Evaluation of the primary mouse hepatocyte model for the prediction of genotoxicity

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Evaluation of the Primary Mouse Hepatocyte Model for the Prediction of Genotoxicity

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Evaluation of the Primary Mouse Hepatocyte Model for the Prediction of Genotoxicity

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Voor bompa...

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Chapter 1

General Introduction

CHEMICAL CARCINOGENESIS

Of the known carcinogenic agents (viruses, ultraviolet and ionizing radiations, and chemicals), chemicals appear to be of major importance in the induction of human cancers. Cancer is one of the leading causes of death worldwide. Nearly 2.9 million incident cases of cancer diagnosis and 1.7 million cancer deaths were reported in 2004 (1). Cancer can be caused by endogenous agents, like hormones, as well as exogenous agents, like chemical carcinogens and ionizing radiation. There is evidence that a considerable part of cancers is caused by exogenous factors and that these factors account for more than 50% of the cancer risk (2). Therefore the prediction of the carcinogenic potential of chemicals is tremendously important.

Chemical carcinogenesis is a multistage process. Initiation, the first stage, occurs rapidly and appears to be irreversible, generally as a result from one or more mutations of cellular DNA. When a compound is able to covalently bind with DNA, the formed adduct can be either incorrectly or not be repaired. This will eventually lead to the formation of a mutation (3, 4). When these adducts are induced at sites with genes involved in regulation of cell growth and cell differentiation, i.e. oncogenes and tumor-suppressor genes, cancer development can be induced (5). Chemicals which are able to, whether or not preceded by biotransformation, covalently bind to DNA and cause DNA damage, and appear to be able to consistently do this in several *in vitro* tests, are called genotoxic compounds (GTX). If this genotoxicity actually develops into carcinogenicity, such chemicals are called genotoxic carcinogens.

The second stage, promotion, occurs over a longer period of time. Promotion is a complex process and has been defined as the interruptible or reversible clonal expansion of the single initiated cell, in order that additional genetic/epigenetic changes can occur. It is followed by a progression stage. The proliferation rate of the cells is increased, enhancing the likelihood of genetic errors to occur which make already mutated cells more cancerous. Also a clinically detectable tumor develops into its terminal, often malignant form, which ultimately overwhelms the host, mainly by invasion and metastasis.

Carcinogens which do not demonstrate chemical reactivity with DNA and hence do not form DNA adducts, but rather promote carcinogenesis by inducing effects that indirectly lead to neoplastic transformation or enhance the development of tumors from pre-initiated cells, are called nongenotoxic compounds (NGTX). When a carcinogen has both initiating and promoting activities, it is called a complete carcinogen.

PREDICTION GENOTOXIC AND OF CARCINOGENIC POTENTIAL

The classification of chemical compounds according to their carcinogenic potential is frequently evaluated through application of chronic rodent bioassays, namely by using rats and mice. These assays are used to estimate cancer risks for humans, but are very expensive and time-consuming as they require the use of many animals and large quantities of the test compound during what is usually a 2-year period of study. These chronic rodent bioassays are also associated with ethical drawbacks. Furthermore the reliability, relevance and effectiveness of these *in vivo* assays are subjected to many uncertainties with regard to the extrapolation from animal to human risk. Therefore demands to develop a reliable way of predicting *in vivo* carcinogenicity in humans with a high predictive value by means of *in vitro* models are increasing (6).

Since many carcinogens are GTX, the testing whether a chemical has GTX properties is the first step in the carcinogenic risk assessment. Most frequently used *in vitro* systems for the identification of the GTX potency of chemicals are the bacterial Ames test, the mouse lymphoma assay, the micronucleus test and the chromosomal aberration test (7). These systems are relatively simple, accurate and much cheaper for the screening of chemical compounds for their GTX potential. The main disadvantage of these classic *in vitro* genotoxicity tests, however, is the generation of an extremely high false positive rate when compared with *in vivo* genotoxicity and carcinogenicity data. (7).

The specificity of the bacterial Ames test, for example was 73.9%, while the mouse lymphoma assay, the micronucleus test and the chromosomal aberration test had very low specificity, even below 45%. Thus the predictive value of *in vitro* experiments for *in vivo* effects is poor. In many cases, compounds that are genotoxic *in vitro* must be further tested using *in vivo* genotoxicity tests (8). Because of this, a large number of chemicals are subject to additional *in vivo* testing without the need for that, which thus results in unnecessary experiments on animals. Therefore, new *in vitro* systems need to be developed which are capable of reliably discriminating chemical compounds according to their GTX potential. This is where alternative cellular models as well as the novel genomics technologies come into the picture.

LIVER MODELS

The liver is the principal organ involved in the metabolism of many compounds including pro-carcinogens, and also represents a major target organ for chemical carcinogens *in vivo* (9, 10). Therefore the use of *in vitro* liver systems might be a relevant experimental approach to screen potentially carcinogenic compounds. Hepatic cell lines, pri-

mary hepatocyte cultures and precision-cut liver slices from various species are well established *in vitro* systems for these studies (9, 10, 11) and are presented in Table 1.

Immortalized cell lines are used frequently for assessing toxicity *in vitro* (12-17). However, these cell lines are usually derived from tumors and have adapted to growth in culture: they lack liver tissue architecture, and cell-cell interactions and liver-specific functions tend to vanish as culture time increases (18, 19). They often acquire a molecular phenotype quite different from liver cells *in vivo*.

Because precision-cut liver slices contain all the cell types present in whole liver and maintain their three-dimensional structure and cell-cell interactions, these are thought to better represent the *in vivo* situation. Therefore they are well accepted as an *in vitro* system to study hepatotoxicity and biotransformation of compounds (20, 21), even after cryopreservation and thawing (22, 23). They remain metabolically active for 2 days, so they are more suited for short-term studies (24). However, poor penetration of compounds into the inner cell layers of slices and inter-assay variability due to different preservation of cells in different slices are reported (24, 25).

Primary mammalian hepatocytes largely retain their liver-specific functions when freshly derived from the animal and are therefore considered to be the most relevant *in vitro* model in hepatotoxicity studies (26). Hepatocytes used to be cultured on cell culture plates, but appeared to lose drug-metabolizing enzymes and other differentiated liver-specific cell functions very rapidly (27). As it became evident later on that hepatocytes need cell-cell and cell-matrix interactions in order to survive, cell culture systems were adapted. Cells cultured on a collagen layer, especially in a sandwich collagen-collagen configuration, appeared to maintain hepatocyte polarity, bile canaliculae formation and liver-specific functions (28, 29; 30, 31; 32).

Thus, for the prediction of *in vivo* toxicity in humans the use of primary human hepatocytes is preferential. The limited availability of donor material and the large variability between the donors, however, has hampered the use of human hepatocytes (11) and resulted in the use of primary hepatocytes isolated from other mammals as an alternative to human tissue. One of these alternatives is represented by primary rat hepatocytes. These hepatocytes, however, show a rapid decline in liver specific functions, in particular cytochrome P450 (Cyp450) enzyme activity (21, 33). Therefore the use of primary mouse hepatocytes might be an alternative to the rat and human systems. Although information about stability of liver specific functions in mice is scarce, the availability of transgenic mouse models and of the complete sequence of the mouse genome, make primary mouse hepatocytes interesting for the purpose mechanistic investigations (34).

Table 1 *In vitro* hepatocyte systems

System	Complexity (level of interaction)	Ability to retain liver- specific functions	Potential duration of culture	Ability to control environment
Immortalized cell lines	some cell to cell*	poor to good*	indefinite	excellent
Primary hepatocyte cultures	cell to cell	fair to excellent**	days to weeks	excellent
Liver slices	cell to cell (all cell types)	good to excellent	good to excellent	good

* varies with cell line; ** varies with culture conditions

TOXICOGENOMICS

The development of new, mechanism-based, *in vitro* assays with a high predictive value for the screening of chemicals for their carcinogenic potential is feasible through the availability of powerful novel technologies for monitoring the complete cellular genome, gene expressions, proteome expressions and metabolite levels. This enables high throughput and multi-endpoints analyses for the effects caused by chemicals, thus presenting opportunities for improved methods to identify and evaluate the carcinogenic potential of chemicals, and for monitoring their effects.

The implementation of these technologies in toxicology together with the required bioinformatics tools is called toxicogenomics and is envisioned to revolutionize current hazard and risk assessment practices. Toxicogenomics provides the opportunity to improve the *in vitro-in vivo* extrapolation, e.g. by developing “fingerprints”, a set of key genes in a gene expression fingerprint for a given exposure to a particular chemical. By defining specific classes of toxicity that need to be discriminated, and by using a range of very well chosen model compounds that are prototypical for those classes, gene expression fingerprints specific for these classes can be generated. The application of microarrays in toxicology is an interesting tool to generate those gene expression fingerprints, which will be useful for prediction of the toxicity of a chemical, such as the carcinogenic potential, and to reveal the mode-of-action of that compound (14, 17, 35-44).

Microarrays

The intensive genome-sequencing and subsequent rapid accumulation of genomic-sequence data has made it necessary to develop novel methods for the high-throughput monitoring of gene expression (45). The monitoring of expression levels of thousands of genes simultaneously was established by using miniaturised hybridization assays, the microarrays (46). Microarrays consist of a platform on which cDNAs or oligonucleotides are immobilized.

In case of a two-colour experiment (Agilent or Operon) RNA from two cell populations, for instance a test sample and a control sample, is extracted and each sample is differentially labelled with a specific dye fluorophore, Cy3 (green) or Cy5 (red). Equal amounts of labelled RNA is hybridized to the microarray. The intensity of both fluorophoric signals is measured and used to determine the ratio of expression for each gene in the test sample compared to the control sample. This will result in the relative expression level (46, 47).

In case of a one-colour experiment (Affymetrix, Illumina, Agilent) the RNA from each sample or control is labelled, fragmented and hybridized to different microarrays. In this case the absolute fluorescence value is determined and, after normalization, compared with other samples or controls to detect variation of expression (48; 49, 50). The procedures of both approaches are compared in Figure 1.

Figure 1 Comparison between a two-colour experiment (Agilent or Operon) and one-colour experiment (Affymetrix).

Toxicogenomics, which combines classical toxicology with high-throughput genomic technologies like microarrays, is focused on the identification of potential human toxicants and their specific alterations in gene expression patterns and subsequent phenotypic responses of cells or organisms, on the identification of useful biomarkers of disease and exposure to toxic substances, and on understanding the molecular mechanisms of toxicity.

The resulting gene expression patterns, so called transcriptomic fingerprints, can be used for predicting toxic properties of chemicals, such as for classification of GTX and NGTX carcinogens or non-carcinogens (12, 17). Furthermore, these gene expression patterns can reveal underlying pathways associated with the compound.

Data analysis

The amount of the data generated with a microarray experiment is immense. Therefore careful data analysis is essential for interpreting toxicological outcomes (45).

For the Affymetrix GeneChip arrays numerous quality control criteria can be applied, such as normalization quality controls, including scaling factors, average intensities, present calls, background intensities, noise, and raw Q values. Their values should be within acceptable limits for all chips. Hybridization controls BioB, BioC, BioD, and CreX, need to be identified on all chips and should yield in the expected increases in intensities as described by Affymetrix.

The raw data are then imported into an integrated software system for managing, mining and visualizing microarray gene expression data. To adjust microarray data for effects which arise from variation within or between chips, data need to be normalized. Most frequently used methods for normalisation are Robust Multi-array Average (RMA) or GeneChip RMA (50). Present-Marginal-Absent calls are used to identify and omit probe sets of poor quality (51). Probe sets are a collection of probes designed to cover a given sequence from a gene. Subsequently, the remaining probe sets are logarithmically (base 2) transformed, corrected for vehicle control if necessary, and subjected to statistical analysis. Identification of differentially expressed probe sets can be by simple fold-change cutoffs or by (complicated) statistical tests. Taking into account the possible false-discovery rate, the chance that a significant finding is false-positive, is crucial for statistical analyses.

Thereafter several methods can be used to interpret and visualize microarray data, including hierarchical clustering, k-means clustering, self-organizing maps (SOM) and principal component analysis (PCA) (52, 53, 54, 55). These methods are used to identify groups of genes with similar expression patterns and are referred to as unsupervised methods. Supervised clustering methods like support vector machines (SVM) or prediction analysis of microarrays (PAM), are used when one has some previous information

about samples that are expected to cluster together according to their gene expression patterns (56). For the PAM software first a training set is made. This software uses a modification of the nearest centroid method, which computes a standardized centroid for each class in the training set. This is the average gene expression for each gene in each class divided by the within-class standard deviation for that gene. Thereafter cross-validation was repeated ten times. Cross-validation imitates how the classifier would work on new data. This will result in a list of genes, i.e. classifiers. These classifiers are then validated with a test set and classes are predicted.

Microarray data can also be analysed in a pathway perspective, which can lead to a higher level of understanding of the system. Several tools have been developed for this purpose.

The Gene Ontology (GO) consortium developed an ontology for genes to describe the roles of genes and gene products in any organism (57). Genes can be listed together according to their biology, function and location with GO analysis, but lacks information about the relation between those genes (58). Therefore other methods, which give information about the connections between genes and gene products, are available. An example is MetaCore, which contains pathways and networks derived from text-mining (59). All these methods require individual genes to be significantly up- or down-regulated in order to contribute to the score of the significance of overlap between pre-defined gene groups, like GO categories, and the subset of induced or repressed genes.

Recently, new pathway analysis methods have been developed for which no subset of genes needs to be selected prior to analysis. T-profiler is such an example of a pathway analysis tool. T-profiler can score GO categories or other pre-defined gene sets without the need to apply cut-offs to the expression level of individual genes (60). It uses the *t*-test to score the difference between the mean expression level of predefined groups of genes and that of all other genes on the microarray. This method has been shown to be useful in several genome-wide studies (61-66). Another pathway analysis tool which does not require pre-selection is Gene Set Enrichment Analysis (GSEA). This tool takes existing pathways and the entire biomolecular network knowledge into account. It uses the statistical non-parametric Mann-Whitney test to calculate the P value to indicate significant enrichment scores (67).

MODEL COMPOUNDS

As mentioned before, the use of well documented model compounds for well specified classes of toxicity is essential to obtain specific transcriptomic fingerprints in order to classify compounds according to their carcinogenic potential.

Based on their mechanisms of action, chemical carcinogens are classified as genotoxic (GTX) or non-genotoxic (NGTX) carcinogens (12). A genotoxic compound may cova-

lently bind with DNA and cause direct damage to DNA by adduct formation. These lesions may not or incorrectly be repaired which leads to mutations and ultimately to the formation of tumors (3). A non-genotoxic compound, on the other hand, lacks the ability to induce DNA damage directly or indirectly. Because NGTX compounds have different features from GTX compounds, it may be hypothesized that GTX and NGTX induce distinct gene expression profiles which consequently may be used for mechanism-based classifying unknown compounds as GTX or NGTX (68, 69).

The compounds used in the studies described in this thesis are chosen to cover both GTX and NGTX classes as well as true GTX compounds and false positive GTX compounds, many taken from the Kirkland databases (70, 70, 71). These compounds and corresponding results from *in vivo* and *in vitro* genotoxicity tests are presented in Table 2.

Genotoxic compounds

Benzo(a)pyrene

Benzo[a]pyrene (BaP) is a widespread environmental pollutant formed during the combustion of organic products (World Health Organization, 1987) with both GTX and NGTX characteristics. This polycyclic aromatic hydrocarbon (PAH) is a preferred substrate for cytochrome P450 (Cyp450)-dependent monooxygenases (72). Those Cyp450 enzymes, together with epoxide hydroxylase, can convert BaP into its ultimate genotoxic carcinogen benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) and forms DNA adducts and DNA damage (73). BaP has been proved to be able to induce both *in vitro* and *in vivo* genotoxicity in several studies and clearly introduces DNA adducts (Table 2).

Aflatoxin B1

Aflatoxins consist of a group of primarily hepatotoxic mycotoxins, produced by some strains of the fungi *Aspergillus flavus* and *A. parasiticus* (74, 75). The fungi are ubiquitous and grow, under appropriate conditions of heat and moisture, on a variety of agricultural products, like milk and other dairy products, intended for human and animal consumption (76). Aflatoxin B1 (AFB1) is one of the most potent hepatocarcinogens. It requires oxidation of the 8,9-vinyl bond to yield the biologically active AFB1-8,9-epoxide, which binds covalently to DNA and thereby forming DNA adducts and chromosomal aberrations (Table 2). Both activation and deactivation of AFB1 are mediated by the microsomal cytochrome P450 system. (77)

2-Acetylaminofluorene

2-Acetylaminofluorene (2-AAF), a synthetic chemical not generally present in the environment, is one of the most intensively studied of all chemical carcinogens. 2-AAF was produced as a synthetic chemical insecticide in the 1940's until its toxicity was revealed (78). Now it is extensively used as a model GTX compound.

