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Detection of genotoxic and non-genotoxic renal carcinogens *in vitro* in NRK-52E cells using a transcriptomics approach

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There is a need to develop quick, cheap, sensitive and specific methods to detect the carcinogenic potential of chemicals. Currently there is no *in vitro* model system for reliable detection of non-genotoxic carcinogens (NGTX) and current tests for detection of genotoxic carcinogens (GTX) can have low specificity. A transcriptomics approach holds promise and a few studies have utilised this technique. However, the majority of these studies have examined liver carcinogens with little work on renal carcinogens which may act via renal-specific NGTX mechanisms. In this study the normal rat renal cell line (NRK-52E) was exposed to sub-toxic concentrations of selected rat renal carcinogens and non-carcinogens (NC) for 6h, 24h and 72h. Renal carcinogens were classified based on their presumed mode of action into GTX and NGTX classes. A whole-genome transcriptomics approach was used to determined genes and pathways as potential signatures for GTX, NGTX and those common to both carcinogenic events *in vitro*. For some of the GTX compounds an S9 drug metabolising system was included to aid pro-carcinogen activation. Only three genes were deregulated after carcinogen (GTX + NGTX) exposure, one *Mdm2* with a detection rate of 67%, and *p21* and *Cd55* with a detection rate of 56%. However, examination of enriched pathways showed that 3 out of 4 NGTX carcinogens and 4 out of 5 GTX carcinogens were related to known pathways involved in carcinogenesis giving a detection rate of 78%. In contrast, none of the NC chemicals induced any of the above genes or well-established carcinogenic pathways. Additionally, five genes (*Lingo1, Hmox1, Ssu72, Lyrm* and *Usp9x*) were commonly altered with 3 out of 4 NGTX carcinogens but not with NC or GTX carcinogens. However, there was no clear separation of GTX and NGTX carcinogens using pathway analysis with several pathways being common to both classes. The findings presented here indicate that the NRK-52E cell line has the potential to detect carcinogenic chemicals, although a much larger number of chemicals need to be used to confirm these findings.

Introduction

Current evaluation as to whether a chemical is carcinogenic is dependent on the outcome of chronic rodent bioassays, which involves administering the compound at maximum tolerated doses for its lifetime, typically two years. At present, a conventional rodent study takes up to five years, costs €1.5 – 3 million per chemical tested and consumes about 800 mice and rats[1].

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The last 50 years of animal data suggests that carcinogenic compounds can be divided, based on their mode of action (MOA) into two classes, genotoxic (GTX) and non-genotoxic (NGTX)[2]. GTX carcinogens are readily detected using a number of short-term bacterial and cultured mammalian cell assays and although these assays are very sensitive, a problem of low specificity exists [3-5].NGTX carcinogens, however, are more problematic and depend in most cases on the outcome of a two-year rodent bioassay. Although some *in vitro* tests are available[6], currently there is no accurate *in vitro* model that can predict the carcinogenic potential of NGTX chemicals. Each year, several thousand new chemicals are entering the world markets and additionally under the Registration, Evaluation, Authorization and restriction of Chemicals (REACH) initiative there is a need to validate the toxicity of an estimated 68,000 chemicals[7]. High-throughput technologies such as whole-genome gene expression profiling have opened the way to understand the systemic response to a toxic insult and also might speed up the process of risk assessment[8-9].

In this study, the Normal Rat Kidney Epithelial cell line (NRK-52E) was exposed to eleven carcinogens and five non-carcinogens (NC) at their IC_{10} concentration at 72h for 6h, 24h

and 72h. The selected renal carcinogens, all known to induce cancer in rats, were classified based on their presumed MOA into GTX, NGTX and NC classes[10]. From the selected GTX compounds, two have been classified by International Agency for Research on Cancer (IARC) as carcinogenic to human (Group 1), aristolochic acid and benzo(a)pyrene. One, dimethylnitrosamine to Group 2A, probable human carcinogen, and three, 2-nitrofluorene, streptozotocin and potassium bromate to Group 2B, possible human carcinogen. The five NGTX carcinogens selected for this study are all chlorinated chemicals. Three of them, bromodichloromethane, chlorothalonil and ochratoxin A are classified as Group 2B while monuron is classified as Group 3, not carcinogenic to humans. S-(1,2-dichlorovinyl)-L-cysteine is a non-classified metabolite of trichloroethylene (which itself belongs to Group 2A). As a control five NC, mainly widely used pharmaceuticals, clonidine, D-mannitol, diclofenac sodium, nifedipine and tolbutamide were used. Many GTX carcinogens undergo some degree of metabolism in order to interact with DNA[11-12]. NRK-52E cells have poor cytochrome P450 drug metabolising capability[13] (HPLC-MS/MS; Bloch, unpublished results), so additional studies were conducted with and without pre-incubation with rat liver β-naphthoflavone-induced S9 fraction. After 6h, 24h and 72h of exposure the total RNA was isolated, purified, quality checked and whole-genome gene expression changes were determined using Affymetrix Rat GeneChip 230 2.0. In addition to alteration in transcriptome profile, DNA damage was determined by conducting the cytokinesis-blocked micronucleus assay[14].

Materials and Methods

Chemicals

All test chemicals were purchased from Sigma-Aldrich, UK, except for S-(1,2-dichlorovinyl)-L-cysteine which was a gift from D. Moore, formerly of Syngenta Central Toxicology Laboratory, Alderley Park, Cheshire. β-naphthoflavone-induced rat liver S9 was purchased from Celsis, In Vitro Technologies, Belgium.

