NF*-κB and the pro-apoptotic genes *BAX* and *BID*, as well as a downregulation of the anti-apoptotic gene *Bcl-2*. These findings suggest stimulation rather than inhibition of apoptosis and, as such, are inconsistent with the proliferative response.

Pathway analyses of the gene expression profiles of the seven remaining NGTXC (set 3) did not yield processes that could be linked to any known mechanism. For CT, HCH, OA, PB and TCPOBOP no significant regulated pathways were found, whereas for CA and TCE the resulting list of 50 pathways was incoherent. Despite the lack of affected pathways, we did find for TCPOBOP an PB (both constitutive androstane receptor (CAR) agonists) upregulation of *Cyp2b10*, known from *in vivo* studies to be a classical target gene of activated CAR (Lempiainen et al. 2011).

Using expression profiles to discriminate modes of action of NGTXC

We used a supervised and unsupervised comparison to dissect modes of action of the 16 NGTXC (Table 2). The 16 NGTXC represent eight different modes of action, with two carcinogens per mode of action (Table 2). A supervised approach was applied to investigate to what extent a similar mode of action resulted in a comparable gene expression profile. For each pair of substances representing a specific mode of action, a set of 100 genes was selected, using the *C* value as parameter (see Materials and Methods for details). Combining the 100 discriminating genes for the eight different modes of action resulted in a set of 795 genes (five genes overlap between two modes of action) (see Supplementary Table S3). A heatmap of the top 30 of genes per pair of substances, *i.e.* per mode of action, is shown in Figure 2. To check the specificity of this method, we performed the same analysis for random pairs of the carcinogens, using 5,000 permutations. The frequency of the maximum *C* values obtained for these random pairs is depicted in Figure 3. Comparison of these results with the maximum *C* values obtained for the correct pairs shows that, for three out of the eight pairs, the discrimination was statistically significant (see Table 3). These three pairs were the peroxisome proliferators (CF and WY), the metalloids (LAC and SAR) and the AhR agonists (ARO and TCDD). These substances had the highest overlap in DEGs (Table 3). When performing the same analysis for the sum of the top100 of *C* values instead of the maximum *C* value, the discrimination of the AhR agonists was no longer significant. This is most likely due to the fact that for ARO and TCDD only the first 2 out of 100 genes had a *C* value reaching statistical significance (Supplementary Table S3).