The metabolic activation of 2-AAF is initiated by the cytochrome P450 dependent N-hydroxylation, leading to the formation of N-OH-AAF, which is the main metabolite of 2-AAF in the liver and can already introduce DNA damage in the liver (79, 80, 81, 82). 2-AAF has proven to be genotoxic in several *in vivo* and *in vitro* studies and is able to introduce gene mutations (Table 2).

Dimethylnitrosamine

Dimethylnitrosamine (DMN) is the most simple and widely occurring nitrosamine and has been shown to be an acute hepatotoxic and potent carcinogenic compound (83-85). It is present in many foods such as cured meat, cooked ham, smoked fish, and alcoholic beverages (86). In order to express its toxic potential DMN needs to be metabolically activated to form its reactive metabolite. The major step in this metabolic activation is the cytochrome P450-dependent (Cyp450) oxidative demethylation of the alpha-carbon to the monomethyl nitrosamine, an unstable metabolite which breaks down to the methyldiazonium ion and eventually to the carbonium ion which alkylates the DNA (87, 88). The most important Cyp450, responsible for the activation of DMN is CYP2E1 in humans or mice (89, 90). DMN shows genotoxic effects in several *in vivo* and *in vitro* studies (Table 2).

Mitomycin C

Mitomycin C (MitC) is an antitumor antibiotic frequently used in cancer chemotherapy (91).

Early studies have demonstrated that enzymatic bioactivation of MitC is NADPH-dependent (92, 93). It has also been shown that NADPH:cytochrome c reductase and xanthine oxidase activate MitC under hypoxic conditions and Cyp450 enzymes might be involved in the anaerobic metabolism of MitC (92, 94, 95). Therefore activated MitC can induce a variety of DNA adducts by cross-linkage and covalent binding to DNA. MitC causes DNA damage and is shown to be genotoxic in several *in vivo* and *in vitro* studies (Table 2).

Non-genotoxic compounds***Cyclosporine A***

Cyclosporine A (CsA), a neutral lipophilic cyclic undecapeptide isolated from the fungus *Hypocladium inflatum* gams, is a potent immunosuppressant widely used to prevent rejections after organ transplantation (107). Despite being highly efficacious for this prevention, the use of CsA as an immunosuppressant is limited by severe side effects including nephrotoxicity, neurotoxicity, and hepatotoxicity (96). It has been shown to induce cell proliferation and cancer progression in different cell lines by a cell-autonomous mechanism (97, 98). However, the precise mechanisms by which CsA stimulates growth of carcinogen-initiated hepatocytes remain uncertain.

2,3,7,8-tetrachlorodibenzo-p-dioxin

Dioxins, like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are ubiquitous environmental contaminants that are inadvertent by-products of various processes including municipal waste combustion and phenoxy acid herbicides production (99, 100, 101). They elicit a broad range of species-specific biochemical and toxic effects in animals such as xenobiotic enzyme induction, tumor promotion and hepatotoxicity (102, 103, 104). Dioxins are considered to exert their toxicity by activating the transcription factor AhR_R(105).

The precise mechanism of action of TCDD remains unclear, but it is found to target mitochondrial transcription and to induce mitochondrial stress signalling *in vivo* and thereby promote tumor progression.

False-positive GTX carcinogens

The false-positive GTX compounds, chosen for this study, are considered to be non-carcinogens, and to show only genotoxicity in *in vitro* tests but not to induce genotoxicity *in vivo*.

o-Anthranilic acid

o-Anthranilic acid (ANAC) is used extensively in electrophoretic separations of oligosaccharides by SDS PAGE (106-108). ANAC is one of the metabolites of L-tryptophan and is excreted in urine after conjugation with glycine or glucuronic acid without further metabolism. However, ANAC can be metabolized to 5-hydroxyanthranilic acid (109) in the presence of NADPH and molecular oxygen, stimulated by superoxide dismutase (110). ANAC has been shown to introduce chromosomal aberrations *in vitro* (Table 2). Also in the mouse lymphoma cell mutation test (MLA) ANAC is shown to be

genotoxic. In the chromosomal aberrations test (CA) *in vivo*, however, ANAC is shown to be equivocal genotoxic in the mouse.

2-(Chloromethyl)pyridine.HCl

2-(Chloromethyl)pyridine.HCl (2-CP) is an aromatic heterocycle frequently used as an intermediate in the preparation of various compounds which have one or more of hypocholesteremic, anti-hypotensive, anti-inflammatory, analgesic, fungicidal or bactericidal properties (111). 2-CP shows *in vitro* genotoxicity in the MLA and Ames test and in the CA test, but shows no *in vivo* genotoxicity in the micronucleus (MN) and CA tests in the mouse (Table 2).

4-Nitro-o-phenylenediamine

4-Nitro-o-phenylenediamine (4-NP) is a nitroaniline dye that has been used exclusively at low levels in some semi-permanent hair colourings (112). It is a very powerful direct-acting mutagen and its mutagenic potency can significantly be enhanced in the presence of plant enzymes (113, 114, 115). Peroxidative oxidation plays a major role in the mutagen enhancement process and endogenous peroxidase activity such as that associated with the plant cytochrome P450-dependent enzyme systems may also play a role in the activation of 4-NP (115). 4-NP shows *in vitro* genotoxicity in the MLA and Ames test, but no in the CA test. In the mouse no *in vivo* genotoxicity was observed in the CA test while equivocal genotoxicity was reported by means of the MN test (Table 2).

8-Hydroxyquinoline

8-Hydroxyquinoline (8-HQ) is a heterocyclic phenol amine used as cosmetic biocides, fungicides, bacteriocides, and oxidation stabilizers in cosmetics. It is also used as reagents for detection of bismuth and as a chelating agent in analysis of trace metals in industry (116). 8-HQ can be metabolized by Cyp450 enzymes to 8-hydroxyquinoline N-oxide or various 8-hydroxyquinoline epoxides. The water-soluble reactive quinoline-2,3-epoxide and 8-hydroxyquinoline sulfate are suggested to be the most important intermediates responsible for the genotoxic activity of 8-HQ (117). 8-HQ shows *in vitro* genotoxicity in the Ames and MLA test. Weak genotoxicity was detected in the CA test. In the *in vivo* MN and CA assays, no *in vivo* genotoxicity was observed in the mouse (Table 2).

Quercetin

Quercetin (Q) is one of the most widely distributed flavonoids, present in fruit, vegetables and many other dietary sources. Many beneficial biological and pharmacological activities for human health have been attributed to quercetin, including antioxidant, anticarcinogenic, anti-inflammatory, and cardioprotective activities (118). Frequently quercetin occurs as aglycone, or sugarless form of rutin, a glycoside (119).

The rapid and extensive metabolism of quercetin might be the cause of the lack of genotoxic responses *in vivo* (120). The gastrointestinal tract and the absorptive gastrointestinal cells are the first sites of quercetin metabolism. Quercetin is, after absorption, partially metabolised *via* *O*-methylation, sulphation, or glucuronidation in the intestinal epithelium (121, 122). Non-metabolized quercetin is conjugated in the liver to form sulphated, glucuronidated, or methylated derivatives (123, 124). The high degree of metabolism lowers the amount of unconjugated quercetin aglycone available in the body. In non-oral *in vivo* exposure and in bacterial and eukaryotic *in vitro* test systems quercetin aglycone levels are higher and this may help to explain the absence of quercetin-related genotoxic activity *in vivo* that is observed *in vitro* (125-134). Q shows *in vitro* genotoxicity in the Ames test and in the MN and CA tests. Q also introduced cytogenetic damage in human and rodent cells. Q is, however, shown to be non-genotoxic by the *in vivo* MN and CA tests (Table 2).

Table 2 The selected compounds and the results from in vivo and in vitro genotoxicity tests (70, 135, 136, 137, 138, 139, 140, 71, 141). V-79 are Chinese hamster cells. CHO and CHLF are respectively Chinese hamster ovary cells and lung fibroblasts.

Chemical	in vitro genotoxicity					in vivo genotoxicity				Carcinogenicity		
	Abbr.	CAS	Ames	MLA	MN	CA	UDS	MN	CA	UDS	TRA	DNA adducts
Benzo[a]pyrene	B(a)P	50-32-8	+	(115)	+ rat hepatocytes (116)	+ IARC Vol.32	+ IARC Vol.32	+	(118)	+	+	+ in vivo & in vitro IARC Vol.32
Aflatoxin B1	AFB1	1162-65-8	+	(115)	+ human cells (116)	+ IARC Vol.32	+ IARC Vol.82	+	(118)	+	+	+ aflatoxin B1-N7- guanine adduct, in vivo (120)
2-acetylaminofluorene	2-AAF	53-96-3	+	(115)	+ CHO cells (115)	+ human fibroblasts (117)	+ human fibroblasts, rat and mouse hepatocytes (117)	+	(118)	+	+	+
Dimethylnitrosamine	DMN	62-75-9	+	(115)	+	(119)	+	(118)	+	(122)	+	+
Mitomycin C	MitC	50-07-7	+	(117), + (118)	+ Bovine L, CHO/CHLF / V79 cells (117)	+ human lymphocytes (117)	hepatocytes/h uman cells (117)	+	(118)	+	+	+
Cyclosporin A												
(79217-60-0)-	CsA	59865-13-3	-	(115)	-	(115)	lymphocytes (115)	-bone marrow rodent (115) (mice + hamsters)	-bone marrow rodent (mice + hamsters), + peripheral blood lymph kidney with prednisolone (115)			
cyclosporine												

Table 2 (continued)

Chemical	Abbr.	CAS	Ames	MLA	in vitro genotoxicity			in vivo genotoxicity			Carcinogenicity				Target organ
					MN	CA	UDS	MN	CA	UDS	TRA	DNA adducts	DNA damage	Mode of action	
2,3,7,8-tetrachlorodibenzo- para-dioxin	TCDD	1746-01-6	- (115)	(115), + (118)		- CHO (115)		- (118)	- (115, 118)	- (118)	-	-DNA, not direct-acting genotoxic agents IARC vol 66	DNA damage mediated by cytochrome P450-catalysed metabolic activation pathways, apoptosis	tumour promotion by Ah receptor-mediated alteration in expression of networks of genes in cell growth	liver
o-Anthrannilic acid	ANAC	118-92-3 / 80206-34-4	- (115)	+ (115)		weakly + (115)			+/- mouse						
2-(chloromethyl)pyridine.HCl	2-CP	6959-47-3	+ (115)	+ (115)		+ (115)		-mouse (115)	-mouse (115)						
4-nitro-o-phenylenediamine	4-NP	99-56-9	+ (115)	+ (115)		- (115)		+/- mouse (115)	-mouse (115)						
8-hydroxyquinoline	8HQ	148-24-3	+ (115)	+ (115)		weakly +CHO (115)		-mouse (115)	-mouse (115)						
[AKA 8-quinolinol]					+ cytogenetic damage in human and + (119)	+ (115)		no genotoxic	IARC Vol 73			+ in vitro	increased the frequency of DNA damage and lipid peroxidation in liver nuclei of rats in vitro		No carcinogen
Quercetin	Q	117-39-5	+ (115)					- (121)	- (121)			(121)			IARC Vol 73

OBJECTIVE AND OUTLINE OF THE THESIS

Various test guidelines using animals have been developed for the evaluation of the toxicological responses of compounds in order to classify them for their genotoxic and carcinogenic potential in humans. These animal studies are mostly long lasting, relatively expensive and are characterized by their low throughput. Furthermore, the predictive value of these animal studies with regard to the extrapolation from animal to human risk is not very high. Combining these disadvantages with the need to assess many chemicals for genotoxicity and carcinogenicity, increases the demand for the development of high-throughput *in vitro* screening assays that are predictive for carcinogenicity in humans. The introduction of the microarray technology in toxicology has led to high throughput monitoring of changes in gene expression of multiple genes. Because the liver is an important organ for the metabolism of many compounds and an important organ for toxicity and carcinogenicity, hepatocyte-based systems are gaining interest in microarray-based toxicity studies. Specifically primary mouse hepatocytes can be very useful because they are metabolic competent and are of additional advantage because of the availability of the complete sequence of the mouse genome and of transgenic mouse models which allow relevant mechanistic studies (34).

Hypothetically, gene-expression fingerprints should be able to discriminate between GTX and NGTX carcinogens and between true and false positive GTX compounds, and should be negative for non-carcinogens.

The objective of this thesis therefore is to combine the use of *in vitro* primary mouse hepatocyte cultures with whole genome gene expression analysis through the microarray technology, in order to identify sets of gene-expression effect markers that are able to discriminate between true genotoxic and false positive genotoxic compounds. Eventually, this may result in a predictive screen which can be applied in chemical safety procedures, initially as a screening test, but eventually as alternative to current rodent testing models and may also lead to commercial applications, i.e. high-throughput systems which are more specific, dedicated, cheaper and societally more acceptable than currently available animal models.

Because gene expression profiling in HepG2 was shown to be able to discriminate GTX from NGTX compounds only at one time point, first experiments were conducted in HepG2 cells in order to investigate time-dependent changes in the cellular responses upon exposure to a well-known carcinogen and in order to further apply this in mouse primary hepatocytes. These initial experiments are described in **chapter 2**. Therefore, the changes in gene expression profiles of HepG2 cells caused by the model carcinogen BaP were investigated at 12 time points after exposure.

Next, the mouse hepatocyte isolation method was set-up and the metabolic competence of sandwich-cultured primary mouse hepatocytes was investigated in **chapter 3**. Pri-

mary mouse hepatocytes were cultured for 0, 42 and 90 h and changes in base line gene expression profiles and Cyp450 enzyme activity were investigated. Thereafter, relevance of the sandwich-cultured primary mouse hepatocyte model was further assessed in **chapter 4** by comparing gene expression profiles of primary mouse hepatocytes and mouse liver *in vivo* after exposure to BaP. Also time- and dose-dependent effects caused by BaP, were investigated. Gene expression profiles were generated from hepatocytes exposed to 10 or 30 μ M BaP for 12, 24, 36 and 48 h and DNA damage was phenotypically evaluated. These generated gene expression profiles were compared to those from mice exposed *in vivo* to 13 mg/kg bodyweight BaP for 7 days.

Chapter 5 focuses on the capacity of the sandwich-cultured primary mouse hepatocyte model to discriminate GTX compounds from NGTX compounds. Again, the optimal treatment time for discrimination was assessed. For that, hepatocytes were exposed to two GTX compounds, e.g. BaP and AFB1, and to two NGTX compounds, e.g. CsA and TCDD. Gene expression profiles and phenotypic measurement of DNA damage were compared between the two groups of carcinogens. PAM was used to classify the compounds and to generate a list of classifiers. For the purpose of validation, two additional GTX compounds were used as test compounds.

Finally, in **chapter 6**, the ability of the sandwich-cultured primary mouse hepatocyte model to discriminate GTX compounds from false-positive GTX compounds was investigated. The hepatocytes were therefore exposed to five GTX carcinogens and to five false positive GTX compounds. Also in this study gene expression profiles and phenotypic measurement of DNA damage were compared between the two groups and PAM was used to classify the compounds and to generate a list of classifiers.

Finally, **chapter 7** presents the summary of the outcome of the work and a general discussion.