Cell culture

NRK-52E cells (ATCC, CRL-1571) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum and penicillin 100 I.U./ml, streptomycin solution 100 µg/ml in a humidified 5% CO_2 incubator at 37^{0}C. For cytotoxicity and transcriptomics studies, cells were sub-cultured in 6-well plates in medium without serum until 80% confluent and then exposed to selected compounds dissolved in DMSO (0.1%v/v) or medium (in case of potassium bromate).

Generation of IC_{10}

A dose-response curve for cytotoxicity using LDH leakage and the MTT assay[15] was generated for each compound after 24h and 72h exposure using cells exposed to medium or DMSO alone as the 100% value. At least three separate experiments were conducted with each compound. The dose that caused approximately 10% cytotoxicity (IC_{10}) at 72h was selected for the transcriptomics studies. The doses selected were as follows: GTX compounds; 1.65µM, aristolochic acid (AA); 40µM, benzo(a)pyrene (BaP); 20µM, 2-nitrofluorene (2NF) and 500µM, potassium bromate ($KBrO_3$). NGTX compounds; 470µM, bromodichloromethane (BDCM); 1.1µM, chlorothalonil (CHL); 3µM, S-(1,2-dichlorovinyl)-L-cysteine (DCVC); 250µM, monuron (MON) and 0.5µM, ochratoxin A (OTA). NC compounds were; 1.12mM, clonidine (CLO); 6µM, diclofenac sodium (DS); 35µM, nifedipine (NIF) and 370µM, tolbutamide (TOL). The GTX compounds; dimethylnitrosamine (NDMA), streptozotocin (STZ) and NC; D-mannitol (MAN) were not cytotoxic to NRK-52E cells and used at a dose of 1mM, 5mM and 10mM respectively. After 6h, 24h and 72h the medium was removed and the cells taken for RNA extraction, three studies were conducted at each time point with each compound.

Incorporating an enhanced xenobiotic capability

The IC_{10} concentration for three GTX compounds was determined in the presence of S9 over 72h as described above. The IC_{10} concentrations were as follows: BaP (87µM), STZ (350µM) and 2NF (70µM). Compounds were pre-incubated in either the presence or absence of rat liver β-naphthoflavone-induced S9 metabolising system for 3h at 37^{0}C. The S9 metabolising system was prepared by diluting the S9 fraction to 10mg protein/ml with ice-cold Tris buffer, (60mM, pH 7.4). The NADPH regenerating system consisted of ice-cold $NaHCO_3$ (2% (w/v), $NADP^+$ (1.7mg/ml), glucose-6-phosphate (7.8mg/ml) and glucose-6-phosphate dehydrogenase (6units/ml) which was prepared immediately before use. The reaction mixture contained S9 fraction 10mg/ml (0.1ml), 60mM Tris buffer, pH 7.4 (0.64ml), NADPH regenerating system (0.25ml) and the reaction was started by the addition of DMSO or tested compound dissolved in DMSO (0.01ml). The reaction was stopped after 3h by heating to 60°C then cooled on ice and added to ~80% confluent NRK-52E cells growing in 1ml medium in 6 well-plates. After 72h the medium was removed and the cells taken for RNA extraction, with three studies conducted with each compound.

Cytokinesis –Block Micronucleus Assay

NRK-52E cells were seeded on glass slides and exposed to GTX or NGTX compounds at the IC_{10} concentration at 72h together with cytochalasin B (3µl/ml of a 1mg/ml stock solution in DMSO) in 1ml medium for 24h. Compounds were dissolved in DMSO, with the exception of $KBrO_3$ which was dissolved in medium. The cells were fixed with 37% (v/v) formaldehyde (0.1ml) in hypotonic solution (0.4% Na-citrate/0.4% KCl 1:1 v/v)(2.4ml) and incubated for 8 min at 4°C. The cells were then washed with a solution containing equal volumes of hypotonic solution (Na citrate/KCl) and fixative (methanol (100%): acetic acid (100%) 4:1v/v). Then 50% of the hypotonic/fixative mixture was removed and fixative alone was gently added. The mixture was then removed and 100% fixative was added and left for at least 3 min. The slides were than air-dried and immersed for 15 min in 10µM DAPI solution in distilled water. After air-drying the slides were mounted with a cover slip for microscopy. Around 3,000 binucleated cells from at least three replicates were analyzed for the presence of micronuclei (MN) using an automated MN scoring system Metafer (MetaSystems). Control cells with DMSO (or medium alone) were compared to treated cells. For some studies, the cells were pre-incubation with rat liver S9 fraction either alone or in the presence of a GTX carcinogen as described above. Statistical analysis was performed between the relevant control and test compound using a paired t-

test (GraphPad InStat) with a p value <0.05 considered significant.

RNA isolation and microarray

Total RNA was isolated from control and compound treated NRK-52E cells with trizol reagent (Invitrogen) and purified using the RNeasy Total RNA Mini Kit (Qiagen) according to manufacturer's instructions. Purified total RNA was checked for purity and integrity using a Agilent 2001 Bioanalyzer (Agilent Technologies GmbH, Germany) before processing. All RNA used in this study had values of 1.9-2.0 as measured by A_{260}/A_{280} ratio, and RIN values from 9-10. The integrity of RNA was also checked by gel electrophoresis with result showing a clear 2:1 ratio of 28S rRNA to 18S rRNA. The amount of RNA was around 1-5µg. Transcriptomics data was generated using Affymetrix GeneChip Rat Genome 230 2.0 Arrays.
Three replicates were used for each compound and control and at each time point..