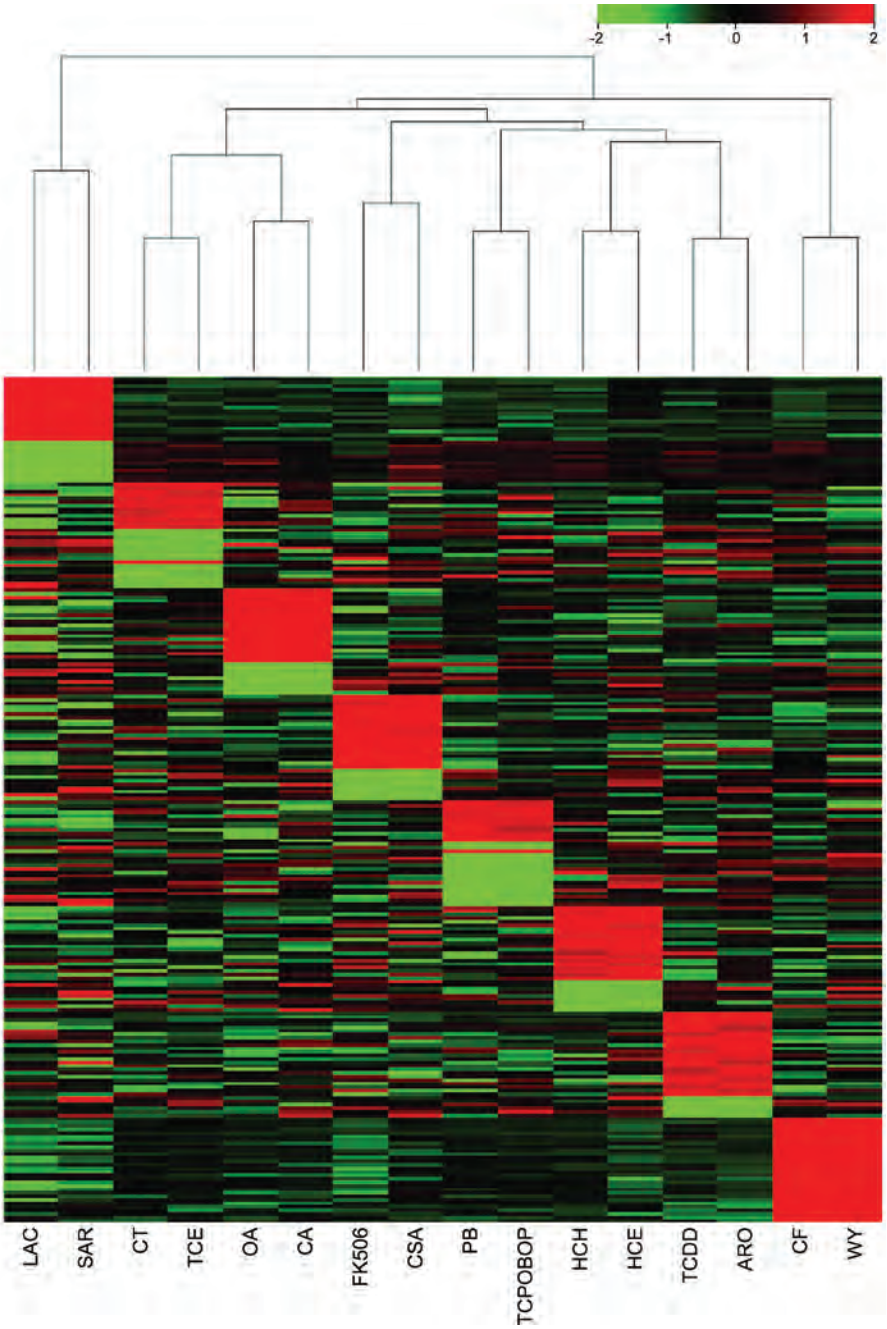

Figure 2. Supervised comparison of the NGTXC based on their modes of action. Heatmap of 240 genes, consisting of the union of a selected set of genes per mode of action. Each lane represents a different substance. In this figure, red indicates upregulation and green downregulation.

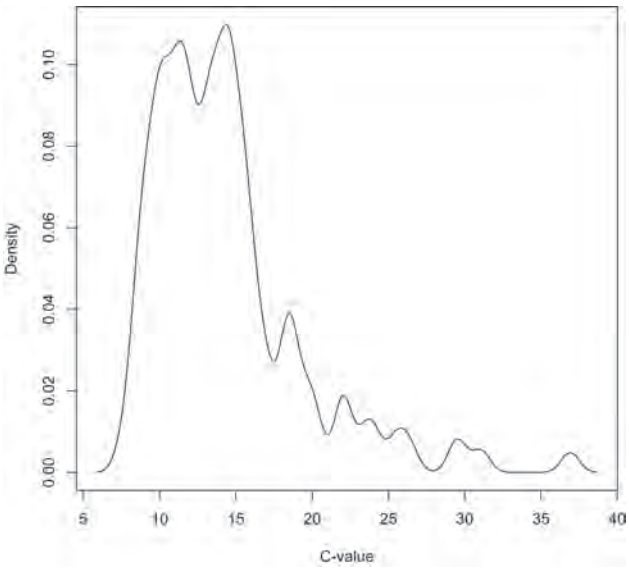

Figure 3. Distribution of the maximum *C* values after supervised comparison of random pairs of substances, using 5,000 permutations.

Table 3. Supervised comparison of the 16 non-genotoxic carcinogens, based on their modes of action

	Max. *C* value		Sum of 100 *C* values		
	***C* value**	***P*-value**	***C* value**	***P*-value**	**Overlay[a] DEGs**
ARO - TCDD	**73.0**	**0.0002**	570.6	0.2470	195
CA - OA	15.1	0.3141	688.2	0.1260	67
CF - WY	**587.0**	**0.0002**	**2903.2**	**0.0002**	244
CSA – FK506	14.2	0.4321	586.5	0.2082	23
CT - TCE	7.1	1.0000	422.9	0.8570	1
HCE - HCH	11.9	0.6351	457.0	0.6789	74
LAC - SAR	**181.0**	**0.0002**	**5959.5**	**0.0002**	3059
PB - TCPOBOP	12.2	0.6140	489.3	0.5011	24

[a] This column shows the number of genes which are differential expressed in both substances.

The second approach to categorize different modes of action was based on an unsupervised comparison. Instead of using pairs of substances as starting point, gene expression patterns were compared individually to find the best matching counterpart for each substance. These analyses were conducted using a GSEA with the DEGs of each NGTXC used as input. This analysis yielded a best match for each substance. For 7 out of 16 NGTXC this analysis resulted in a match with a carcinogen having the same mode of action; for three modes of action this was

a perfect match in both ways (see Table 4). The NGTXC that could be matched using this approach were CA and OA, LAC and SAR, and WY and CF. The match of the AhR agonists was not perfect. ARO did induce a gene signature that was comparable to the one of TCDD, whereas HCE fitted best with the signature of ARO. Since the GSEA resulted in enrichment scores (ES) for each combination of substances, it was possible to rank the 15 NGTXC in terms of similarity. The complete ranking of all substances can be found in Supplementary Table S4A. Since the number of DEGs for the 16 NGTXC varied from 40 to > 5,500 (Table 2), we repeated the GSEA with an equal number of genes as input. Per substance, the input consisted of the top 200 of upregulated genes and the top 200 of downregulated genes. Using these 400 genes per substance, we found almost the same counterparts as for the DEGs (Table 4). Again, 7 out of 16 were a match between carcinogens having the same mode of action and these seven were exactly the same using the DEGs as input. The complete ranking of all NGTXC can be found in Supplementary Table S4B.