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Chapter 2

Time Series Analysis of Benzo(a)Pyrene-Induced Transcriptome Changes Reveals Temporary Effects on DNA Repair, Metabolism and Cell Cycle

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Abstract

Chemical carcinogens may cause a multitude of effects inside cells, thereby affecting transcript levels of genes. Such as by direct activation of transcription factors or through the formation of DNA-damage. As the profiles of these responses may be profoundly different, examining temporal changes may provide new insights in mechanisms underlying carcinogenesis. Therefore, we investigated in human hepatoma cells gene expression changes caused by the carcinogen benzo(a)pyrene at 12 time points after exposure, in relation to DNA-adduct and cell cycle. Temporal profiles, demonstrating both early and late effects in up- and downregulation, were identified. Dioxin-responsive genes show the earliest response. Most interesting are the slower effects, such as upregulation of DNA repair pathways. Expression increase for several genes in transcription-coupled nucleotide excision repair last for ca 30h, until $\pm 50\%$ of the DNA adducts has been repaired, whereas for global genomic nucleotide excision repair and base excision repair upregulation is up to 60h. The data also indicate that when S-phase cell levels and DNA adduct levels are high, the DNA-damage responses are large, while gene expressions related to metabolism of amino acids and lipids are low. After S-phase levels decrease and those for G2/M increase, this metabolism is upregulated. Noteworthy are also the sequential periods during which several reaction pathways in gene expression, transcription and translation are upregulated. Thanks to the temporal dimension, this study of changes in gene expression profiles following exposure to a chemical carcinogen provides more and more robust insights in the affected molecular processes.

Introduction

Chemicals can cause cancer through a variety of mechanisms. Genotoxic carcinogens induce damage to the genetic material, either directly by covalently binding to DNA or indirectly by interfering with the mitotic machinery, ultimately leading to mutations in genes or large aberrations in chromosomes (1). If tumour suppressor genes are inactivated or proto-oncogenes are activated by this process, this may lead to cancer formation. The mode of action of non-genotoxic carcinogens does not imply damage to DNA or chromosomes, and, can be very diverse, ranging from stimulation of cell proliferation, suppression of apoptosis, induction of oxidative stress, to activation of biotransformation enzymes, suppression of the immune system, etc. (2).

Polycyclic aromatic hydrocarbons (PAH) comprise a large group of structurally related compounds, including many carcinogens, to which humans are daily exposed through the environment and food. PAH are well known carcinogens, especially for lung, but also for many other tissues including liver. Many carcinogenic PAH, such as benzo(a)pyrene (BaP), share both genotoxic and non-genotoxic properties. During several enzymatic steps involving cytochrome P450 enzymes and epoxide hydroxylase, BaP is converted into the ultimate genotoxic carcinogen benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE). BPDE can form DNA adducts, mainly BPDE-N2deoxyguanosin (BPDE-dG) (3). If not repaired, these adducts lead to mutations (4). BaP can also be transformed into radical cations (5) and reactive and redox-active o-quinones, which also can form DNA adducts (6,7). Auto-oxidation of intermediates and/or redox cycling of o-quinones generates reactive oxygen species (ROS) capable of modifying DNA, leading to oxidised nucleotides like 8-hydroxy-deoxyguanosine (8-OHdG) (8). These processes all comprise the genotoxic properties of BaP.

The non-genotoxic properties of PAH are assumed to act through activating the transcription factor *AhR*, the aromatic hydrocarbon receptor, which thereupon dimerizes with the AhR nuclear translocator (*ARNT*). The *AhR-ARNT* complex binds to the dioxin-responsive elements (DRE) in the promoter regions of genes, thereby inducing their transcription (9). Many genes are involved in metabolism of carcinogens, including phase I enzymes like *CYP1A1*, *CYP1A2* and *CYP1B1* and several phase II enzymes (10).

In addition, PAH-mediated oxidative stress leads via the activation of transcription factor Nrf2 to the induction of multiple phase I and II biotransformation enzymes, including aldo-keto reductases, glutathione *S*-transferase-P, NAD(P)H:quinone oxidoreductases and UDP-glucuronosyl transferase 1A6 (11). *Nrf2* protects against induction by BaP of DNA adducts, mutations and tumours in mice (12).

In order to protect the cells from the deleterious effects of DNA damage, cells are capable of repairing damaged DNA by many different mechanisms. In the case of BaP, the most relevant are nucleotide excision repair (NER) for the BPDE-DNA adducts and

base excision repair (BER) for the oxidised damages (13,14). In order to allow for more time for repair, cell proliferation can be blocked at several phases of the cell cycle, such as at G1-S transition, S-phase and G2-M transition. Also, instead of this proceeding into cell cycle arrest, apoptosis can be induced, especially at high DNA damage levels (15).

Whereas exposure of cells to a full carcinogen such as BaP is likely to induce multiple molecular responses, their time dependencies and possible functional interactions are not well understood. Revealing successive waves of related gene expression changes linked with relevant phenotypical markers, may provide more insight into this. The objective of our study therefore is to investigate time-dependent changes in cellular responses upon exposure to a full carcinogen, thereby using BaP as a model compound. This is achieved by extensive time series analyses of global gene expression changes in human hepatoma cells (HepG2) in relation to phenotypic events such as DNA adduct formation and cell cycle progression. HepG2 cells represent the liver as target organ for PAH-related carcinogenesis *in vivo*, are metabolically competent with respect to biotransformation of mutagens and carcinogens, carry no p53 mutations, and are frequently used in toxicology and gene expression studies (16-19).

Materials and methods

Cell culture and treatment

HepG2 cells were cultured and treated with 3 μ M BaP (purity 97%, CAS no. 50-32-8; Sigma-Aldrich, Zwijndrecht, the Netherlands) or vehicle control (DMSO, 0.1%) as described previously. This is the lowest BaP dose with a maximum accumulation of cells in S-phase (20). The cells were exposed for 3 to 60 h where after they were either fixed with cold methanol and stored at -20°C for FACS analysis or media was removed from the culture flasks and Trizol (Gibco/BRL, Breda, The Netherlands) was added for RNA and DNA isolation. Two independent experiments were conducted.

BaP in culture medium

BaP levels in culture medium were determined following extraction with hexane, evaporation of hexane, dissolving the residue in acetonitrile, and analyses on an HPLC gradient system (Supelcosil LC-PAH (column 25cm*4.6mm, 5 μ m); solvent gradient at 1.5 ml/min: 3 min 64% Acetonitril - a linear gradient to 100% acetonitril in 20 minutes – a 15 min hold at this) with fluorescence detection (ex/em at 280/>350nm).

γ H2AX foci analysis

HepG2 cells cultured and exposed on cover slips were analysed for DNA double strand breaks by immunofluorescence staining of γ H2AX foci as described (21). Following

fixation, foci were stained with the anti-phospho-Histone H2A-X (ser139) Clone JBW 301 antibody (Upstate Biotechnology, Lake Placid, NY) and Alexa fluor 488 goat anti-mouse IgG antibody (Invitrogen, Breda, The Netherlands). Cells were scored manually using a damage score, ranging from 0 to 4, and sample scores (100 cells/sample) are given as percentage of the maximal damage.

Flow cytometric analyses for cell cycle and apoptosis

Analyses of cell cycle profiles and apoptosis was performed as previously described using a FACSsort (Becton Dickinson, Sunnyvale, USA) (20). For DNA content cells were stained with propidium iodide; apoptotic cells were visualised with the primary antibody M30 CytoDeath (Roche, Penzberg, Germany) and FITC conjugated anti-mouse Ig as secondary antibody (DakoCytomation, Glostrup, Denmark). For each sample 10,000 cells were analyzed. Cells in the G0-1, S or G2-M phase were expressed as a percentage of the total number of cells.

RNA and DNA isolation

RNA was isolated from the Trizol solutions according to the producer's manual and purified with the RNeasy mini kit (Qiagen Westburg bv., Leusden, The Netherlands). After isolation, the remaining phases were used for DNA isolation according to manufacturer's protocol. RNA and DNA quantity was measured on a spectrophotometer and RNA quality was determined on a BioAnalyzer (Agilent Technologies, Breda, The Netherlands). Only RNA samples which showed clear 18S and 28S peaks and with a RIN level higher than 8 were used.

DNA adduct analyses

DNA adduct levels were determined by ³²P-postlabelling according to the procedure originally described by Reddy and Randerath (22) with modifications described by Godschalk et al (23).

Gene expression analyses with DNA microarrays

Labelling and hybridization

Labelling and hybridisation of RNA samples was done according to Agilent's manual for microarrays (Agilent Technologies, Breda, The Netherlands). Samples from BaP or vehicle treated cells was labelled with Cyanine 3 (Cy3) or Cyanine 5 (Cy5). cRNA of the time-matched treated and control samples was applied on the G4110B Agilent 22K Human Oligo Microarray, hybridized and washed according to Agilent's manual and slides were scanned on a ScanArrayExpress (Packard Biochip Technologies, Perkin Elmer life sciences, Boston, USA) with fixed laser power (100%) and PMT gain (55% for Cy5 and 60% for Cy3). For each biological experiment, two hybridizations per time

point were conducted, with swapped Cy3 and Cy5 dyes. In total this resulted in 48 hybridisations.

Image analysis and processing

The images (10 micron resolution; 16 bit tiff) were processed with ImaGene 6.0 to quantify spot signals (BioDiscovery Inc., Los Angeles, USA). Irregular spots were automatically flagged and excluded from data analysis. Data from ImaGene were further processed with GeneSight 4.1.6 (BioDiscovery Inc., Los Angeles, USA). For each spot, background was subtracted and flagged spots as well as spots with a net expression level below 20 in both channels were omitted. Data were log base 2 transformed and LOWESS normalized. If for a gene more probes were on the array, replicates were averaged while omitting outliers (>2 standard deviations). Raw data are available at ArrayExpress (accession numbers E-TOXM-22 and E-TOXM-23, for the first and second experiment respectively) (www.ebi.ac.uk/arrayexpress/).

Gene expression data analyses and data mining

Differentially expressed genes

First genes were selected for which all four replicate hybridizations gave an expression difference of >0.2 or all four <-0.2 (meaning all replicates in the same direction; on a normal scale, this is a 15% increase or decrease of transcript level). This resulted in 5211 genes. Second, genes were selected by ANOVA using the plug-in “Time series analysis” from BRB Array Tools (version 3.4 and R version 2.2.0; <http://linus.nci.nih.gov/BRB-ArrayTools.html>) with “Time” as descriptor. This resulted in 2808 genes with a False Discovery Rate (FRD) <0.1 . All further analyses on significantly differentially expressed genes, start with these 2808 genes.

Time series analyses

For identification of genes, reactions, pathways or gene-sets co-regulated time-dependently and correlating with a biological parameter, the software tool “Short Time-series Expression Miner” (STEM, version 1.1.2b; <http://www.cs.cmu.edu/~jernst/stem/>) was used (24,25). STEM finds statistically significant patterns from time series microarray experiments and can compare data sets across experiments. The clustering algorithm assigns each gene, reaction, pathway or gene-set to these profiles based on correlation coefficients (also between the two independent experiments) and permutation analyses ($n=50$). For all parameters, log-transformed data were used, normalised such that treated-control ratios ranged from +1 to -1, with 0 for no change.

Functional interpretation of significantly differentially expressed gene sets

Reactome (<http://www.reactome.org>; Version 25, November 2008) (26) is used to examine the biological processes that may be affected at the level of reactions and reaction pathways, a level of detail that cannot be obtained by gene ontology annotations, and KEGG and BioCarta pathways. Reactions are grouped into causal chains to form pathways. The tool Skypainter is used to determine per time point which events (reactions

and/or reaction pathways) harbour differentially expressed genes (down- and upregulated genes separately), selecting only events with at least 3 genes. The proportion of these number of differentially genes per event (time point / experiment) with total numbers present in the set of 2808 genes, are calculated for each pathway.

Functional interpretation of all expressed genes

T-profiler is used for functional annotation of pathways and processes in the complete data set of genes without pre-selecting modulated genes. T-profiler uses the unpaired *t*-test to score the difference between the mean expression level of predefined sets of genes and that of all other genes within the complete data set (27). Pathways and processes included gene sets from GO terms, KEGG pathways, GenMAPP pathways, Motifs, Broad/MIT pathways, (manually curated) gene sets and gene sets retrieved from literature, i.e. lists of genes modulated by BaP or TCDD in HepG2 cells (28-30) and a set of genes with known antioxidant response elements (AREs), also known as Nrf2 binding sites (11). Per array *t*-values were determined for each gene set. Only gene sets with an absolute *t*-value > 1 at at least 2 succeeding time points in all four replicate arrays were selected for further analyses.

Results

Effects on BaP levels, DNA adduct levels , cell cycle and apoptosis

Clearance by HepG2 cells of BaP from the culture medium was fast, with 1% remaining after 30-36 h, levels being not detectable after 48 h. BaP-DNA adduct levels increased between 3-12 h, were stable until 18/30 h, where after they slowly declined (Fig. 1). For the second experiment, adduct levels reached a lower maximum and faster decreased then for the first experiment. Adduct levels stabilised to 15-25 % of the highest level, even after 60 h, suggesting a diminished repair of adducts during the last 12-30 h. Similar results were obtained by *in situ* staining for γ H2AX foci, a marker for double strand DNA breaks (31). At 9h this was induced, at 24h it was maximal, and at 48h it was decreased to ca. 25%. Analyses of cell cycle distributions shows no changes upto 12 h. At 18 h BaP causes a drastic increase in S-phase cells with a concomitant decrease of cells in G1 and G2 phases (Fig. 1). This S-phase accumulation lasts 6-18 h, where after the number of S-phase cells decrease with a concomitant increase of G2/M-phase cells. Similar as for BaP-DNA adducts, the effects on cell cycle in the second experiment last shorter than in the first experiment. After about 54 h, the distribution of BaP-treated cells becomes similar to that of the controls. In the control cultures, the % of cells in S-phase increases after 18-24 h together with a decrease of G1-phase cells. This may be due to depletion of the culture medium for essential compounds. Apoptotic cells levels did not increase due to BaP treatment, varied between 0.5-3% for control and BaP cultures, and were slightly higher in the first experiment (data not shown).

Effects on gene expression

Differentially expressed genes

Gene expression profiles were determined by DNA microarrays, which resulted in 48 hybridisations, 4 per time point. Differentially expressed genes were identified by reproducible similar changes over the 4 replicates, combined with ANOVA, resulting in 2808 differentially expressed genes. The same approach on randomised arrays resulted in not a single significantly affected gene, which indicates that few false-positives were obtained. Visualisation of the log base 2 expression ratios for these 2808 differentially expressed genes and all arrays in a heat map, shows a high reproducibility in the expression profiles between the various replicate experiments and arrays. A few genes were verified by quantitative RT-PCR, namely CYP1A1, CYP1B1 and CDKN1A. With both methods the patterns are similar, although the effects on gene expression as measured by RT-PCR are larger.

Pathway analyses by Reactome

In order to examine the responses that may occur at the level of reactions and reaction pathways, the sets of differentially expressed genes per time point were analysed in Reactome. An overview of the reaction pathways is in Fig. 2, thereby focusing on reaction pathways / processes that are considered most relevant for chemical carcinogenesis. Functional annotation based on over representation analyses using GO-terms and other gene-sets was also done using FatiGO+ (<http://babelomics.bioinfo.cipf.es>) (32). Results added limited knowledge and thus, are not presented.

Pathway analyses by T-profiler

T-profiler was applied to identify affected functions using the complete data set, thus without pre-selecting modulated genes. Besides gene sets based on GO annotation etc, also gene sets retrieved from literature were incorporated. Following an analysis per time point, in total 155 gene sets were identified as being modulated. Hierarchical clustering was used to group these gene sets (Fig. 3). The main functional annotations of these clusters show upregulation of TP53 and NRF2 signalling pathways, apoptosis-related gene sets, and responses induced by genotoxic stressors. Downregulation consists of metabolisms of amines, sterols and lipids, (regulation of) transcription and nucleosome assembly. This confirms the Reactome data; although T-profiler presents less in-depth information it provides a better statistical evaluation.

Figure 1 Levels of BaP-DNA adducts and cell cycle distribution in HepG2 cells. Cells are analysed after various exposure times in two independent experiments (Circles and triangles are for BaP treated and control cells, respectively. Closed and open symbols are for experiment 1 and 2, respectively).

Time-related effects of the functional data

Numerous different approaches can be used to identify co-regulated genes with time-related patterns in time series experiments on microarray studies, including hierarchical clustering, or dedicated tools such as CAGED (33), GQL (34) and STEM (24,25). All these tools have been applied on our gene expression data set. Although STEM resulted in the most comprehensible information, this mainly confirmed the data from the functional annotation analyses mentioned above which is thus not presented here.

STEM was also used to uncover time-related patterns in the reactions, pathways and biological processes as identified by Reactome and T-profiler (Fig. 2 and 3), in combination with the phenotypical parameters (BaP levels in medium, BaP-DNA adducts, and

various phases of the cell cycle). In these analyses experiment 1 and 2 were used independently. Both positive and inverted correlations are observed, showing temporal changes in pathways and reactions in DNA repair, DNA damage response, cell cycle and general metabolisms. These are summarised in Table 1 and further discussed below.