Microarray Hybridization

Target preparation

cDNA was prepared using the Affymetrix IVT express kit (Affymetrix, Santa Clara). cDNA synthesis and labelling was performed according to the manufacturer's procedures. Subsequent labelling of the samples was conducted by synthesis of Biotin-labelled complementary RNA (cRNA) using the GeneChip IVT labelling kit (Affymetrix). Purified cRNA was quantified using a spectrophotometer, and unfragmented samples were checked on the Bioanalyzer. Subsequently, cRNA samples were fragmented for target preparation according to the Affymetrix manual and checked on the Bioanalyzer. Samples were stored at −20°C until ready to perform hybridization.

Table 1 Frequency of MN formation in NRK-52E cells treated with GTX, GTX/S9 and NGTX chemicals

Class	Compound name and compound dose	% MN formation
Control	DMSO	0.26 ± 0.04
	Medium	0.27 ± 0.03
	S9 alone	0.27 ± 0.15
GTX	AA (1.65µM)	0.54 ± 0.11 *
	BaP (40µM)	0.54 ± 0.04 **
	BaP (87µM)	1.40 ± 0.97
	BaP/S9 (87µM)	4.00 ± 0.70 *
	2NF(20µM)	0.18 ± 0.07
	2NF (70µM)	0.50 ± 0.35
	2NF/S9 (70µM)	0.90 ± 0.72
	$KBrO_3$ (500µM)	6.63 ± 1.37 *
	NDMA (1mM)	0.32 ± 0.15
	STZ (350µM)	0.20 ± 0.10
	STZ/S9 (350µM)	0.80 ± 0.56
NGTX	BDCM (0.47mM)	0.18 ± 0.10
	CHL (1.1µM)	0.20 ± 0.20
	DCVC (3µM)	0.16 ± 0.16
	MON (250µM)	0.32 ± 0.34
	OTA (0.5µM)	0.23 ± 0.10

Results are mean ± SD with at least three different cell cultures per measurement.
Statistically significantly different from appropriate control, * p< 0.05, ** P<0.01

Hybridization

cRNA targets were hybridized on high-density oligonucleotide gene chips (Affymetrix GeneChip Rat Genome 230 2.0 Arrays) according to the Affymetrix Eukaryotic Target Hybridization manual. The gene chips were washed and stained using the Affymetrix Fluidics Station 450 and Genechip Operating Software and scanned by means of an Affymetrix GeneArray scanner.

Microarray analysis

The intensity values of different genes (probe sets) generated by Affymetrix GeneChip Operating Software were imported into Genedata Expressionist® version 7.0 and GeneSpring version 11 software (Agilent), respectively, for data analysis. The raw data files (.CEL files) containing signal values for individual probes were pre-processed to generate one value per probeset. Pre-processing of arrays was done using GC-RMA (Robust Multiarray Analysis algorithm). To identify the differentially expressed genes, the condensed data were subjected to pairwise comparisons, using paired t-test. Comparisons were made between control and treated samples. Only genes with >2-fold change, using a $p < 0.001$ threshold value were analyzed.

Functional annotation

To understand the biological meaning behind the list of differentially expressed genes, KEGG (Kyoto Encyclopaedia of Genes and Genomes database) pathways analysis and GO (Gene Ontology) from DAVID website (The Database for Annotation, Visualization and Integrated Discovery) v 6.7 was used (http://david.abcc.ncifcrf.gov/)[16]. The list of differentially expressed probe sets (DEPs) obtained from the GeneSpring v.11 was uploaded and run for enriched GO and KEGG pathways. Only GO and pathways with p <0.05 were analysed and discussed.

Results

Selection of exposure dose

These studies was aimed at detecting the early carcinogenic potential of eleven renal carcinogens *in vitro*, thus NRK-52E cells were exposed to a single dose that caused 10% cytotoxicity (IC_{10}) at 72h. Three exposure time points were selected 6h, 24h and 72h exposure effects. Cytotoxicity studies showed that 81% of the selected compounds (13/16) were cytotoxic to NRK-52E cells, the cytotoxicity being time and dose-dependent. All NGTX compounds were toxic to NRK-52E cells, whereas two compounds from the GTX group (STZ and NDMA) and one from the NC group (MAN) exhibited no cytotoxicity at the highest concentration tested. The most toxic compounds were OTA followed by CHL > AA > DCVC > DS > BaP > NIF > 2NF > MON > TOL > BDCM > $KBrO_3$ > CLO. In addition to cytotoxicity, the *in vitro* cytokinesis-block micronucleus assay was used to assess if the dose used had any effect on DNA. The assay confirmed the lack of clastogenicity with NGTX compounds, as none of the five NGTX compounds induced micronuclei (MN) formation (Table 1). In the GTX group chromosomal damage was increased with $KBrO_3$, AA and BaP (Table 1). In addition, MN formation was examined after

exposure to three GTX compounds BaP, 2NF and STZ in the presence of a rat liver β-naphthoflavone-induced S9 drug metabolising system. S9 alone after heat-incubation step caused 10% cytotoxicity CHECK IT. A statistically significant increase in MN formation was seen after exposure to BaP in the presence of S9 (Table 1), while in the absence of S9 there was a small increase which was not statistically significant (Table 1). There was a trend for an increase in MN formation with 2NF and STZ in presence of S9 but this was not statistically significant (Table 1).