Table 4. Unsupervised comparison of the 16 non-genotoxic carcinogens

	Best match	
NGTXC	**Based on DEGs[a]**	**Based on 400 genes[b]**
ARO	HCE	HCE
CA	***OA***	***OA***
CF	***WY***	***WY***
CSA	TCPOBOP	CT
CT	PB	HCH
FK506	LAC	LAC
HCE	ARO	ARO
HCH	PB	TCPOBOP
LAC	***SAR***	***SAR***
OA	***CA***	***CA***
PB	ARO	ARO
SAR	***LAC***	***LAC***
TCDD	***ARO***	***ARO***
TCE	HCH	TCPOBOP
TCPOBOP	HCH	HCH
WY	***CF***	***CF***

A row with ***bold-italic*** letters indicates a match with a substance sharing the same mode of action.
[a]The best match based on DEGs.
[b]The best match based on the top 200 up- and top 200 downregulated genes.

Discussion

The aim of the present study was to find an approach by which NGTXC can be categorized according to their mode of action, to be used as part of a test battery to detect NGTXC. The study was performed to provide proof of concept, and was not meant to be a human risk analysis. As such, the selected substances represented not only human but also rodent carcinogens. Besides hepatocarcinogens, which are more likely to give a positive response at the gene expression level in primary hepatocytes, we also included carcinogens with other target organs than the liver. The various modes of action were chosen arbitrarily and the selected carcinogens are known to induce direct responses (e.g. by receptor binding) and indirect responses (e.g. via the activated metabolite). Testing such a wide range of NGTXC enabled us to assess the suitability of primary mouse hepatocytes as an *in vitro* test system.

To distinguish substances by their mode of action, we employed two different approaches to recognize similarities in gene expression signatures for each pair of substances. The supervised approach resulted in correct grouping of the peroxisome proliferators (CF and WY) and the metalloids (LAC and SAR). The results for the AhR agonists (ARO and TCDD) were only significant when the maximal *C* value was used. More substances need to be tested to increase the number of substances per mode of action as well as the number of different modes of action. In our view, the gene sets representing different modes of action resulting from the present and future studies can possibly be used in an *in vitro* screening assay to detect the mode of action of an unknown substance having comparable features.

Besides the supervised approach, we employed an unsupervised approach, which is based on GSEA without taking existing modes of action into account. Using the DEGs as input, this analysis yielded 7 matches with the same modes of action, with a perfect match for 6 out of 7 substances. These matches included the peroxisome proliferators (CF and WY), the metalloids (LAC and SAR) and the skin tumor promoters (CA and OA). The finding that peroxisome proliferators (and AhR agonists) could be categorized in a hepatic setting is not novel (see also Flaveny et al. 2010; Tamura et al. 2006). However, discrimination of the metalloids, and more importantly, the skin tumor promoters is quite new.

Performing the unsupervised analysis with an equal number of genes as input resulted in an outcome comparable to the results based on the DEGs. The use of an equal number of genes as input has the advantage that it harbors the potential to compare the degree of similarity in expression profiles between substances. However, the application of GSEA to compare clustering results in a quantitative manner requires further research and development of the method. Both methods of data analysis have advantages for their use in screening assay procedures in the future. The supervised approach may bring forward the mode of action of an unknown substance. The unsupervised approach is, unlike the supervised method, not restricted to the modes of action, but indicates whether the profile of an unknown substance matches to the one of a known substance in a general database. The unsupervised approach appears to be more robust and objective and, therefore, we favor the use of the unsupervised method for future use in mode of action detection of NGTXC.

Next, an important step is to investigate the relevance of the *in vitro* identified gene sets for the *in vivo* situation. Ideally, the gene sets found *in vitro* consist of genes *in vivo* known to be involved in the primary processes which in the end will lead to tumor induction. For the peroxisome proliferators responsive genes appeared to be genes of the cytochrome P450 4A family (*cyp4a10, cyp4a14, cyp4a31*) and genes encoding thioesterases (*Acot1,2,3 and 5*), indicating their response recapitulates the *in vivo* situation (Fidaleo et al. 2009; Peters et al. 2005). We, however, do not believe that these processes are directly underlying the carcinogenic features of these substances.
The most responsive genes for the metalloids included, several RIKEN cDNA genes, *Adrb2*, *Pi4k2a* and *Angprl3*. The latter is involved in lipid metabolism via FXR and LXR (Ge et al. 2005). The presence of *Angprl3* suggests a hepatotoxic effect, which is enhanced by the presence of *Cyp17a1*, a target gene of FXR (Anakk et al. 2011), and *Scp2*, a members of the intracellular cholesterol transport pathway (Atshaves et al. 2009). These hepatotoxic effects are possibly overruling the regulation of pathways responsible for the carcinogenicity of LAC and SAR, like, for instance, the induction of oxidative stress and the interaction with DNA repair processes (Beyersmann and Hartwig 2008). Consequently, the metalloid-specific gene set cannot be directly linked to mechanisms identified thus far in an *in vivo* setting.