Figure 2 Reactions / reaction pathways from Reactome affected in HepG2 cells by BaP treatment. Red indicates an enrichment of upregulated genes, blue of downregulated genes. Enrichment is relative to the number of genes in that pathway as present in the set of 2808 modulated genes.

Figure 3 Hierarchical clustering of gene sets affected in HepG2 cells following treatment with BaP. The *t*-values provided by the functional annotation tool T-profiler, were used for clustering with centroid linkage and euclidean correlation. The tree was cut at 12 clusters, and the main annotations for these are presented. Red and green mean up- and downregulation, respectively.

Table 1 Summary of the correlation analyses between biological parameters and pathways, processes, gene sets from the functional annotation tools T-profiler and Reactome (the data from Fig. 2 and 3) as given by the time-series analysis tool STEM.

Positive correlation with BaP levels T-profiler-4 - TCDD, xenobiotics metabolism
Inverted correlation with BaP levels React_474 - metabolism of carbohydrates T-profiler-11 - oxidoreductase activity T-profiler-5 – various
Positive correlation with adduct levels React_1059 - extrinsic pathway for apoptosis React_1104 - base excision repair React_1628 - transcription-coupled NER (TC-NER) React_1698 - nucleotide metabolism React_2254 - G1/S DNA damage checkpoints React_964 - intrinsic pathway for apoptosis T-profiler-1 - ARE-Nrf2, many genotoxic stressors (e.g. UV, BaP), oxidative stress, apoptosis
Inverted correlation with adduct levels T-profiler-8 – chromatin
Positive correlation with G1 cells T-profiler-8 – chromatin
Inverted correlation with G1 cells React_1590 - G1 phase React_2253 - global genomic NER (GG-NER) T-profiler-12 - p53, genotoxic stressors
Positive correlation with S cells React_1590 - G1 phase React_2253 - global genomic NER (GG-NER) T-profiler-12 - p53, genotoxic stressors
Inverted correlation with S cells
Positive correlation with G2/M cells T-profiler-10 - (regulation of) immune response, amino acid metabolism, steroid metabolism, lipid metabolism T-profiler-7 - developmental process
Inverted correlation with G2/M cells React_1014 - translation React_11052 - metabolism of non-coding RNA React_1725 - M/G1 transition T-profiler-2 - (regulation of) transcription

Discussion

Chemical carcinogens, and especially full carcinogens like BaP which possess both genotoxic as well as non-genotoxic properties, cause a multitude of effects in cells. These range from altering the cellular metabolic capacity, to inducing DNA damage and repair, mutagenesis, and influencing cell growth and viability. As many of these processes are exerted through changing the expression of genes at the level of mRNA, genome-wide transcriptome profiling over an extensive period and multiple time points can provide detailed insight into these processes and their regulations and interdependencies.

In our study, transcriptome responses HepG2 cells were time-dependently compared to BaP exposure levels, DNA adduct levels, distribution of cells over various phases of the cell cycle and apoptosis. We show clear temporal functional changes and temporal profiles for many genes, demonstrating both early and late effects in up- and downregulation, and for clusters of correlating genes. We delineated these gene expression changes in numerous alterations of biological pathways and processes, by several tools/approaches. Eventually, we identified by Reactome altered biological processes at the level of reactions and reaction pathways, and by T-profiler modulated biological pathways and processes.

What do all these changes teach us about the biological effects in these carcinogen-exposed cells? To understand that, we finally correlated changes in phenotypical/biological parameters (BaP levels in medium, BaP-DNA adducts, and various phases of the cell cycle) with those in reactions, pathways and gene sets by the time-series analyses tool STEM (Table 1 for more detailed information).

One and three pathways/processes show a clear positive or inverted correlation, respectively, with BaP levels in medium (Table 1). The correlation with TCDD responsive genes is as expected, since also PAHs like BaP activate *AhR* (9). A inverted correlation of BaP is observed with various diverse gene sets, namely “React_474 - metabolism of carbohydrates”, “T-profiler-11 - oxidoreductase activity”, and “T-profiler-5 – various”.

BaP-DNA adducts show a positive correlation with 7 pathways/processes/reactions (DNA repair related gene sets, many DNA damage and stress response related gene sets; Table 1), and with 1 an inverted correlation (T-profiler-8 – chromatin).

Most interesting are the effects on DNA repair pathways: both base excision repair (BER) and transcription coupled nucleotide excision repair (TC-NER) correlate with DNA adducts and are induced from 9/12h until 48h (Fig. 2). Reactive oxygen species (ROS) is expected to be formed during the metabolic activation of BaP. ROS leads to DNA damage, such as 8-OHdG, which is repaired by BER (14). Although at 30/36h most BaP has been consumed, its metabolites and intermediates are still present and thus may cause an extended oxidative stress. Indeed, the induction of BER agrees very well with the period that the gene set “T-profiler_3 - NRF2-regulated gene sets + mo-

tifs” is induced. Through the activation of transcription factor Nrf2, ROS induces the expression of many genes with anti-oxidant responsive elements in their promoter regions (11). The BER genes modulated by BaP are FEN1, MBD4, POLD4 and SMUG1, some of which are also affected by ultraviolet light (35).

Repair of bulky DNA damages – like BaP-DNA adducts – is by NER (13) and most of that is strand-specific: the transcribed strand is faster repaired than the non-transcribed strand (36,37). This is well reflected by the gene expression alterations we observed: the TC-NER reaction pathway is upregulated for a shorter period than the GC-NER reaction pathway, namely from 9-42 and 9-60h, respectively (Fig. 2). Six out of the nine NER genes modulated by BaP are in both GG-NER and TC-NER. DDB2 is specific for GG-NER and upregulated from 9-60h; POLR2A and POLR2D are specific for TC-NER and upregulated at early time points or downregulated at late time points, respectively. Effects by BaP on DDB2 have been described before in MCF-7 cells but not in HepG2 cells (28,38).

Taken together, our data suggest that the DNA damage repair system is regulated to some extent at the level of transcription. To our knowledge, this coordinated differential transcriptional regulation of various DNA damage repair pathways upon exposure to a genotoxic stressor, represents a novel finding. Till now, regulation of DNA damage repair pathways has been considered to occur mostly at post-translational level through protein modifications rather than gene expression (39,40).

The correlation of BaP-DNA adducts with many pathways representing the DNA damage response, like “REACT_1059 - extrinsic pathway for apoptosis”, “React_2254 - G1/S DNA damage checkpoints”, “React_964 - intrinsic pathway for apoptosis” and “T-profiler-1 - ARE-Nrf2, many genotoxic stressors (e.g. UV, BaP), oxidative stress, apoptosis” is as expected, since PAHs like BaP cause genotoxic damage and induce the DNA damage response pathway (41,42). Although many pathways/processes/reactions related to apoptosis are induced, BaP causes only a marginal induction of apoptosis at the doses used here. Apparently, the apoptotic machinery is activated, but BaP-induced damages are repaired sufficiently to halt that process before execution. At higher BaP doses and by other PAHs, apoptosis is induced in HepG2 cells (20).

The set of genes identified by Hockley et al. to be affected by BaP in HepG2 cells (28), is part of “T-profiler-1 - ARE-Nrf2, many genotoxic stressors (e.g. UV, BaP), oxidative stress, apoptosis”. The largest overlap in affected genes for that study with ours is at 9-30h.

BaP-DNA adduct levels show an inverse correlation with “T-profiler-8 – chromatin”, which also shows a positive correlation with the number of cells in G1-phase. When DNA adduct levels are high, cell numbers in G1-phase are low and in this period also GO gene sets on chromatin assembly and chromosome organization are down regulated. Numbers of cells in G1-phase of the cell cycle are decreased from 18 to 24/36 h. G1-cell count shows a positive correlation with one set of pathways (see previous para-

graph) and an inverse correlation with 3 others, namely “React_1590 - G1 phase”, “React_2253 - global genomic NER (GG-NER)” and “T-profiler-12 - p53, genotoxic stressors. All of these were also found to correlate with the number of cells in S-phase, but in the positive direction. During the time that counts for S-phase cells are high and for G1-phase cell are low, also the DNA adduct levels are increased. This explains the correlations of these with gene sets for DNA damage repair and DNA damage response. Interestingly, the G1-cells show an inverted correlation with “React_1590 - G1 phase”, indicating that upregulation of this reaction pathway does not necessarily agree with the actual cell cycle phase.

The concomitant increase of cells in S-phase show with a decrease of cells in G1 phase (Fig. 1) suggests an arrest of cells in S-phase and/or a stimulation of G1-cells to go into S-phase. Kinetic analyses of progression of cells into various phases of the cell cycle at various time points after BaP exposure are required to clarify this further. BaP is known to induce the G1/S-phase arrest and also to block DNA synthesis at replication forks (20,41-43).

Two pathways/processes/reactions have been shown to correlate with G2/M-cells, which are decreased from 18/24/36h and then increased from ca 24/36 to 54h (Fig. 1), namely with “T-profiler-10 - (regulation of) immune response, amino acid metabolism, steroid metabolism, lipid metabolism” and “T-profiler-7 - developmental process”. Within the T-profiler-10 cluster, many gene sets relate to metabolism and they also show this downregulation followed by an upregulation (see Fig. 3). This suggests that in the period when DNA adduct and numbers of cells S-phase are high and when G1 and G2/M cell levels are low, the general metabolism of the cells is downregulated. When thereafter S-phase levels decrease and G2/M increase, the demand of the cells for general metabolism is high. Once cell cycle distribution is back to baseline and DNA adducts have mostly been repaired, metabolic processes return to their normal levels.

G2/M cell levels show an inverted correlation with four pathways. Noteworthy, the inverted correlation is mainly with reactions involved in gene expression, transcription and translation. Most of these show an initial upregulation followed by a downregulation thereafter. An order is apparent: the induction period increases from 6h for “React_11052 - metabolism of non-coding RNA” (such as miRNAs), ca 27h for “React_1014 - translation” and 39h for “React_1788 - transcription” (see Fig. 2). This suggests that the regulation of these reaction pathways is strictly organised. G2/M cell levels also correlate inversely with “React_1725 - M/G1 transition”, which is not understood.

Our data point out that a full carcinogenic compound like BaP, with a genotoxic component through the induction of DNA adducts and mutations and a non-genotoxic component via the *AhR* transcription factor, may also influence many other reactions / pathways that affect the carcinogenic process. Examples are the various DNA repair processes (BER, TC-NER and GG-NER), gene expression process (e.g. metabolism of non-

coding RNA, which includes miRNAs), and many signalling pathways (see Fig. 2). The factual relevance of these modulations for cancer formation is not clear yet, which thus represents a challenge for future investigations to further unravel the chemical carcinogenesis process.

Effects of BaP on gene expression changes as measured by DNA microarrays in HepG2 cells have been published before, but on maximally 4 time points (19,20,28,44). This study, however, is the first with so many time points in a toxicogenomic evaluation of a chemical carcinogen. Therefore, our data set may serve as benchmark for others, in order to compare with responses induced by other carcinogenic compounds or in other cells or organisms, to develop new bioinformatic tools for time-series analyses, functional annotation and correlation analyses of transcriptome sets with phenotypic parameters, etc.

In the current detailed time series study, numerous temporal changes in clusters of genes upon exposure of cells to the carcinogen BaP have been observed, thereby providing an unprecedented insight in time-dependent interactions of the affected processes and pathways. As could be expected, TCDD responsive genes, many of them regulated through *AhR*, are those showing the earliest effect. Most interesting are the slower effects, however, such as on DNA repair. Transcription-coupled nucleotide excision repair lasts ca 18 h, until $\pm 50\%$ of the DNA adducts have been repaired, whereas global genomic nucleotide excision repair and base excision repair is upregulated up to 60 h, at which time point adducts are still present. To our knowledge, this transcriptional regulation of various DNA repair pathways upon exposure to a genotoxic stressor, represents a novel finding. The data also suggest that when S-phase cell levels and DNA adduct levels are high, the metabolism of nucleotides is low. After S-phase levels decrease and those for G2/M increase, this general metabolism is high. Once cell cycle distribution has returned to normal and most adducts are repaired, metabolic processes also return to normal levels. Also noteworthy are the sequential periods during which several reaction pathways in gene expression, transcription and translation are upregulated. All these new observations indicate that the cellular transcriptome is strictly regulated, with main responses once DNA damages are generated, and adapts to the changing needs of cells following acute exposure to a carcinogen.

Due to the temporal dimension, this study of changes in gene expression profiles at many time points after exposure to a chemical carcinogen provides more and more robust insight in the affected molecular processes and mechanisms than any previous study. This knowledge will aid in further understanding chemical carcinogenesis and in identifying the carcinogenic properties of chemicals.

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Chapter 3

Assessing the Metabolic Competence of Sandwich-Cultured Mouse Primary Hepatocytes

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Abstract

Primary human and rat hepatocyte cultures are well established *in vitro* systems used in toxicological studies. However, while transgenic mouse models provide an opportunity for studying mechanisms of toxicity, mouse primary hepatocyte cultures are less well described. The potential usefulness of a mouse hepatocyte-based *in vitro* model was assessed in this study by investigating time-dependent competence for xenobiotic metabolism and gene expression profiles. Primary mouse hepatocytes, isolated using 2-step collagenase perfusion, were cultured in a collagen sandwich configuration. Gene expression profiles and the activities of various Cyp450 enzymes were determined after 0 h, 42 h and 90 h in culture. Principal component analysis of gene expression profiles shows that replicates per time point are quite similar. Gene expression levels of most phase I biotransformation enzymes decrease to ca 69 % and 57 % of the original levels at 42 h and 90 h, respectively, while enzyme activities for most of the studied Cyp450s decrease to 59 % and 34 %. The decrease for phase II gene expression is only to 96 % and 92 % of the original levels at 42 h and 90 h, respectively. Pathway analysis reveals initial effects at the level of proteins, external signaling pathways and energy production. Later effects are observed for transcription, translation, membranes and cell cycle related gene sets. These results indicate that the sandwich-cultured primary mouse hepatocyte system is robust and appears to maintain its metabolic competence better as compared to rat hepatocytes.

Introduction

Toxicological studies designed to assess safety and possible toxicity of compounds in human populations predominantly rely on the use of animal systems. As animal experiments are expensive, time-consuming and connected with ethical drawbacks, numerous attempts have been made to find a reliable way of predicting *in vivo* toxicity in humans by means of *in vitro* models in recent years.

Traditionally, since the liver plays a major role in the metabolism of many compounds and also represents an important target organ in systemic toxicity, hepatic models are frequently used as an *in vitro* alternative in pharmacological, toxicological and metabolic studies (1, 2). Hepatic cell lines, primary hepatocyte cultures and precision-cut liver slices from various species are well established *in vitro* systems for these studies (1, 2, 3). As precision-cut liver slices have a very short live span and immortalized cell lines have lost many liver specific functions, primary cultures of hepatocytes are preferentially used for *in vitro* studies on liver toxicity (3, 4, 5).

Primary mammalian hepatocytes largely retain their liver-specific functions when freshly derived from the animal. Long-term cultures of functional hepatocytes however are difficult to establish. To increase the longevity and maintain differentiated functions of hepatocytes in primary cultures, cells are cultured in a sandwich configuration of collagen-collagen and in serum-free culture medium (6). Hepatocytes cultured in a sandwich configuration reorganize to form an architecture similar to that found in the liver and are able to form functional bile canalicular networks and gap junctions. In such a sandwich configuration, hepatocytes can be cultured for a longer time period compared to cultures on single layers of collagen (7, 8, 9, 10).

The use of primary human hepatocytes is preferential in order to predict *in vivo* toxicity in humans but is hampered by the limited availability of donor material and the large variability between the donors (3). Therefore, primary hepatocytes isolated from other mammals are used as an alternative to human tissue. However, the rapid decline in liver specific functions, in particular cytochrome P450 (Cyp450) enzyme activity, in rat liver *in vitro* systems limits the use of these in studies for testing chemicals for which metabolism depends on the Cyp450 enzyme system (9, 11). Human hepatocytes also tend to show a decrease in most Cyp450 enzymes, but some levels of Cyp450 gene expressions can be restored after a few days (12, 13). A mouse *in vitro* hepatocyte system might be an alternative to the rat and human systems, especially if the metabolic competence is preserved. In addition, since the complete sequence of the mouse genome is known (14) and transgenic mouse models are widely available, primary mouse hepatocytes are usable for the purpose of performing mechanistic investigations of liver toxicity. However, information about stability of liver specific functions in mice is scarce.