Table 2 Number of DEPs and enriched KEGG pathways identified after GTX carcinogen, NGTX carcinogen and NC exposure to NRK-52E cells for 6h, 24h and 72h

CLASS	COMPOUND NAME AND IC10 DOSE	NUMBER OF DEPS			ENRICHED KEGG PATHWAYS (P<0.05)
		6h	24h	72h	
GTX	AA (1.65μM)	3	8	78	p53 signalling, Pathways in cancer, Ubiquitin mediated proteolysis
	BaP (40μM)	11	1	1	-
	$KBrO_3$ (500μM)	1	8	57	-
	2NF (20μM)	6	0	0	-
	NDMA (1mM)	10	0	21	-
	STZ (350μM)	-	-	9	-
NGTX	BDCM (0.47mM)	0	0	2	-
	CHL (1.1μM)	174	324	317	MAPK signalling, Toll-like receptor signalling, Cell cycle, Steroid biosynthesis, Biosynthesis of unsaturated fatty acids, Proteasome assembly, Pathways in cancer, Terpenoid backbone biosynthesis, Focal adhesion, Ubiquitin mediated proteolysis
	DCVC (3μM)	0	1	1448	Aminoacyl-tRNA biosynthesis, Cell cycle, Ubiquitin mediated proteolysis, Regulation of actin cytoskeleton, Endocytosis, Pyrimidine metabolism, Pathways in cancer, Protein export, spliceosome, Focal adhesion, p53 signalling, Valine, leucine and isoleucine biosynthesis, mTOR signalling
	MON (250μM)	6	24	123	Mevalonate kinase pathway
	OTA (0.5μM)	170	1057	723	Pyrimidine metabolism, Purine metabolism, Cell cycle, WNT-signalling, p53 signalling, Spliceosome, Steroid biosynthesis, Fructose and mannose metabolism, Pathways in cancer, Renal cell carcinoma, Sphingolipid metabolism, Lysosome
NC	CLO (1.12mM)	42	16	46	Steroid biosynthesis
	DS (16μM)	7	1	22	-
	MAN (10mM)	1	2	94	Focal adhesion
	NIF (35μM)	4	1	0	-
	TOL (0.37mM)	0	4	31	Tight junction

Table 3 DEPs commonly altered after GTX or NGTX carcinogen exposure to NRK-52E cells

ENTREZ GENEID	*GENE*	*DEREGULATION AFTER EXPOSURE*
314856	***Mdm2*** (p53 binding protein homolog (mouse)	AA (72h), BaP/S9 (72h), 2NF/S9 (72h), STZ/S9 (72h), CHL (24h), DCVC (72h)
114851	***p21*** (Cyclin-dependent kinase inhibitor 1A)	AA (72h), $KBrO_3$ (24h), BaP/S9 (72h), 2NF/S9 (72h),OTA (24h)
64036	***Cd55*** (Decay accelerating factor for complement 5)	$KBrO_3$ (72h), BaP/S9 (72h), 2NF/S9 (72h) STZ/S9 (72h), OTA(72h)
25240	***Aqp1*** (Aquaporin 1)	AA (72h), BaP/S9 (72h), DCVC (72h), OTA (72h)

Transcriptomics studies

Transcriptome analysis revealed that the response of the NRK-52E cells to GTX carcinogens and NC compounds was far less pronounced than to NGTX carcinogens (Table 2). BDCM (NGTX) and NDMA (GTX) had a marginal effect on the transcriptome profile and as both compounds were volatile at 37^0C, the exact exposure concentration was uncertain and thus both chemicals were not included in the analysis. In total, transcriptomic analysis has been conducted on five GTX carcinogens, four NGTX carcinogens and five NC compounds. Exposure to the five GTX carcinogens altered 214 genes in total over 6h, 24h and 72h, while exposure to the five NC altered a total of 271 genes. In contrast, exposure to the four NGTX carcinogens altered the expression of > 4,000 genes (Table 2). In agreement with previous studies, there was generally a time-dependent increase in the number of differentially expressed genes[17-18].

Carcinogenic versus non-carcinogenic compounds

There were no common genes altered by all of the renal carcinogens (GTX+NGTX), including the three GTX compounds tested in the presence of a S9 metabolising system. However, three genes *Mdm2* (MDM2 p53 binding protein), *p21* (Cyclin-dependent kinase inhibitor 1) and *Cd55* (Decay accelerating factor for complement), were commonly altered by 6 out of 9 compounds (67%) in case of *Mdm2* and by 5 out of 9 compounds (56%) in case of *p21* and *Cd55* (Table 3). The deregulation primarily occurred at the later time point (Table 3). In addition, *Aqp1* (Aquaporin 1) was altered by two GTX and two NGTX compounds after 72h exposure (Table 3). None of these genes were altered at any time point by the five NC compounds.

Non-genotoxic compounds

. No commonly deregulated genes were induced by all four NGTX carcinogens at 6h, 24h or 72h. However, five genes with the same pattern of expression (except *Usp9x*) were commonly deregulated after 72h exposure to CHL, DCVC and OTA namely: *Lingo1* (Leucine rich repeat and Ig domain containing 1), *Hmox1* (Heme oxygenase, decycling 1), *Ssu72* (Ssu72 RNA polymerase II CTD phosphatase), *Lyrm1* (LYR motif containing 1) and *Usp9x* (Ubiquitin specific peptidase 9, X-linked) (Table 4).