Major genes for both CA-OA and OA-CA in the unsupervised approach were *Tlcd1*, *Ptpn14* and *Map1lc3a*. We were not able to link these genes to the modes of action of the skin tumor promoters in their role of inhibitors of protein-serine/threonine phosphatases (Fujiki and Suganuma 2009). Exposure to the skin tumor promoters CA and OA is usually preceded *in vivo* with an initiation step by a genotoxic carcinogen (Fujiki and Suganuma 2009). These two-stage experiments lead to tumor formation whereas exposure to the skin tumor promoter alone does not result significantly in tumor development (Fujiki and Suganuma 2009). This may be an explanation for the lack of response in hepatocytes at the individual substance level. Possibly, an initiation step is also required *in vitro* to mimic carcinogenic features. Nevertheless, the match of CA and OA in the unsupervised approach remains striking.

Another aspect for *in vitro* carcinogen testing is how to detect the right concentration. Finding the appropriate test concentrations is a general problem of *in vitro* test systems. In our study, we applied the criterion of a low level (*i.e.* up to 10%) of cytotoxicity as defined as cell growth measured in MTT tests. For some of the NGTXC tested, this may not have been the most ideal criterion. The immunosuppressive substances CSA and FK506 illustrate the importance of appropriate concentration selection procedures. Both substances are, besides being immunosuppressive, known to cause cholestasis in the liver as a side effect (Kostrubsky et al. 2003; Stieger et al. 2000). Analysis of the genes regulated by CSA revealed that immune-related pathways (e.g. OSM signaling, Th17 cell differentiation) were indeed affected. In contrast, the overall effect of FK506 was more hepatotoxic, indicated by regulation of pathways involved in bile acids regulation of glucose and lipid metabolism via FXR. We hypothesize that FK506 induces an immunosuppressive response at lower concentrations. Additional studies are needed to define useful procedures in order to obtain reliable, physiologically relevant (comparable to *in vivo*) concentrations for *in vitro* exposure studies. It is reasonable to assume that more concentrations per carcinogen or substance are needed to assess reliable substance specific responses.

Even with improved concentration selection procedures, primary hepatocytes may not be suited to detect all NGTXC, all having specific modes of action. Despite the fact that hepatocytes are metabolically competent, not all cytochrome P450

enzymes are still active. For example, CT, one of the carcinogens tested, is *in vivo* mainly metabolized by CYP2E1 to form a trichloromethyl radical, which in turn induces several types of toxicity (Weber et al. 2003). In primary hepatocytes, however, CYP2E1 mRNA as well as CYP2E1 protein levels decrease shortly after culturing the cells (Mathijs et al. 2009; Sakurai et al. 1996; Weber et al. 2003). We also observed (not shown) low CYP2E1 mRNA levels, which appears to be in most cases not inducible (except for CSA).
Next, NGTXC may trigger target cells through cellular receptors, which are absent in primary mouse hepatocytes. In this study, the Ah receptor appeared to be functional because both ARO and TCDD did induce their target genes *Cyp1a1*and *Cyp1a2*. However, many cellular receptors are only expressed in specific cell types and may be absent in primary mouse hepatocytes, which may be the cause of a lack of response to certain substances, e.g. HCE and HCH acting through the estrogen receptor.

In conclusion, we have shown that gene expression profiling in primary mouse hepatocytes is a useful approach to detect various modes of action of NGTXC. Recent literature data point out the importance of recognizing and characterizing the mode of action of substances in carcinogenicity screening assays, especially for the NGTXC (Fielden et al. 2011; Hattis et al. 2009; Hernandez et al 2009; Waters et al. 2010). Presumably, in the end a test battery of multiple *in vitro* cellular systems will be required to detect all modes of action. In view of the societal and ethical pressure to reduce the number of experimental animals, we consider the approach presented here to be of added value. One of the first follow up experiments to be done is using human hepatocytes in a similar setting to see whether rodent carcinogens will no longer be classified as a carcinogen in the assumption that these substances are harmless (false positives) to humans.

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Supplemental information

Supplementary data is available at the journal's website.
http://link.springer.com/article/10.1007%2Fs00204-012-0883-6

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