It was the aim of the current study to investigate the robustness of a mouse hepatocyte-based *in vitro* model, especially with respect to the biotransformation functions (evalu-

ated by assessing gene expression and enzyme activities) in order to get more insight in its potential usefulness in toxicology.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin/streptomycin, Hanks' calcium- and magnesium-free buffer, insulin and Trizol were obtained from Invitrogen (Breda, The Netherlands). Glucagon, hydrocortisone (50-23-7), collagenase type IV, dimethylsulfoxide (DMSO; 67-68-5) and Trypan blue (72-57-1) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Collagen Type I Rat Tail was obtained from BD BioSciences (Bedford, MA).

The RNeasy minikit was obtained from Qiagen, Westburg B.V. (Leusden, The Netherlands). The 5x MegaScript T7 Kit was obtained from Ambion (Austin, TX). The GeneChip® Expression 3'-Amplification Two-Cycle cDNA Synthesis Kit and Reagents, the Hybridization, Wash and Stain Kit and the Mouse Genome 430 2.0 Arrays were purchased from Affymetrix (Santa Clara, CA). The CYP450 AL4 cocktail was delivered by Advanced In Vitro Cell Technologies (Valencia, Spain).

Animals

Permission for animal studies was obtained from the Animal Ethical Committee. Adult male C57BL/6 mice (Charles River), weighing 20-25 g, were obtained from Charles River GmbH, Sulzfeld, Germany. This mouse strain was chosen because it is frequently used in toxicological and pharmacological investigations, and it is a common background for transgenic mouse strains. The animals were housed in macrolon cages with sawdust bedding at 22°C and 50-60% humidity. The light cycle was 12 h light/12 h dark. Feed and tap water were available *ad libitum*.

Isolation of hepatocytes

Hepatocytes were isolated from adult male C57BL/6 mice by a two-step collagenase perfusion method according to Seglen and Casciano (15, 16), with modifications. The liver was perfused after cannulation of the abdominal inferior vena cava. The hepatic portal vein was cut through and the thoracic inferior vena cava was occluded with forceps. The organ was washed with Hanks' calcium- and magnesium-free buffer for 3 min. After the liver had been freed of blood the calcium-free buffer was replaced by a collagenase buffer (0.5 mg/ml) for 7-10 min. A perfusion rate of 7 ml/min and a temperature around 39°C was maintained for both perfusates during the entire procedure. After the perfusion had been terminated, the liver was rapidly excised from the body cavity and transferred to a sterile Petri dish. The gall bladder and remnants of the dia-

phragm were removed and cells were released by disrupting the liver capsule mechanically and by shaking the cells into attachment medium. The cells were separated from undigested tissue with a sterile 50- μ m mesh nylon filter. After washing by low-speed centrifugation at 50 g for 3 min at 4°C several times, cell viability and yield were determined by trypan blue exclusion.

Cell culture

Cells from three independent biological replicates with viability >85% were used and cultured on collagen gel precoated 6-well plates at a density of 6.5×10^5 cells/ml (17). Cells were allowed to attach for 2-4 h at 37°C in a humidified chamber with 95%/5% air/CO₂ in DMEM supplemented with 10% FCS, insulin (0.5 U/ml), glucagon (7 ng/ml) and 2% penicillin/streptomycin (5000 U/ml penicillin; 5000 μ m/ml streptomycin). After attachment, medium containing unattached cells and debris was removed by washing and the cultures were overlaid with a second collagen layer to form a collagen-collagen sandwich culture. Primary cultures of mouse hepatocytes were cultured at 37°C in a humidified chamber with 95%/5% air/CO₂ in serum-free culture medium supplemented with insulin 0.5 U/ml, glucagon (7 ng/ml), hydrocortisone (7.5 μ g/ml) and 2% penicillin/streptomycin (5000 U/ml penicillin; 5000 μ m/ml streptomycin). Cells from three independent biological experiments (each from a different animal) were harvested at 0, 42 and 90 h after isolation for gene expression analysis.

RNA isolation

Total RNA was isolated from cultured mouse hepatocytes using Trizol reagent with the RNeasy kit according to the manufacturer's protocol. Total cellular levels were measured on a spectrophotometer and the quality of each RNA preparation was determined with a bio-analyzer (Agilent Technologies, The Netherlands). Extracted RNA was stored at -80°C.

Target preparation and microarray hybridization

cRNA targets were prepared according to the Affymetrix protocol. The targets were hybridized according to the manufacturer's recommended procedures on high-density oligonucleotide genechips (Affymetrix Mouse Genome 430 2.0 GeneChip arrays). The genechips were washed and stained using a fluidics station by Affymetrix and scanned in an Affymetrix GeneArray scanner.

A total of nine targets (3 time points from 3 animals) were prepared, and each preparation was analyzed using one GeneChip array. Normalization quality controls, including scaling factors, average intensities, present calls, background intensities, noise, and raw Q values all were within acceptable limits. Hybridization controls BioB, BioC, BioD, and CreX, were called present on all chips and yielded the expected increases in intensities.

Data analysis

Nine datasets were obtained from this experiment. The raw data were imported into ArrayTrack (18) and converted and normalized by Robust Multi-array Average (RMA, integrated into ArrayTrack) (19). Spots of poor quality and control spots were omitted. Subsequently, the remaining probe sets (25,084) were logarithm (base 2) transformed. Processed and normalized gene expression values were evaluated for identifying differentially expressed probe sets with the multiclass significance analysis of microarrays (SAM, integrated into ArrayTrack; p -value < 0.05) (20). Multiclass SAM resulted in 6560 remaining differentially expressed probe sets and these were used for principal component analysis (PCA).

Pathway analysis

Gene expressions at 42 vs 0 h, 90 vs 0 h and 90 vs 42 h were uploaded in T-profiler (<http://www.t-profiler.org>; 21) to identify transcriptional regulation of biochemical pathways and biological processes which included genes sets from Gene Ontology (GO) terms, curated gene sets, Motifs and Kyoto Encyclopedia for Genes and Genomes (KEGG) pathways. T-profiler uses the T test to score the difference between the mean expression level of predefined groups of genes and that of all other genes without any statistical pre-selection of modulated genes (21). Significance was determined by generating an E value, a Bonferroni corrected P value. Pathways and processes were determined significant if E values were below 0.05, and were thereupon used for hierarchical clustering and gene grouping in GenePattern (<http://www.broad.mit.edu/cancer/software/genepattern/>).

In order to increase specificity of the transcriptomics changes, the 6560 differentially expressed probe sets, determined by Multiclass SAM, were uploaded into MetaCore (GeneGo, San Diego, CA) for identifying the involvement of differentially expressed genes in specific cellular pathways by overrepresentation analyses compared to the total amount of genes involved in the particular pathways.

Analysis of CYP450s enzyme activities

Cyp450 activity assays at 0 h, 42 h and 90 h were conducted by the direct incubation of three independent sandwich-cultured mouse hepatocyte preparations with a cocktail of Cyp450 substrates (22). Substrates mixture stock was prepared in DMSO, and conveniently diluted in incubation media to obtain the optimal assay concentrations (Table 1). The final concentration of DMSO during incubation was 0.5% (v/v). After 2h of incubation enzymatic reactions were stopped by aspirating the incubation medium. Samples were subsequently centrifuged at 2500 rpm for 5 min. The supernatants were transferred to clean tubes and frozen at -80°C until analysis. Sample analysis and cocktail preparation was carried out in the Unidad Mixta Hospital La Fe-Advancell, Valencia, Spain. Samples were subjected to hydrolysis before analysis by incubation with

β -glucuronidase and arylsulfatase for 2h at 37°C. One volume of ice-cold acetonitrile was added and centrifuged samples were transferred to clean vials for further analysis. Metabolites formed and released into the culture medium were quantified by high performance liquid chromatography tandem mass spectrometry (23). Enzymatic activities were expressed as pmol of metabolite formed/incubation time/number of cells. For the statistical analysis the T-Test was used with a significance level of 5%.

Table 1 Substrates in the CYP450 AL cocktail for measurement of CYP450 activities.

CYP	Substrate	Concentration (μ M)	Metabolite
1A2	Phenacetin	10	Acetaminophen
2A4	Coumarin	5	7-HO-Coumarin
2B9/10/13/19/23	Bupropion	10	HO-Bupropion
2C37	Mephenytoin	50	4'-HO-Mephenytoin
2C39-40/44/50/54-55/65-70	Diclofenac	10	4'-HO-Diclofenac
2D9-13/22/26/34/40	Bufuralol	10	Hydroxybufuralol
2E1	Chlorzoxazone	50	6-HO-Chlorzoxazone
3A11	Midazolam	5	1'-HO-Midazolam

Results

Principal component analysis of all genes

A PCA on the 6560 differentially expressed probe sets, identified with the multiclass SAM analysis (p -value < 0.05), was used to visualize inter-individual and time-dependent differences in gene expression profiles of primary mouse hepatocytes cultured in sandwich configuration (Figure 1). PCA shows a clear discrimination between the three time groups and only limited differences between replicates of the 42 h and 90 h groups are observed, while more variance in gene expression is seen between the 0 h replicates.

Pathway analysis

T-profiler was used for identifying gene groups that were differentially expressed. The gene groups significantly altered in hepatocytes from at least two experiments and modified in the same direction in liver cells from all experiments were selected for each time period. In total, 384 gene sets were identified as being modulated.

Hierarchical clustering was used to generate more comprehensible groups of these genes (Figure 2). Most gene sets were either only downregulated or only upregulated. A small amount of these gene sets appeared to be upregulated during the first 42 h, and downregulated in the period from 42 to 90 h.

An additional analysis of affected cellular pathways was conducted using MetaCore, which showed similar results compared to the results from T-profiler (data not shown). Most prominent pathways significantly altered in MetaCore were involved in several catabolic, metabolic and biosynthetic processes, immune responses and cellular, nuclear and macromolecule related processes.

Figure 1 Principal component analysis using the data of all differentially expressed genes from primary mouse hepatocytes at 0, 42 and 90 h cultivation.

Biotransformation enzymes

Gene expression and activity of phase I and phase II drug-metabolizing enzymes were quantified to assess the metabolic capacity of the primary mouse hepatocyte system (see Table 2 and Table 3). For the majority of the Cyp450 genes, the gene expression level decreased during culturing. For a small group of Cyp450s, however, the expression levels remained relatively constant while for an even smaller group, gene expression levels increased in all time periods.

For the phase II genes, large variations were seen in their expression levels between the different time points, even within groups of phase II genes belonging to the same family. Most phase II enzyme groups were down-regulated or hardly changed over time. Only genes involved in glutathione metabolism and N-acetyltransferases were generally up-regulated.

Cyp450 enzyme activities of 8 selected enzymes were measured in each sample. These activities are presented as Log2 mean $\pm$ SD values in Figure 3. Enzyme activities for Cyp2a4 and Cyp2a1 were not detected. For the majority of the studied Cyp450 en-

zymes, a significant decrease in enzyme activity was measured in both time periods. Only one group of enzymes (Cyp2B9-10/13/19/23) showed an increase in activity and expression levels. After 90 h in culture, all Cyp450 enzymes remained active in the primary hepatocytes.

These Cyp450 enzyme activities and their gene expression are presented in Figure 3. In all cases gene expression and enzyme activity changed in the same directions, and mostly in the same order.

Figure 2 Hierarchical clustering of gene sets significantly modified in primary mouse hepatocytes in the time periods from 0 to 42 h, from 42 to 90 h and from 0 to 90 h. Clustering is based on the t-values, as provided by T-profiler. Yellow and blue mean up- and downregulation, respectively.

Table 2 *Expression of Cyp450 genes in primary mouse hepatocytes at 42 and 90 h as percentages of the expression at 0 h.*

GENENAME	42h	90h
Cyp17a1*	2659	107
Cyp1a1	119	117
Cyp1a2*	29	7
Cyp1b1	109	104
Cyp20a1	155	213
Cyp21a1	114	108
Cyp26a1*	24	10
Cyp27a1*	66	56
Cyp2a12*	65	128
Cyp2a4-5	42	71
Cyp2b10	1546	1460
Cyp2b13	102	95
Cyp2b19	90	86
Cyp2b9	126	110
Cyp2c29*	32	13
Cyp2c37	24	1
Cyp2c37-50-54*	19	1
Cyp2c38	54	65
Cyp2c44*	27	2
Cyp2c55*	50	16
Cyp2c70*	75	39
Cyp2d10*	88	59
Cyp2d13*	40	20
Cyp2d22*	74	33
Cyp2d26*	69	56
Cyp2d9*	59	22
Cyp2e1*	56	3
Cyp2f2*	22	4
Cyp2g1*	40	37

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Cyp2j5*	10	6
Cyp2j6*	99	83
Cyp2r1	103	76
Cyp2s1*	266	83
Cyp2u1	76	80
Cyp39a1*	197	421
Cyp3a11*	77	76
Cyp3a13*	203	175
Cyp3a25*	21	18
Cyp3a41*	66	65
Cyp3a44*	82	77
Cyp46a1*	83	67
Cyp4a10*	32	38
Cyp4a12*	16	1
Cyp4a14*	19	7
Cyp4b1	202	100
Cyp4f13	70	58
Cyp4f14*	19	5
Cyp4f15*	26	9
Cyp4f16*	381	341
Cyp4v3*	41	22
Cyp51	115	108
Cyp7a1	76	42
Cyp7b1*	17	12
Cyp8b1*	109	7
	69	57

*

Significant ($p < 0.05$) in multiclass SAM

Table 3 Expression of phase II genes in primary mouse hepatocytes at 42 and 90 h as percentages of the expression at 0 h.

GENENAME	42h	90h
Aldo-keto reductases		
Akr1a4	92	101
Akr1b3*	208	321
Akr1b7*	267	285
Akr1b8	131	127
Akr1c12	96	85
Akr1c13	103	107
Akr1c14*	29	47
Akr1c19	125	84
Akr1c20*	27	22
Akr1c21*	86	71
Akr1c6*	4	0
Akr1d1*	20	13
Akr1e1	112	103
Akr7a5	93	83
Sulfotransferases		
Chst10	81	72
Chst11	92	81
Chst4	83	80
Chst9	98	95
D4st1*	151	239
Gal3st1	175	128
Gal3st4	96	81
Hs1bp3*	120	205
Hs2st1*	167	229
Hs3st1	93	88
Hs3st2	88	75
Hs3st3b1*	88	76
Hs3st6	83	73

Hs6st1*	86	59
Hs6st2	94	89
Ndst1	99	100
Ndst2	165	113
Ndst3	94	92
Ndst4	93	84
Sult1a1	110	119
Sult1b1*	67	18
Sult1c2	76	50
Sult1d1*	457	86
Sult1e1*	1231	397
Sult5a1*	67	60
Epoxide hydrolases		
Ephx1	140	133
Ephx2*	47	35
Flavin-Containing Monooxygenase		
Fmo1*	31	19
Fmo2	102	96
Fmo3	99	84
Fmo4	133	108
Fmo5	369	96
Glutathione reductases		
Gsr	146	129
Gss*	153	329
Glutathione peroxidases		
Gpx1*	70	47
Gpx4*	139	161
Gpx6*	58	44
Gpx7*	113	222
Glutathione transferases		
Gsta1-2*	526	409
Gsta2*	194	142

Gsta3*	39	42
Gsta4*	82	47
Gstm1	113	127
Gstm2*	364	383
Gstm3*	378	593
Gstm4	127	125
Gstm5*	197	205
Gstm6	110	85
Gstm7*	64	51
Gsto1	117	97
Gsto2	115	115
Gstp1	98	107
Gstt1*	49	38
Gstt2	78	105
Gstt3	69	55
Gstz1*	94	92
Mgst1*	63	60
Mgst2*	94	76
Mgst3	141	150
N-acetyltransferases		
Gcnt1	110	107
Gcnt2	76	63
Nat10*	153	239
Nat11	114	174
Nat12	145	196
Nat2*	179	122
Nat5	240	332
Nat6	82	114
Nat9	134	135
Glucuronosyl transferases		
Ugt1a1-2-5-6a-6b-7c-9-10*	59	38
Ugt2a3*	30	4

Ugt2b1*	13	1
Ugt2b34	127	112
Ugt2b35	99	147
Ugt2b36*	42	59
Ugt2b37*	40	28
Ugt2b38*	11	5
Ugt2b5*	35	39
Ugt3a1*	20	6
Ugt3a2*	26	4
	96	92

* Significant ($p < 0.05$) in multiclass SAM

Figure 3 Cyp450 enzyme activities and gene expression in primary mouse hepatocytes after 0, 42 and 90 h in culture. Values are represented as percentages from activities and gene expression 0 h and as means $\pm$ SD of 3 animals for each time point.