Table 4 List of DEPs commonly deregulated after 72h exposure of NGTX carcinogens to NRK-52E cells

ENTREZ GENEID	***GENE NAME***	***REGULATION PATTERN (FOLD CHANGE TREATED V CONTROL)***		
		CHL	***DCVC***	***OTA***
24451	***Hmox1*** (Heme oxygenase, decycling 1)	39.53 ↑	40.97 ↑	3.14 ↑
298681	***Ssu72*** (Ssu72 RNA polymerase II CTD phosphatase)	2.45 ↑	2.3 ↑	2.15 ↑
365361	***Lyrm1*** (LYR motif containing 1)	3.68 ↑	7.0 ↑	3.57 ↑
315691	***Lingo1*** (Leucine rich repeat and Ig domain containing 1)	6.52↓	18.11↓	2.38↓
363445	***Usp9x*** (Ubiquitin specific peptidase 9, X-linked)	2.87↓	5.47↓	2.25 ↑

↑ up-regulation ↓down-regulation

Pathways analysis in the NGTX class

It is widely acknowledged that tumorogenesis is driven by disruption in pathways rather than individuals genes[19], thus similarities in the pathways affected by NGTX carcinogen exposure were investigated. Exposure to NGTX carcinogens predominantly up-regulated genes involved in signalling pathways, whereas genes involved in metabolism were predominantly down-regulated.

Three NGTX carcinogens affected cell cycle pathways, at different time points CHL, 6h; OTA, 24h and DCVC 72h, with common deregulation of genes from the GADD45 family (Growth arrest and DNA-damage-inducible). Intriguingly, both CHL and DCVC up-regulated *Gadd45* genes whereas OTA exposure down-regulated them. Additionally, three genes were commonly altered after DCVC and OTA exposure these were, *Mad2l2* (MAD2 mitotic arrest deficient-like2), *Rad21* (RAD21 homolog, S. pombe) and *Rbx1* (Ring-box 1). *Mad2l2* is a component of the mitotic spindle assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate. Interestingly, *Mad2l2* and *Rad21* were up-regulated and *Rbx1* down-regulated after DCVC exposure, while with OTA, *Mad2l2* and *Rad21* were down-regulated and *Rbx1* was up-regulated. In addition to the cell cycle pathway, CHL (72h), DCVC (72h) and OTA (24h) exposure also significantly enriched cancer pathways, with three genes commonly deregulated from the Frizzled and Integrin family and *Tcf* (Similar to transcription factor 7-like 2, T-cell specific). The canonical p53 signalling pathway was also enriched after 72h exposure to DCVC and 24h exposure to OTA, with two commonly altered genes, *Rrm2* (Ribonucleotide reductase M2) and *Gadd45*. CHL and DCVC enriched the focal adhesion pathway with two commonly deregulated genes; *Pak1* (p21 protein (Cdc42/Rac)-activated kinase 1) and *Akt3* (V-akt murine thymoma viral oncogene homolog 3). Genes from the Integrin and Laminin family were also commonly deregulated by CHL and DCVC.

In addition, OTA (24h) enriched WNT-signalling and the renal cell carcinoma pathway, while DCVC (72h) altered the mTOR pathway and CHL (6h) enriched the MAPK signalling pathway (Table 2). MON did not alter any canonical cancer pathways, but down-regulated the mevalonate kinase pathway (Table 2).

Table 5 Number of DEPs and enriched KEGG pathways identified after 72h exposure to GTX and GTX/S9 in NRK- 52E cells

COMPOUND NAME AND DOSE	***NUMBER OF DEPS***	***ENRICHED KEGG PATHWAYS(P<0.05)***
BaP (87µM)	1	-
BaP/S9 (87µM)	93	MAPK signalling, Focal adhesion, Pathways in cancer
2NF (70µM)	87	p53 signalling pathway
2NF/S9 (70µM)	110	p53 signalling, Aminoacyl-tRNA biosynthesis, Porphyrin metabolism, Glycine, serine and threonine metabolism, Cell cycle
STZ (350µM)	9	-
STZ/S9 (350µM)	58	p53 signalling pathway

Genotoxic compounds

. In the GTX class there were no commonly genes identified at any time point with AA, BaP, 2NF, STZ and $KBrO_3$ or BaP, STZ or 2NF in the presence of S9.

AA was the only compound (from the GTX without S9) that enriched cancer related pathways (Table 2). BaP and 2NF had a very small effect on the transcriptome profile, whereas exposure to $KBrO_3$ altered 66 genes over 72h (Table 2). As BaP and 2NF are known pro-carcinogen's that need to be metabolized to DNA reactive proximate carcinogens to exert their carcinogenic potential, an external metabolic activation system, β-naphthoflavone-induced rat liver S9 was used. Exposure of NRK-52E cells to S9 alone altered genes involved mainly with immunological functions: NOD-like receptor and chemokine signalling suggestive of an immune response. No genes involved

in cancer-related pathway were altered in the presence of S9. AA was not studied in the presence of S9 as nitro-reduction is primarily involved in the activation while oxidation via CYP1A1 has been shown to protect against the carcinogenic effect of AA[20]. In general, exposure to GTX compounds (BaP, 2NF and STZ) together with induced S9 increased the number of DEPs and helped to predict their carcinogenic potential (Table 5).

Five genes with the same pattern of expression were found to be commonly deregulated by BaP, 2NF and STZ after prior external metabolic activation; *Mgmt* (O-6-methylguanine-DNA methyltransferase), *Epoh1* (Epoxide hydrolase 1), *Scarb2* (Scavenger receptor class B, member 2), *Jam3* (Junctional adhesion molecule 3) and *Cd55* (Table 6). However, from these five deregulated genes, three were also deregulated by NGTX carcinogens. *Cd55*, in addition to being down-regulated by BaP, STZ and 2NF after metabolic pre-incubation, was also down-regulated by $KBrO_3$ and by the NGTX carcinogen OTA, whereas *Mgmt* and *Scarb2* were up-regulated by DCVC exposure. Thus it is much more difficult to find a set of specific genes deregulated by the GTX carcinogens.