*Significant (P value < 0.05) compared to 0 h.

#Significant (P value < 0.05) compared to 0 and 42 h.

Discussion

As human and rat sandwich-cultured hepatocytes have their disadvantages because of the limited availability of donor material and/or a rapid decline in Cyp450 enzyme activities, primary mouse hepatocytes may be an option to overcome this shortcoming (3, 11). Moreover, the growing availability of transgenic models can be a reason to use mouse primary hepatocytes. As information on the stability of liver specific functions in mouse hepatocytes is scarce, the present study investigated the stability of a mouse hepatocyte-based *in vitro* model, especially with respect to the biotransformation functions (gene expression and enzyme activities), in order to get more insight in its potential usefulness in toxicology.

PCA was used to elucidate changes in gene expression profiles between donor animals and between cultivation times. This revealed that profiles are very different between the three time points. These time-dependent changes can be attributed to the adaptation of the primary hepatocytes to their *in vitro* environment. Noteworthy, the hepatocytes at time point 0 h show larger inter-animal differences compared to those at later time points. This observation might be caused by initial stress induced by the enzymatic separation and isolation of hepatocytes (24). This inter-animal variability is less clear in the later time points, indicating that the stress responses are fading.

Pathway analysis, used to identify biochemical pathways and biological processes that were differentially expressed, showed again that differences exist between the two cultivation periods. Some gene sets were affected in both periods, whereas others in only the first 42h or only in the 48h thereafter. During only the first 42 h in culture, gene sets in mitochondrial processes, hormonal processes and chemotaxis were downregulated, whereas those in protein related processes are upregulated during only this period. Pathways mostly affected after the first 42h are downregulation of membrane related processes, and upregulation of transcription and replication and cell cycle related processes. Gene sets that are continuously downregulated are on humoral and cell mediated immunity, CYP450 related responses and liver-specific genes. A continuous upregulation is observed in nuclear and nucleic acid related metabolism, cellular organization, and in parts of cell cycle related processes. The overall picture based on these pathway analyses, is that liver-specific functions are continuously decreasing and that gross cellular organizations are continuously upregulated. Initial effects are more at the level of protein synthesis, external signaling pathways and energy production, while later on transcription, translation, membranes and cell cycle related gene sets are affected.

Microscopic observations showed that in this 42 h period, cells were changing their periphery, cellular components and structures, and forming aggregates (not shown). These morphological changes at microscopic level confirm previously described studies on human hepatocytes and on the histology of rat and mouse hepatocytes. The morphological changes include attachment to the collagen layer, the remodeling of sandwich-

cultured hepatocytes to a polygonal shape, the appearance of a clear, less granular, cytoplasm and the aggregation of the cells including the formation of gap junctions for intercellular communication (6, 25, 26, 27). Membrane and mitochondrial changes have also been demonstrated before in freshly isolated hepatocytes and hepatocytes cultured for 3 days (28, 29). Unfortunately, all these morphological changes were poorly represented by changes at the level of pathways as analyzed by T-profiler or MetaCore. These morphological changes were perhaps too specific and the annotation of genes with respect to that is too limited. In human and rat hepatocytes cultured in a sandwich configuration, others have found genes and pathways involved in cell structure to be highly expressed after 24 h in culture (30, 31, 32, 33).

To investigate the metabolic competence of sandwich-cultured mouse hepatocytes in more detail, gene expression profiles from phase I and phase II biotransformation enzymes were evaluated during the cultivation period. In general the expression values of all phase I biotransformation enzymes are decreasing over the whole cultivation period. During the first 42 h these phase I enzyme gene expression values are decreasing by 31% (ca 69 % remaining) from the original values at 0 h. After 90 h in culture the hepatocytes exhibit clearly lower levels (ca 57%) of Cyp450s compared to the original values at 0 h.

It was described before that many cultures of mammalian hepatocytes exhibit low levels of Cyp450 but still possess the ability to respond to Cyp450 inducers (34). The expression of Cyp1a1 and Cyp1a2 during cultivation has been studied in detail by Northern blotting (35). In primary mouse hepatocytes cultured in dishes without collagen, Cyp1a1 expression increased during cultivation. Cyp1a2 was constitutively expressed in these cells, but insulin suppressed this expression slightly. Our results show a steady level in Cyp1a1 gene expression and a down-regulation in Cyp1a2 gene expression. In human hepatocytes, most Cyp450s also showed a decrease in expression levels with the exception of a few Cyp450s which had elevated levels of expression (36). Cyp1A2 appeared to be more stable compared to the mouse model during cultivation, while Cyp2E1 and Cyp3A4, which is the homologue for Cyp3a11 in mice (37), were shown to be decreasing in time, comparable to our results (38). Most Cyp450s in conventional sandwich-cultured rat hepatocytes appeared to be decreasing in a time-dependent manner (10). Even within the short time period of 24 h after isolation, the expression levels of many Cyp450s decreased tremendously in rat hepatocytes (9). While in our study gene expression levels showed a slower decrease over time indicating that mouse hepatocytes remain more stable with respect to Cyp450 gene expression as compared to rat hepatocytes but not to human hepatocytes.

As several rat studies suggest that phase II enzyme activities are better preserved in culture than phase I enzyme activities, we decided to only measure enzyme activities of most important phase I enzymes involved in xenobiotic biotransformation to strengthen our gene expression results (39, 40). These results show a general decrease in Cyp450

enzyme activity, with only one exception. Activity levels were decreasing the first 42 h to 59% from the original activities. After 90 h, activity levels were decreased to 34 % in comparison to 0 h. Compared to our results, Cyp450 enzyme activities in rat hepatocytes are decreasing very rapidly. Already after 6 h, less than 50% of the initial Cyp450 enzyme activity was left in rat hepatocytes (41, 42). These rat hepatocytes, however, were cultured on a collagen monolayer, which may affect the stability of the cells.

Phase II biotransformation enzymes demonstrate a less clear pattern. Enzymes were either up- or down-regulated or remain unchanged. Even within a group of enzymes, differences in gene expression can be seen. In general, the expression levels are decreasing to 96 % and 92 % as compared to levels at 0 h after 42 h and 90 h, respectively. In rat hepatocytes, however, phase II enzymes in general showed a decrease in gene expression level after 72 h compared to gene expression in liver (10). For rat hepatocytes, it is known that the phase II enzymes are better preserved in culture than phase I enzymes (39, 40). Our data confirm these findings, thus showing that mouse hepatocytes are also stable in expression of genes encoding phase II enzymes.

An overall comparison of the gene expression levels from phase I and phase II enzymes from rat and mouse hepatocytes demonstrated that mouse hepatocytes exhibit a more stable gene expression pattern than primary rat hepatocytes.

Conclusion

In summary, our findings show that the sandwich-cultured primary mouse hepatocyte *in vitro* model has little or no inter-animal variations, demonstrating its reproducibility. The system is still metabolic competent even after 90 h culturing, despite that phase I enzyme gene expression and activity are generally decreasing. In rat hepatocytes, this decrease in phase I and II gene expression appears to be faster. Human hepatocytes, on the other hand, also show a decrease in these phase I enzymes, but are able to restore the gene expression levels of some Cyp450s. Taken together, our results indicate that the primary mouse hepatocyte system is relatively stable and might maintain its metabolic competence longer than rat hepatocytes, and roughly similar to that of human hepatocytes. Based on our gene expression data, we recommend applying a 42 h recovery period before using the mouse hepatocytes.

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Chapter 4

The Relevance of Primary Mouse Hepatocytes for Toxicity Profiling: Responses by Benzo(a)Pyrene

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Abstract

The availability of transgenic models has advanced mice as useful experimental models for assessing chemical safety. Consequently, mouse *in vitro* models are also gaining interest in the field of toxicology. Here, we aim to further determine the relevance of the sandwich-cultured primary mouse hepatocyte model for assessing genotoxic and carcinogenic properties of chemicals by investigating time- and dose-dependent effects on gene expression profiles caused by benzo(a)pyrene (BaP). Furthermore, the data were compared with transcriptomic responses in liver from mice exposed to BaP.

Primary mouse hepatocytes from male C57BL/6 mice were treated for 12, 24, 36 or 48h with various non-toxic concentrations of BaP (0, 10, or 30 μ M). As phenotypic markers for genotoxic effects, immunostaining of γ H2AX foci was used to detect double-strand DNA (dsDNA) breaks and 32 P-postlabeling was applied for analyzing the presence of BaP-DNA adducts. Whole genome gene expression modifications were analyzed by means of Affymetrix mouse genome 430 2.0 microarrays. Datasets were normalized using RMA and analyzed using SAM, MetaCore and T-profiler.

Levels of both DNA adducts and dsDNA breaks increased time- and dose-dependently. Furthermore, the number of significantly modulated gene expressions and pathways increased with time and dose. Genes and pathways significantly altered after exposure to BaP, appear mainly involved in biotransformation, metabolism, response to DNA and cell damage, cell survival, and tumor development and progression.

The results from this study indicate that primary mouse hepatocytes are able to generate expected transcriptomic effects of BaP in a time- and dose-dependent manner. *In vitro* – *in vivo* comparison revealed some overlapping transcriptomic responses to BaP, which are of interest, and suggest that primary mouse hepatocytes might represent a relevant *in vitro* model for toxicogenomics studies.

Introduction

Since mouse models are used frequently in toxicological studies, interest is raised in the relevance of *in vitro* mouse models, such as for liver. Where rodent bioassays have been accepted for the assessment of chemical toxicity, mice in particular have advantages because of the availability of transgenic models (1).

Because the liver is an important organ for the metabolism and biotransformation of many toxic compounds and consequently represents a target organ for toxicity, hepatic models are widely used as an *in vitro* alternative in pharmacological, toxicological and metabolic studies (2, 3). Well-established hepatic *in vitro* systems comprise hepatic cell lines, primary hepatocyte cultures and precision-cut liver slices from various species (2, 3, 4). The use of primary human hepatocytes is considered as the “gold standard” but this is hampered by a limited availability of donor material (4). Therefore, primary mouse hepatocytes may be considered as an alternative. Sandwich-cultured hepatocytes are in favor compared to other hepatocyte-based *in vitro* models, because the longevity and differentiated functions of hepatocytes in primary cultures are maintained and this configuration has been shown to maintain *in vivo* characteristics, like cellular polarity and the distribution of actin filaments, for several weeks (5, 6, 7,8). Furthermore, the primary mouse hepatocyte system has been proven to be robust and appears to maintain its metabolic competence better as compared to rat hepatocytes (8).

Here, we aim to investigate time- and dose-dependent effects caused by benzo(a)pyrene (BaP), a well known carcinogen, in primary mouse hepatocytes and to determine the relevance of this hepatocyte model by comparing these results with data obtained from the liver of BaP-exposed C57BL/6 mice. The C57BL/6 mouse is a well-established mouse strain routinely used in toxicology, pharmacology and biomedical sciences.

Benzo[a]pyrene (BaP), a well-known carcinogen belonging to the class of polycyclic aromatic hydrocarbons (PAHs) with both genotoxic and non-genotoxic properties, was used in this study as the model compound. BaP binds to the Arylhydrocarbon receptor, and this complex thereupon translocates to the nucleus and induces the expression of several genes (9). An important group of these genes is represented by genes coding for Cytochrome P450 enzymes (e.g. Cyp1A1 and Cyp1A2) which are responsible for metabolizing BaP into its genotoxic dihydrodiol epoxide derivatives (e.g. benzo[a]pyrene-7,8-diol-9,10-epoxide). These intermediates can covalently bind to DNA, RNA or proteins. (10, 11) When these DNA adducts are not properly removed or repaired, mutations can occur during cell division and cancer development can be induced (12).

In order to protect cells from the deleterious effects of DNA damage, cells can repair the damaged DNA by a many different mechanisms, like nucleotide excision repair and base excision repair (13, 14). In order to allow more time for repair, cell proliferation can be blocked at several phases of the cell cycle, such as at G1-S transition, S-phase

and G2-M transition or apoptosis can be induced, especially at high DNA damage levels (15).

For the purpose of further determining the relevance of the *in vitro* primary mouse hepatocyte sandwich-cultured system, we investigated such dose- and time- effects of BaP by analyzing whole genome gene expression modification, and by associating these with analysis of DNA adduct formation and γ H2AX foci formation as relevant phenotypic parameters (16).

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), Hanks' calcium- and magnesium-free buffer, Alexa fluor 488 goat anti-mouse IgG antibody, insulin and Trizol were obtained from Invitrogen (Breda, The Netherlands). Glucagon, hydrocortisone, collagenase type IV, BaP, Trypan blue, dimethylsulphoxide (DMSO), bovine serum albumin (BSA), 4',6-diamidino-2-phenylindole (DAPI) and Tween-20 were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Triton X-100, NaCl, Na₂HPO₄·2H₂O and NaH₂PO₄ were obtained from Merck (Darmstadt, Germany) and paraformaldehyde from ICN biomedical (Aurora, Ohio). The anti-phospho-Histone H2A-X (ser139) Clone JBW 301 antibody was purchased by Upstate Biotechnology (Lake Placid, NY). Vectashield® Mounting Medium was supplied by Vector Labs (Burlingame, CA). Collagen Type I Rat Tail was obtained from BD BioSciences (Bedford, MA). The iScript cDNA Synthesis kit en iQ SYBR Green Supermix were purchased from BioRad (Hercules, CA). Primers were designed using Primer Express 2.0 from Applied Biosystems (Foster City, CA) and purchased from Operon (Cologne, Germany). The RNeasy minikit was obtained from Qiagen, Westburg B.V. (Leusden, The Netherlands). The 5x MegaScript T7 Kit was obtained from Ambion (Austin, TX). The GeneChip® Expression 3'-Amplification Two-Cycle cDNA Synthesis Kit and Reagents, the Hybridization, Wash and Stain Kit and the Mouse Genome 430 2.0 Arrays were purchased from Affymetrix (Santa Clara, CA).

Animals

Permission for animal studies was obtained from the Animal Ethical Committee. For the *in vivo* study all mice were housed specific pathogen-free (SPF) in a climate-controlled room. For the *in vitro* study adult male C57BL/6 mice (Charles River), weighing 20-25 g, were obtained from Charles River GmbH, Sulzfeld, Germany. The animals were housed in macrolon cages with sawdust bedding at 22°C and 50-60% humidity. In both studies the light cycle was 12 h light/12 h dark. Feed and tap water were available *ad libitum*.

In vivo treatment

Eight week old male C57BL/6 mice were treated by gavage with 13 mg/kg body weight BaP dissolved in sunflower oil. This dose was chosen to compare results with those from previously conducted *in vivo* studies on carcinogenicity in wild type and p53^{+/-} Xpa^{-/-} mice (17). During 7 days four animals were treated 4 times (at start of the experiment, day 2, day 4, day 6. with BaP or solvent. The animals were sacrificed at day 7. At autopsy, the liver was isolated from each animal and RNA was freshly isolated.

Primary hepatocyte culture and treatment

Hepatocytes were isolated from adult male C57BL/6 mice by a two-step collagenase perfusion method as described before (8). Cells were cultured in a collagen-sandwich configuration as described before (8). Prior to treatment, primary cultures of mouse hepatocytes were allowed to recover for 40-42 h at 37°C in a humidified chamber with 95%/5% air/CO₂ in serum-free culture medium supplemented with insulin 0.5U/ml, glucagon (7 ng/ml), hydrocortisone (7.5 µg/ml) and 2% penicillin/streptomycin. Culture medium was refreshed every 24 h. After the recovery period, the culture medium was replaced by culture medium containing 10 or 30 µM B(a)P; the highest dose is based on a cytotoxicity assay with 80% viability (MTT test, data not shown), or 0.5% DMSO as a vehicle control. Cells were incubated with 10µM BaP for 24 or 48 h and with 30 µM BaP for 6, 12, 24, 36 or 48 h before being used for detecting double-stranded (ds) DNA breaks or harvested for RNA and DNA isolation by adding Trizol reagent. Three independent experiments were conducted using cells from different mice.