In addition, no commonly deregulated genes were found to be altered by GTX carcinogens exposure, that were positive for MN induction (AA, $KBrO_3$ and BaP/S9) after 72h exposure.

Table 6 List of genes common deregulated after 72h of BaP/S9, 2NF/S9 and STZ/S9 exposure in NRK-52E cells

ENTREZ GENEID	***GENE NAME***	***REGULATION PATTERN (FOLD CHANGE TREATED VS CONTROL)***		
		BaP/S9	***2NF/S9***	***STZ/S9***
25332	***Mgmt*** (O6-methylguanine DNA methyltransferase)	3.78↑	2.74↑	2.98↑
117106	***Scarb2*** (Scavenger receptor class B, member 2)	2.34↑	2.2↑	2.14↑
25315	***Epoh1*** (Epoxide hydrolase 1)	3.87↑	5.33↑	3.53↑
315509	***Jam3*** (Junctional adhesion molecule 3)	3.0↑	3.21↑	2.67↑
64036	***Cd55*** (Decay accelerating factor for complement 5)	4.92↓	6.27↓	8.07↓

↑ up-regulation ↓down-regulation

Pathway analysis of GTX carcinogens after S9 exposure

BaP exposure after metabolic activation (BaP/S9) increased of number of DEPs and significantly enriched pathways associated with cancer such as the MAPK signalling and focal adhesion (Table 5). STZ after metabolic activation (STZ/S9) also increased the number of DEPs as compared to STZ alone and significantly enriching the p53 signalling pathway (Table 5). Results with 2NF were more ambiguous, with a slight increase in the number of DEPs and enrichment of more diverse set of pathways including cancer pathways.

Discussion

The rat renal normal epithelial cell line (NRK-52E) was selected for this work with the main aim being to find an alternative model system to classify compounds according to their carcinogenic potential to the kidney. As the majority of the current carcinogenic data derives from rodents (mainly rats), NRK-52E cells were the preferred cell line. The current evaluation of carcinogenicity of chemicals relies heavily on different animal *in vivo* and *in vitro* methods, with NGTX carcinogens being especially problematic.

In this work eleven chemicals that cause renal tubule tumours in rats have been evaluated. Six of them have previously been shown to act *via* a GTX mechanism and five chemicals are thought to act by a NGTX mechanism[10]. Additionally five NC had been used as controls. Cytotoxicity studies were conducted using all 16 chemicals on NRK-52E cells, to assess the effect that these chemicals had on the cells. Determination of cytotoxicity is an initial step toward the characterisation of chemicals. Although cytotoxicity assays *per se* does not provide mechanistic data, they shed light on cell response/susceptibility to investigated cytotoxins. Furthermore, knowledge of the concentration range that induces cytotoxicity enables better definition of concentrations to be employed in more in-depth descriptive and mechanistic studies such as gene expression profiling. As the main aim of the project was to try to detect an early carcinogenic potential of the selected compounds, the endpoint of interest was carcinogenicity not cytotoxicity, the selected dose that cause only 10% cytotoxicity (IC_{10}) and thus have small but visible effect on the cell line was used. For transcriptomics, only one dose was investigated, but with different times of exposure. The reason for this being the analysis of previous published studies, showing that in *in vitro* models, treatment time has a much larger impact on gene expression than the chemical's dose[21-22]. Three time points were selected to provide additional data 6h, 24h and 72h . The early exposure time point (6h) was selected as GTX chemicals have been shown to induce DNA damage and repair in a matter of few hours after administration[23-24]. As the main aim of this study was to look at the carcinogenicity of the chemical and 1/3 of the chemicals used are known to be DNA-reactive, the *in vitro* micronucleus assay was used to assess if the dose used (IC_{10}) had any effect on DNA. The assay confirmed the lack of clastogenicity with NGTX chemicals (Table 1). In the GTX group, chromosomal damage was proven for $KBrO_3$, AA and BaP. The clastogenicity after $KBrO_3$ exposure is believed to be due to oxidative stress, increased reactive oxygen species, glutathione depletion[25] and 8-OHdG formation leading to double strand breaks in the DNA[26-27]. AA has also been shown to increase MN formation in mouse bone marrow cells[28], human lymphocytes exposed to the plant extract from Aristolochia[29] and HepG2 cells[30]. A small, but statistically significant, induction of MN was seen with BaP (Table 1). BaP is known to require metabolism by cytochrome P450 to undergo metabolic activation[31] and as NRK-52E cells do not possess functional cytochrome P450 (as quantified by HPLC-MS/MS with a range of different substrates, Bloch, unpublished results) this finding was unexpected, but may be explained by photoactivation of the chemical[32]. A statistically significant increase in MN formation was observed after the addition of a xenobiotic drug metabolising system in case of BaP and a small but not statistically significant increase was noted after 2NF/S9 and STZ/S9 (Table 1).

Carcinogen detection

Although it was not possible to find a set of genes that was commonly deregulated by all five GTX carcinogens and by all four NGTX carcinogens, *Mdm2, p21* and *Cd55* were commonly altered by five or more carcinogens (Table 3). *Mdm2* and *p21* were proposed by Ellinger-Ziegelbauer[33] as a gene signature for GTX exposure in rat liver, however we observed that CHL, OTA and DCVC also altered the expression of the aforementioned genes (Table 3). CD55 is one of the complement regulatory/inhibitory proteins and is altered in a wide range of solid tumours[34]. Interestingly *Aqp1* was also altered by GTX and NGTX carcinogens and recently *Aqp1* has been proposed as a renal cancer biomarker[35].

Additionally examination of pathways enriched by chemical exposure, showed that 3 out of 4 (75%) NGTX carcinogens and 4 out of 5 (80%) GTX carcinogens (AA, BaP/S9, 2NF/S9, STZ/S9, with $KBrO_3$ being the only chemical that did not enriched any pathway) were related to known pathways involved in carcinogenesis. In contrast, none of the NC chemicals induce any of the well-established carcinogenic pathways. Mannitol and BaP/S9 both increased 4 and 5 genes respectively in the focal adhesion pathway, however there was only one common gene coll1α1. So far gene expression profiling studies using cancer related pathways has shown a detection rate of 78% for renal carcinogens (7/9) under the conditions of these studies.

The enriched pathways that were affected by two or more carcinogens include; p53 signalling pathway (AA, 2NF/S9, STZ/S9, DCVC and OTA), pathways in cancer (AA, BaP/S9, CHL, DCVC and OTA), cell cycle (2NF/S9, DCVC and OTA), focal adhesion (BaP/S9, CHL and DCVC), MAPK signalling pathway (BaP/S9 and CHL). Thus, this study has shown that NRK-52E, rat renal cell model has a potential for detecting renal carcinogens *in vitro*. MON was the only NGTX compound that did not altered any canonical cancer related pathways. However, using a less stringent p value of <0.01 MON increased pathways in cancer, renal cell carcinoma and the cell cycle 72h after exposure..

As mentioned above there is a real problem of early detection of NGTX carcinogens. In NRK-52E cells, exposure to three of the four compounds (except for MON) commonly deregulated five genes, *Lingo1, Hmox1, Ssu72, Lyrm1* and *Usp9x* (Table 4)[36]. *Lingo1* is currently implicated in oligodentrocyte differentiation and axonal myelination and no study so far have shown its involvement in carcinogenesis. HMOX1 which is under the regulation of Nrf2, plays an important role in oxidative injury[37] and has been shown to regulate cell proliferation, modulate inflammatory response and facilitate angiogenesis[38]. *Ssu72* encodes a protein involved in RNA processing and termination. Little is known about the function of LYRM1, although Qiu *et al*.[39] has shown that the protein is involved in modulation of cell growth and apoptosis in pre-adipocytes. USP9X is a member of the peptidase family that regulate the production and recycling of ubiquitin which are involved in the control of cell growth, differentiation and apoptosis[40]. NGTX carcinogens predominantly up-regulated genes involving in signalling pathways, whereas genes involved in metabolism were predominantly down-regulated.

This finding agrees with studies analysing gene expression in human clear cell renal carcinoma, where loss of normal renal function, downregulation of metabolic genes and alteration in multiple canonical cancer-associated pathways occurs, including cell cycle, focal adhesion, ECM-interaction and disruption in amino-acid metabolism[41-42]. Additionally as mentioned above three NGTX carcinogens commonly deregulated the cell cycle pathway with common deregulation of genes from the GADD45 family. Proteins encoded by these genes are DNA-damaging sensor proteins, with up regulation of expression after a DNA-damaging event. They interact with both CDK1 (Cyclin-dependent kinase 1) and CCNB1 (Cyclin B1), resulting in inhibition of the kinase activity of the CDK1/CCNB1 complex, and function as a negative growth control. In addition, all three NGTX deregulated different cancer pathways such as WNT-signalling, renal cell carcinoma pathway, mTOR pathway and MAPK signalling pathway (Table 2).

GTX compounds, except from AA, without prior metabolic activation altered a much smaller number of genes as compared to NGTX compounds and did not alter cancer related pathways. Thus an external metabolising system was used to improve the metabolic capabilities of the NRK-52E cells. Pre-incubation with an induced rat liver S9 fraction was shown to improve the detection of carcinogenic potential of GTX compounds (Table 5). Following BaP/S9, 2NF/S9 and STZ/S9 exposure five genes were found to be commonly deregulated; *Mgmt, Scarb2, Epoh1, Jam3* and *Cd55* (Table 6). *Mgmt* encodes an enzyme that repairs alkylated guanine in DNA. STZ is a known alkylating agent, while BaP and 2NF are not, however Ellinger-Ziegelbauer and coworkers reported an increase in *Mgmt* after *in vivo* exposure to 2NF and other GTX compounds[43]. While Grombacher and coworkers showed that p53 regulates *Mgmt* expression and that *Mgmt* is induced after GTX stress, being one of the first DNA repair gene to be up-regulated[44]. *Epoh1* is a critical enzyme in xenobiotic detoxification, which catalyzes the hydrolysis of arene and aliphatic epoxides to less reactive and more water soluble dihydrodiols. However, EPOH1 has been shown to activate BaP to mutagenic and carcinogenic products and *Epoh1* polymorphisms have been associated with the onset of numerous cancers[45-47]. *Scarb2* encodes a transmembrane glycoprotein that is located in lysosome and endosome membranes and may participate in membrane transportation and the reorganization of endosomal/lysosomal compartments. Huang and coworkers reported the loss of heterozygosity in *Scarb2* in human hepatocellular carcinoma[48]. *Jam3* encodes a protein that is expressed in close proximity to tight junctions of polarized endothelial and epithelial cells and also mediates cell adhesive events between tumour cells and the endothelium[49].