Detection of dsDNA breaks. Detection of dsDNA breaks

Double-stranded DNA breaks were detected by immunostaining of the phosphorylated histone H2AX (γH2AX) as described before (18). Hepatocytes, used for the detection of dsDNA breaks, were cultured in a sandwich configuration on top of a cover slip. After desired exposure times, medium was aspirated and the cells were rinsed with 1x Phosphate-buffered saline (PBS) before fixation in 4% Paraformaldehyde for 10 min at ambient temperature. The cells were washed again with 1xPBS and made permeable with 0.2% Triton X-100 at 4°C for 5 min. Thereafter cells were washed two times with 1xPBS for 5 min and blocked during 1 h at 37°C with blocking buffer (1% BSA+0.5% Tween-20 in 1X PBS). Samples were washed two times with 1xPBS for 5 min and incubated with the first antibody (Mouse monoclonal γH2AX (Ser-139)) 1:1000 in dilution buffer (0.5% BSA + 0.5% Tween-20 in 1X PBS) during 2 h at 37°C. After two washing steps with 1xPBS cells were incubated with the second antibody (Alexa 488 labelled) 1:1000 in dilution buffer during 1 h at 37°C and washed again two times with 1xPBS. Samples were incubated with DAPI for 15 min at room temperature, dehydrated in series of ethanol solutions (70, 90 and 100%) for 3 min and air-dried. Vectashield® Mounting medium was used to stick the cover slips onto microscopic glass slides. Mi-

croscopic photographs were taken and samples were scored manually. A damage score, ranging from 0 to 4, determined by the grade of damage, was given to 100 cells per sample. The damage scores were multiplied by the amount of cells with that score and all together calculated to get a total percentage of damage in each sample. For the statistical analysis the T-Test was used with a significance level of 5%.

DNA isolation and ^{32}P -Postlabelling

DNA was isolated from primary mouse hepatocytes using Trizol reagent according to the manufacturer's protocol. DNA adduct levels were determined using the procedure described by Reddy and Randerath, with some modifications (19, 20). The DNA adduct levels were quantified by including samples with known BaP-DNA adduct levels. The detection limit was 1 adduct per 10^8 nucleotides. The radio-labeled adducted nucleotide biphosphates were separated by multi-directional thin layer chromatography (TLC). The adduct spots on the chromatograms were quantified using Phosphor-Imaging technology (FLA-3000, Fuji, France) and AIDA/2D densitometry software. The average and standard deviation was calculated for all three experiments and for the statistical analysis of time- and dose-dependent effects the one-way ANOVA was used with a significance level of 5%.

RNA isolation

Total RNA was isolated from the livers from the *in vivo* study and from cultured mouse hepatocytes using Trizol reagent with the RNeasy kit according to the manufacturer's protocol. RNA concentrations were measured on a spectrophotometer and the quality of each RNA preparation was determined with a bio-analyzer (Agilent Technologies, The Netherlands). Only samples with a good quality (clear 18S and 28S peaks and RIN>6) were used for hybridization. Extracted RNA was stored at -80°C until it was used as template for cDNA synthesis.

Whole genome gene expression analysis

Target preparation and hybridization

cRNA targets, prepared according to the Affymetrix protocol, were hybridized on high-density oligonucleotide gene chips (Affymetrix Mouse Genome 430 2.0 GeneChip arrays) according to the manufacturer's procedures. The gene chips were washed and stained using an Affymetrix fluidics station and scanned in an Affymetrix GeneArray scanner.

From the *in vivo* study eight samples were collected (from 4 treated animals and 4 controls) and from the *in vitro* study thirty samples were collected (from incubations in triplicate with 2 concentrations at 24 and 48 h, and with 1 concentration at 12 and 36 h as well as from controls at each time point).

A total of thirty-eight GeneChips was run. Normalization quality controls, including scaling factors, average intensities, present calls, background intensities, noise, and raw Q values, were within acceptable limits for all chips. Hybridization controls BioB, BioC, BioD, and CreX, were identified on all chips and yielded the expected increases in intensities.

Data analysis

Thirty-eight datasets were obtained from this experiment. Raw data were imported into ArrayTrack (21) and normalized using Robust Multi-array Average (RMA, integrated into ArrayTrack) (22).

Present-Marginal-Absent calls were used to identify and omit probe sets of poor quality (23). Subsequently, the remaining probe sets (24872 *in vitro* and 23011 *in vivo*) were logarithm- (base 2) transformed and corrected for their DMSO vehicle control as described below, and used for statistical analysis. The significance analysis of microarrays (SAM, integrated into ArrayTrack) (24) was used for identifying significant changes in expression levels (paired two class, fold change 1.5) and multiclass SAM was used for identifying dose- or time-dependent differentially expressed probe sets. ArrayTrack was also used for principal component analysis (PCA) and Euclidian (complete) hierarchical clustering.

Log2 of the ratios for treated-control were calculated for the *in vitro* data set. For the *in vivo* data set, the average of all controls was used as common control to calculate the log2 of the ratios for treated-common control. These two data sets were uploaded in T-profiler (25) for identifying transcriptional up- or down-regulation of biochemical pathways and biological processes, which included genes sets from Gene Ontology (GO), curated gene sets, Motifs and Kyoto Encyclopedia for Genes and Genomes (KEGG) pathways. T-profiler uses the T-test to score the difference between the mean expression level of predefined groups of genes and that of all other genes without any pre-selection of significantly modulated genes (25). Significance was determined by generating an E value, a Bonferroni corrected P value. Pathways and processes were significant when E values were below 0.05.

Interesting modulated probe sets, were further analyzed for functional annotation by MetaCore using the shortest path algorithm (GeneGo, San Diego, CA). The total list of probe sets as described before (24872 *in vitro* and 23011 *in vivo*) and were used as background list. A false discovery rate of 0.4 was used to select significant MetaCore maps.

Correlation analysis

Expressions of significantly modulated genes (as revealed by SAM), and significantly altered pathways (obtained by T-profiler) from primary mouse hepatocytes, that correlated with DNA adducts or γ H2AX foci formation, were identified by means of the gene expression profile analysis suite (GEPAS 4.0; CIPF, Valencia, Spain). Spearman corre-

lation coefficients were calculated and correlating genes and pathways were selected by $P < 0.05$, a false discovery rate of 0.32 and multiple testing correction (fwer.holm 1) and correlating genes were further analyzed for functional annotation by MetaCore as described above. A false discovery rate of 0.4 was used to select significant MetaCore maps.

Results

1. *In vitro* results

Detection of dsDNA breaks

The generation of foci of phosphorylated γ H2AX in primary mouse hepatocytes after 24 and 48 h exposure to 10 and 30 μ M BaP is shown in Figure 1. The number of foci showed a significant dose-dependent increase as more foci were formed in BaP-exposed cells compared to DMSO controls at all doses at both time points.

Figure 1 Levels of dsDNA breaks (γ H2AX foci) in primary mouse hepatocytes exposed BaP or solvent after 24 or 48 h (average with SD of 3 experiments)
 * Significant (ANOVA, p -value < 0.05) compared to solvent controls.

BaP-DNA adduct formation

The formation of DNA adducts in primary mouse hepatocytes treated with 10 and 30 μ M BaP was investigated by 32 P-Postlabelling. At both incubation concentrations, mainly one adduct spot was observed, coinciding with the reactive metabolite anti-benzo(a)pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide (BPDE) bound to the N^2 position of guanine. As shown in Figure 2, BaP exposure resulted in significant time- and concentration-dependent increase in DNA adduct formation. Only at 6 h of exposure, no significant dose-dependency was detected.

Figure 2 DNA adduct levels in primary mouse hepatocytes exposed to BaP for several periods as measured by 32 P-postlabelling. The mean number of DNA adducts per 10^8 nucleotides with SD (3 experiments) is shown.

* Significant influence of time (ANOVA, p -value < 0.05)

[#] Significant influence of dose (ANOVA, p -value < 0.05)

Gene expression analysis

Gene expression data were generated for 30 samples. Gene expression data from one of the 12h 30 μ M treatments could not be discriminated from the DMSO treated samples; therefore, these results were not used for further analyses. Analysis of the data by SAM resulted in a list of differentially expressed probe sets at each time point. We found 5 altered genes after 12 h of exposure to 30 μ M BaP, 11 and 627 genes after 24 h of exposure to 10 and 30 μ M BaP respectively. 10 out of 11 probe sets, differentially expressed after 24 h of exposure to 10 μ M were common with those from exposure to 30 μ M. After 36 h of exposure to 30 μ M BaP 337 probe sets were differentially expressed. After 48 h of exposure to 30 μ M BaP 693 were found to be differentially expressed and 92 out of 118 probe sets which were differentially expressed after 48 h of exposure to 10 μ M were common to those from treatment with 30 μ M BaP. Multiclass SAM re-

sulted in 127 time-dependent probe sets, and 16 and 68 dose-dependent probe sets for respectively 24 and 48 h. Combining all these differentially expressed probe sets at all time points resulted in a list of 1231 differentially expressed probe sets.

Principal component analysis of differentially expressed genes

To visualize time- and dose-dependent effects of BaP on gene expression, PCA was performed, thereby using the expression values of all 1231 differentially expressed probe sets (Figure 3a). The treated and control hepatocytes were discriminated by principal component (PC) #2, containing a variance of 28.872 %. PC #1, which contains most of the variance (33.485 %), discriminates the different time points. A minor discrimination between samples exposed to 10 or 30 μ M was observed. The log₂ expression ratios for the differentially expressed probe sets were also analyzed by PCA (Figure 3b). Early time points, 12 and 24 h were discriminated from the late time points, 36 and 48 h by PC#1 (37.128%). PC#2 (22.604%) discriminated dose groups, with the exception of 12 h treatments, which were grouped together with 10 μ M treatments of 24h.

Pathway analysis

T-profiler was used for identifying BaP-induced biochemical pathways and biological processes. Pathways and processes significantly altered in hepatocytes from at least two experiments and altered in the same direction in all replicates, were considered significant and selected for each time period. 7 pathways were significantly changed after 12 h exposure to 30 μ M BaP, which were all overlapping with those from 24 h exposure to 30 μ M BaP. After 24 h treatment with 30 μ M BaP 98 pathways and processes appeared significantly altered. Four out of the five significantly altered pathways by 24 h treatment with 10 μ M BaP were also responding at 30 μ M BaP. Exposure for 36 h with 30 μ M BaP resulted in 60 significantly altered pathways and processes. After 48 h, 95 and 115 pathways were significantly changed after treatment with 10 and 30 μ M, respectively. Only 36 of these were common between both dose groups. Comparison of the treatments with 30 μ M BaP for all time points is presented in Figure 4. This resulted in one common pathway. Both pathways significantly changed after 24 and 48 h exposure to BaP appeared to have the most time point unique pathways, and greatest overlap is observed between 36 and 48 h of exposure.

A

B

Figure 3 *Principal component analysis of in vitro experiments (all 30 chips) using (A) the expression values of the differentially expressed probe and (B) the log2 values of the expression ratios for the differentially expressed probe sets after exposure to BaP. The gray-scale of the symbols increases with exposure time*

Figure 4 Venn diagram representing the number of significantly modulated biochemical pathways and biological processes after treatment of primary mouse hepatocytes with 30 μ M BaP.

Hierarchical clustering was used to generate more comprehensible groups of these pathways (Figure 5). Pathways generated at early time points during BaP exposure seem to cluster separately from those at late time points, showing a certain time-dependent response. Gene sets involved in metabolism, catabolism and membrane- and extracellular related processes and immune responses were downregulated at the early time points and upregulated at the later time points. Gene sets involved in biotransformation and metabolism are mainly continuously upregulated as treatment with BaP elongated. Tumor development and progression, cell damage and nucleus and nucleic acid related process were mainly upregulated during the first 24 h of treatment, but were downregulated at later time points.

Figure 5 Hierarchical clustering of significantly altered pathways in primary mouse hepatocytes after treatment with BaP for 12, 24, 36 and 48 h, based on the *t*-values generated by T-profiler. Yellow and blue mean up- and downregulation, respectively.

Correlation analysis

In order to link gene expression changes with phenotypic effects, DNA adduct and γ H2AX foci levels, following BaP exposure, we performed Spearman's correlation analyses. DNA adducts formed at all time points of exposure and all incubation concentrations were correlated with the gene expression changes of all significantly modulated probe sets (1231 probe sets). This resulted in 489 correlating probe sets at $P < 0.05$. Correlation analyses of the γ H2AX foci formation at 24 and 48 h of BaP treatment for all concentrations with the gene expression changes, resulted in 189 correlating probe sets with $P < 0.05$. Of these, 140 probe sets were in common with that for the associations with DNA adduct levels.

These two lists of correlating probe sets were analyzed for enrichment in biological processes or pathways using MetaCore. This demonstrated that these genes are mainly involved in several metabolism processes, cell adhesion and organisation, cell signaling, responses to several stimuli, immune responses and apoptosis.

Table 1 Overlapping differentially expressed probe sets from BaP-exposed mouse hepatocytes in vitro and mouse liver in vivo.

GENENAME	LOCUSID	SPOTID	BaP in vitro										BaP in vivo				Same effect direction								
			12 h		24 h 10 μ M		24 h 30 μ M		36 h		48 h 10 μ M		48 h 30 μ M		Mouse 1	Mouse 2		Mouse 3	Mouse 4						
	exp1	exp2	exp1	exp2	exp3	exp1	exp2	exp3	exp1	exp2	exp3	exp1	exp2	exp3	exp1	exp2	exp3	exp3	16	7					
In common	20	18.18	1.28	2.37	4.24	1.73														20.5					
Percentage of all DEPs common	20	18.18	1.28	2.37	4.24	1.73														20.5					
Abcg5	27409	353693	0.72	0.76	0.58	0.63	1.03	0.55	1.10	0.76	0.26	1.34	2.54	1.34	0.83	0.56	1.13	0.89	1.36	0.81	0.77	0.65	0.95	x	
Bhlh1p1	68041	354993	-0.14	0.24	-0.15	-0.55	-0.41	-0.22	-0.85	-0.70	-0.69	-1.05	-0.81	-0.93	-0.92	-1.01	-0.79	-1.32	-0.96	0.85	0.91	1.04	0.45		
Mid1p2	20893	357340	-0.11	-0.31	-0.24	-1.25	-0.17	-0.76	-0.65	-0.16	-0.86	-0.85	-0.67	-0.91	-0.90	-1.25	-0.55	-0.91	-1.51	-1.12	0.80	0.70	0.85	0.46	
Fabp2	14079	358144	0.84	-0.20	1.12	1.60	1.24	1.38	1.19	0.97	1.76	1.06	1.91	1.84	0.57	1.05	1.95	1.49	1.83	0.60	0.78	0.60	0.66	x	
Lima1	65970	359850	-0.32	-0.07	-0.01	0.65	0.03	0.32	0.11	-0.33	-0.36	-0.29	-0.55	-0.33	-0.33	-0.81	-0.88	-1.03	-0.93	0.64	0.65	0.87	0.61		
Aco13	171281	360749	0.61	1.14	0.88	0.32	0.32	0.83	0.52	0.87	1.14	0.25	1.15	0.83	1.13	1.35	0.47	0.85	0.51	0.55	0.74	0.63	0.51	x	
Rassf5	54354	360148	-0.26	-0.38	-0.63	-0.13	-0.42	-0.87	-1.17	-0.90	-1.68	-0.27	-0.63	-0.25	0.50	-0.27	-1.27	-0.15	-0.73	1.08	1.13	0.66	0.65		
Cyp1a2	13077	374226	2.81	3.25	4.06	4.63	4.15	3.91	4.22	3.82	3.90	4.70	5.81	5.24	5.30	6.38	4.78	5.12	6.42	1.74	1.76	1.82	1.51	x	
Tiparp	99929	377169	1.93	2.43	2.30	1.48	1.67	2.28	2.17	1.87	2.41	2.37	1.58	1.80	0.26	0.91	2.04	2.04	2.22	0.72	0.58	0.61	1.10	x	
Phldb2	208177	382549	0.08	-0.63	0.30	0.21	-0.31	-0.19	0.21	-0.04	-0.74	-0.93	-1.76	-1.17	-0.85	-1.34	-1.84	-1.00	-1.54	0.46	0.80	0.56	0.63		
Lmo7	380928	382501	0.77	1.04	1.59	0.48	0.98	1.48	0.88	1.03	0.54	0.81	0.15	0.38	0.72	-0.01	0.32	0.58	0.33	0.72	0.91	0.70	0.96	x	
Lima1	65970	386374	-0.53	0.02	0.21	0.59	-0.16	-0.14	0.21	-0.09	-0.49	-0.32	-0.60	-0.93	-0.31	-0.98	-1.37	-0.63	-1.13	0.80	0.82	0.96	0.56		
E030004N02Rik	319905	387958	-0.24	-0.89	0.72	-0.07	-0.80	0.26	-1.22	-1.58	-2.08	-1.63	-1.46	-1.54	-0.30	-0.83	-2.87	-1.21	-1.63	1.11	0.74	1.02	0.86		
Dcamk13	245038	387975	1.96	2.30	2.02	1.05	1.17	2.07	1.36	0.82	1.66	2.37	1.75	2.99	1.81	1.30	2.21	1.72	1.44	1.07	1.27	0.99	0.95	x	
AK451557	102084	389254	0.19	-0.09	-0.01	-0.08	-0.23	0.03	0.47	-0.09	-0.31	-0.15	-0.56	-0.85	0.01	-0.18	-0.95	-0.58	-0.66	0.89	1.20	1.07	1.31		
Bact2	12014	390221	0.35	-0.97	-0.15	0.77	-0.69	-0.10	-0.39	-1.28	-1.20	-1.32	-1.04	-1.42	-0.32	-0.92	-1.79	-1.52	-1.50	0.72	0.47	1.19	1.45		

Significant genes are indicated in italic and bold

2. *In vivo* results

Gene expression analysis

Gene expression data were generated for liver samples from 8 animals. Analysis of the data by SAM resulted in a list of 78 differentially expressed probe sets.