In addition, cancer–related pathways were also enriched with induction of the p53 signalling pathway after STZ/S9 and 2NF/S9 exposure and MAPK signalling after BaP/S9 exposure. Enrichment of MAPK signalling pathway after BaP exposure has also been reported by others [50-52]. MAPK signalling pathway was also enriched after 6h exposure to CHL (NGTX), although only one gene *Gadd153* was commonly up-regulated by both compounds. Although the addition of the S9 fraction greatly improved the response to BaP and STZ, with 2NF/S9 the results were more ambiguous. 2NF (40μM) alone altered 6 genes over

72h, however increasing the dose to 70μM led to 87 genes being altered while the addition of S9 to 2NF (70μM) led to 110 genes being altered. This suggests in the case of 2NF that the dose was more important than metabolic pre-incubation. However, closer examination revealed that although the higher concentration induced more DEPs, the DE genes altered after exposure to 2NF alone were involved in apoptosis in contrast to 2NF/S9 exposure where the genes were primarily involved in metabolism (amino acid transport, amino acid activation and cholesterol biosynthesis). In addition, there were some similarities after exposure to 2NF (70μM) with and without S9; ten commonly deregulated genes associated with cancer and a significant enrichment of the p53 signalling pathway. In addition, exposure to 2NF/S9 enriched four pathways namely aminoacyl-tRNA biosynthesis, glycine, serine and threonine metabolism and porphyrin metabolism, all being down-regulated (Table 5).

Table 7 KEGG pathways enriched after GTX exposure

KEGG Pathway	No of genes involved in the pathway	P-value
p53 signalling pathway	9	4.1E-5
Pathways in cancer	17	4.6E-4
Small cell lung cancer	7	5.8E-3
ErbB signalling pathway	7	6.6E-3
Melanoma	6	1.2E-2
Cell cycle	8	1.2E-2
MAPK signalling pathway	12	1.6E-2
Glycine, serine and threonine metabolism	4	2.5E-2
Terpenoid backbone biosynthesis	3	3.0E-2
Ubiquitin mediated proteolysis	7	3.9E-2
Aminoacyl-tRNA biosynthesis	4	4.4E-2

Table 8 KEGG pathways enriched after NGTX exposure

KEGG Pathway	No of genes involved in the pathway	P-value
Cell cycle	41	7.2E-8
Steroid biosynthesis	11	1.5E-5
Aminoacyl-tRNA biosynthesis	17	2.7E-5
Spliceosome	34	3.8E-5
Pyrimidine metabolism	27	1.3E-4
p53 signalling pathway	21	2.8E-4
Terpenoid backbone biosynthesis	8	1.1E-3
Ubiquitin mediated proteolysis	30	2.2E-3
Sphingolipid metabolism	13	7.7E-3
Pathways in cancer	58	1.2E-2
RNA polymerase	9	1.8E-2
Porphyrin metabolism	10	1.8E-2
Fatty acid metabolism	12	2.0E-2
Glycine, serine and threonine metabolism	10	2.2E-2
Protein export	5	2.4E-2
Amino and nucleotide sugar metabolism	12	2.4E-2
Small cell lung cancer	19	2.6E-2
RNA degradation	14	3.7E-2
DNA replication	10	3.8E-2
Renal cell carcinoma	16	3.9E-2
Colorectal cancer	18	4.0E-2
Focal adhesion	36	4.4E-2
Arginine and proline metabolism	13	4.6E-2
Regulation of actin cytoskeleton	38	4.7E-2
WNT signalling pathway	28	4.8E-2

Genotoxic versus non-genotoxic carcinogens

Overall, many more genes were deregulated after NGTX exposure. The reason for this could be that NGTX have a more diverse MOA, or simply do not need metabolic activation. However, genes traditionally connected with GTX exposure like *Gadd45*, *Gadd153*, *Mdm2*, *p21* and *Mgmt* were up-regulated by exposure to NGTX carcinogens CHL, DCVC and OTA. When the list of all differentially expressed genes induced by exposure to the four NGTX carcinogens, at the three time points and the six GTX carcinogens with and without previous metabolic activation at the three time points were pooled, pathway enrichment analysis showed that there was no major differences between these two groups (Tables 7 and 8). In general, the pathways affected by GTX carcinogens were also affected by NGTX carcinogens, although not all pathways enriched by NGTX were enriched after GTX exposure. The unaffected pathways were mainly intermediary metabolism pathways for amino acids, carbohydrates and lipids. This may be explained by a more diverse MOA and thus the larger number of genes affected after NGTX exposure. The motivation behind classification into GTX and NGTX class is risk assessment based. NGTX carcinogens are thought to be less hazardous to human health than GTX, with a threshold for NGTX exposure and no threshold value for GTX carcinogen exposure. However, some studies have suggested that there might also be a threshold for GTX exposure[53-54]. This study using NRK-52E cells has demonstrated that it is difficult to separate GTX from NGTX carcinogens. This might be due to incorrect classification of a compound(s) *e.g.* OTA[55], inherent limitation of the *in vitro* model system used, or the small number of compounds tested. Despite these limitations, the data presented here shows that gene expression profiling in NRK-52E cells is a promising model tool which used in conjunction with an external metabolising system can provide valuable information on the potential carcinogenicity of chemicals.

In summary, we have been able to detect renal carcinogens using pathway analysis with a success rate of 78% for 9 carcinogens, using an S9 drug metabolising system for the GTX compounds. In addition we could clearly separate these carcinogens from 5 NC. With regard to separating GTX from NGTX carcinogens we identified several common pathways for both classes, making it difficult to separate direct acting carcinogens from the indirect acting of carcinogens.

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