Pathway analysis

T-profiler was again used for identifying BaP-induced alterations in biochemical pathways and biological processes. Pathways and processes significantly altered in at least two animals and in the same direction in all four replicates, were selected for each time period. Only 8 pathways were found to be significantly changed, mainly involved in liver injury and immune responses.

3. *Mouse in vivo-in vitro* comparison

Transcriptomics and T-profiler data from BaP-exposed primary mouse hepatocytes and from the livers of BaP-exposed mice *in vivo* were compared, both at the level of modulated genes and that of altered biochemical pathways and biological processes. Differentially expressed probe sets from BaP-exposed mouse hepatocytes *in vitro* (1231 probe sets) and mouse liver *in vivo* (78 probe sets) show an overlap of 16 probe sets (Table 1), only 7 probe sets had their effects in the same direction. Also probe sets from BaP-exposed mouse hepatocytes *in vitro* generated by multiclass SAM (127 time-dependent probe sets, and 16 and 68 dose-dependent probe sets for respectively 24 and 48 h) were compared with those from mouse liver *in vivo* and showed an overlap of only one probe set (Cyp1a2), with the same effect direction.

To compare the effects of BaP on biochemical pathways and biological processes, the T-profiler data were used. The selected gene groups from the hepatocytes (272 gene groups) were compared with those from the *in vivo* study (8 gene groups). Only 1 gene group appeared commonly expressed between BaP-exposed hepatocytes and the *in vivo* study (ICHIBA_GVHD). It was, however, down-regulated *in vitro* system and up-regulated *in vivo*.

Commonly shared probe sets between the two models were further analyzed for enrichment in biological processes or pathways by MetaCore. These probe sets appeared to have a predominant role in several metabolism processes, chemotaxis, chemokine related pathways, cell adhesion, immune responses and transport.

Discussion

As mouse *in vitro* models are gaining interest in the field of toxicology, it was the aim to further determine the relevance of the sandwich-cultured primary mouse hepatocyte model for assessing genotoxic and carcinogenic properties of chemicals by investigating time- and dose-dependent effects on whole genome gene expression caused by benzo(a)pyrene (BaP), a well known carcinogen, and comparing obtained results *in vitro* with liver responses generated in mice exposed to BaP *in vivo*. Primary mouse hepatocytes were treated with two doses and analyzed at multiple time points, to identify time- and dose-dependent effects on gene expression profiling. Furthermore effects of BaP on primary hepatocytes were also investigated at the phenotypic level by measuring γ H2AX foci formation and DNA adduct formation. In order to determine the relevance of the primary mouse hepatocytes, gene expression profiles were compared with those from an *in vivo* study.

Gene expression analysis reveals clear time- and dose-dependent effects induced by exposure to BaP *in vitro*, as visualized in the PCA plot (Fig. 2) and by the increasing number of significantly modulated genes. Time-dependent effects of BaP seem to have more influence on gene expression profiles compared to dose. That time- and dose have an effect on the whole genome gene expression was observed before *in vitro*, where time-dependent waves of gene expression changes were observed (26, 27, 28, Chapter 2). As BaP is known to cause cell cycle arrest in proliferating cells in order to allow for damaged DNA to be repaired, it is not surprising that effects of BaP differ between proliferating cells such as HepG2 cells, and non proliferating-cells like primary mouse hepatocytes (15, 26, 27, 28, Chapter 2). Indeed, in primary mouse hepatocytes, hardly any cell cycle-related genes were found to be significantly altered after treatment with BaP, but DNA damage and metabolism related genes, however, were found to be significantly altered.

In order to unravel time and dose-related effects on pathways and biological processes, T-profiler was used. Clustering of these pathways reveals that two main clusters are formed, namely early and late time points (Figure 4). Dose seems to have minor influence on clustering. T-profiler results shows that some gene groups are progressively up-regulated during exposure to BaP. These pathways are mainly involved in biotransformation and glutathione metabolism. Most likely this is due to the activation of the Aryl hydrocarbon receptor by these compound (29, 30). However, many gene groups are upregulated during the first 24 h and downregulated later on: namely tumor development and progression, responses to DNA and tissue damage and nucleus and nucleic acid and replication related process. Also many gene groups are downregulated during the first 24 h and upregulated later on: metabolism, catabolism and membrane- and extracellular related processes and immune responses. Responses to DNA damages and tumor development related pathways are expected to be up-regulated after treatment

with a GTX carcinogen. They are subsequently down-regulated at later time points, indicating that BaP levels in the culture medium are declining and therefore the level of DNA damage is stable at later time points during incubation.

The number of BaP-induced biochemical pathways and biological processes, considered significantly altered *in vitro*, increases in a time- and dose-dependent manner. At 24 h of BaP treatment four out of five pathways found after exposure to 10 μ M BaP were also significantly altered after exposure to 30 μ M. At 48 h of exposure 36 out of 95 pathways which were significantly altered after exposure to 10 μ M BaP were also altered at 30 μ M, thus indicating that dose-dependent effects are larger at that time point. A possible explanation for this larger dose-dependent effect at later time point is that due to metabolism, BaP levels in culture medium decrease over time, which obviously has a larger effect for the lower dose. From these results, it is difficult to decide which time point is optimal for investigating effects of compounds on transcriptomic profiles in the primary mouse hepatocyte model.

Histone H2AX is phosphorylated on Ser-139 by ATM kinase in response to damage that induces dsDNA breaks (31). Immunocytochemical detection of phosphorylated H2AX foci (γ H2AX) thus reveals the presence of dsDNA breaks in chromatin and was developed by us to study the introduction of dsDNA breaks in carcinogen-treated primary mouse hepatocytes. BaP shows a time- and dose-dependent induction of DNA damage. These time- and dose-dependent effects of BaP on γ H2AX were observed before in Human amnion FL and HepG2 cells (32, Chapter 2). In the Human amnion FL cells a time- and dose-dependent induction of γ H2AX foci was observed (32). Also in the HepG2 cells a time-dependent effect on the formation of γ H2AX foci was found, however the amount of foci increased during the first 24 h of exposure, but decreased again after 24 h due to repair of the DNA damages (Chapter 2). It is unknown whether BaP induces dsDNA breaks directly. The formation of dsDNA breaks can be a result of repair processes, and replication of damaged DNA in S-phase cells (32). However, in our study, the latter is less likely as primary hepatocytes are hardly dividing (33).

Formation of BaP-DNA adducts was analyzed to investigate the capability of primary mouse hepatocytes to metabolize BaP to its DNA reactive metabolites, which is the critical step in the initiation of chemical carcinogenesis (34). The adduct levels increased dependent on exposure time and dose, which demonstrates that these primary mouse hepatocytes are capable of metabolizing BaP to its DNA binding reactive metabolites. These results are in line with the study of Kim et al (35) for rat, where they showed the same time effect of DNA adduct formation in primary hepatocytes during 48 h of treatment with BaP. Also in HepG2 cells, BaP introduced DNA adducts in a time-dependent manner (Chapter 2). Adduct levels in these HepG2 cells increased during the first 12 h, were stable until 18-30 h, where after they slowly declined again. This was also observed in the primary hepatocytes, where adduct levels were only increasing during the first 24 h and stable until 48 h.

Upon correlating these BaP-induced DNA adduct levels and γ H2AX foci with gene expression modifications, it is shown that processes such as metabolism processes, cell adhesion and organisation, cell signaling, responses to several stimuli, immune responses and apoptosis, are involved. Many of these pathways are related to apoptosis, which can be caused by DNA damage. This was expected because DNA adduct levels and γ H2AX foci are phenotypic measures of DNA damage, showing the good correlation between phenotypic anchoring and gene expression patterns.

To further determine the relevance of a primary mouse hepatocyte *in vitro* system to test the toxicity of chemicals and drugs, transcriptomics results are compared to those from an *in vivo* mouse study. In the primary mouse hepatocytes 1231 probe sets were affected, whereas 78 probe sets were modulated *in vivo*, indicating that the effect of BaP on gene expression is larger in primary hepatocytes as compared to liver *in vivo* which is most probably related to differences in routes of exposure (direct exposure of hepatocytes *in vitro*, via blood flow in mice orally treated *in vivo*) and treatment design (once *in vitro* with multiple time points for sample collection, and *in vivo* on multiple days with one time point).

Some mouse *in vivo* studies with BaP showed that BaP is cleared from the blood very fast, even within a few hours, while this does not happen in primary mouse hepatocytes because cells are directly exposed to BaP (36, 37). Exposure to BaP might therefore last longer in primary mouse hepatocytes which might cause more severe effects of BaP *in vitro* compared to *in vivo*. In several mouse *in vivo* studies DNA adduct level increased as exposure time elongated, and eventually reached a plateau (37, 38). This is comparable to our data on primary hepatocytes. The adduct levels measured in these *in vivo* studies, however, were much lower than those from our primary mouse hepatocytes, indicating that the dose in the primary mouse hepatocytes is much higher compared to *in vivo*.

Comparing both studies reveals that only 16 common probe sets are commonly affected, and that the number of commonly affected probe sets increases in a time- and dose-dependent manner. It is therefore difficult to consider one time point as the optimal time point for comparison with the *in vivo* situation. From these common probe sets only 7 appeared to have the same effect direction. These were mainly genes involved in transcription and regulation of cell cycle and organisation. These genes are expected to be modulated after exposure to BaP because BaP is known to have effects on cell cycle in order to allow DNA damaged to be repaired (39).

Also the effects of BaP on biochemical pathways and biological processes in mice hepatocytes and mouse liver *in vivo* were compared. Again more gene groups are significantly modulated in the primary mouse hepatocytes (272 gene groups) compared to those from the *in vivo* study (8 gene groups). The amount of gene groups is increasing time- and dose-dependent in the primary mouse hepatocytes. Only 1 gene group is common between the *in vitro* and *in vivo* study. This gene group is ICHIBA_GVHD, involved in graft versus host disease in the liver. The induction of graft versus host

disease is a three-phase phenomenon (40). The first phase involves damage to host tissues by inflammation due to tissue damage. In the second phase, antigen-presenting cells as well as inflammatory cytokines triggering the activation of T cells, which expand and differentiate into effector cells. In the third phase, the effector phase, activated T cells mediate cytotoxicity against target host cells (40). Tissue damage by exposure to BaP might therefore induce graft versus host disease. Furthermore it is observed before in graft versus host models, that tumor necrosis factor (TNF), a cytokine produced by cells, is an important factor in graft versus host disease (41, 42). TNF is part of the extrinsic pathway for triggering apoptosis and can be produced by hepatocytes (43).

The significant pathways found in the *in vivo* study are all involved in the immune system and Cyp450 and DNA damage related pathways. Also in primary mouse hepatocytes pathways involved in the immune system and Cyp450 and DNA damage related pathways were found. Although they were not the same pathways as *in vivo*, most were deregulated in the same direction.

Although experimental design differences between the *in vitro* and the *in vivo* study are obvious and *in vitro/in vivo* extrapolation consequently have only limited value, the overlapping transcriptomic responses to the BaP challenges are of interest, and suggest that primary mouse hepatocytes indeed, represent a relevant *in vitro* model for toxicogenomics studies.

In summary, our results show that primary mouse hepatocytes are able to respond profoundly to exposure to BaP as the expected effects of BaP, like DNA damage and xenobiotic metabolism, are observed. Primary mouse hepatocytes are able to metabolize BaP, which cause DNA adducts and changes in gene expression profiles. Time appears to be a major contributor to that, whereas dose seems to have a minor contribution.

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Chapter 5

Discrimination for Genotoxic and Non-genotoxic Carcinogens by Gene Expression Profiling in Primary Mouse Hepatocytes Improves with Exposure Time

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Abstract

Assessing the potential carcinogenicity of chemicals for humans represents an ongoing challenge. Chronic rodent bioassays predict human cancer risk at only limited reliability while simultaneously being expensive and long-lasting. In order to seek for alternatives, the ability of a transcriptomics-based primary mouse hepatocyte model to classify carcinogens by their modes of action was evaluated. As it is obvious that exposure will induce a cascade of gene expression modifications, in particular, the influence of exposure time *in vitro* on discriminating genotoxic (GTX) carcinogens from non-genotoxic (NGTX) carcinogens class discrimination was investigated. Primary mouse hepatocytes from male C57BL/6 mice were treated for 12, 24, 36 and 48 h with two GTX and two NGTX carcinogens. For validation, two additional GTX compounds were studied at 24 and 48 h. Immunostaining of γ H2AX foci was applied in order to phenotypically verify DNA damage. It confirmed significant induction of DNA damage after treatment with GTX compounds but not with NGTX compounds. Whole genome gene expression modifications were analyzed by means of Affymetrix microarrays. When using differentially expressed genes from datasets normalized by RMA, the two classes and various compounds were better separated from each other by hierarchical clustering when increasing the treatment period. Discrimination of GTX and NGTX carcinogens by Prediction Analysis of Microarray improved with time, and resulted in correct classification of the validation compounds. The present study shows that gene expression profiling in primary mouse hepatocytes is promising for discriminating GTX from NGTX compounds and that this discrimination improves with increasing treatment period.

Introduction

The carcinogenic potential of chemical compounds is currently evaluated through application of chronic rodent bioassays. This strategy, however, generates a high false positive rate and is therefore not quite reliable (1) while simultaneously being expensive and time-consuming, as it requires the use of many animals and large quantities of the test compound during what is usually a 2-year period of study. Therefore, the usefulness of *in vitro* assays for predicting human carcinogenicity *in vivo* is increasingly explored (2). As the liver is the main organ for metabolism of many compounds including pro-carcinogens, and also represents a major target organ for chemical carcinogens *in vivo*, *in vitro* models based on hepatocyte systems are frequently considered for the prediction of toxicity. Among these, primary hepatocytes, precision cut liver slices and hepatic cell lines are well established *in vitro* models (2, 3, 4). Combining such *in vitro* models with powerful genomic-based methods might provide high throughput screening methods for predicting toxic risks in humans. The application of DNA microarray technology enables examining differential gene expression for many genes simultaneously. These DNA microarray technologies have the potential to both enhance our understanding of the mechanism underlying a compound's carcinogenic effects and to identify a characteristic set of genes from a database of reference profiles, which may allow the prediction of an unknown compound's mode of action (5, 6).

Based on their mechanisms of action, chemical carcinogens are classified as genotoxic (GTX) or non-genotoxic (NGTX) carcinogens (7). A genotoxic compound may covalently bind with DNA and cause direct damage to DNA by adduct formation. These lesions may not or incorrectly be repaired which leads to mutations and ultimately to the formation of tumors (6). A non-genotoxic compound, on the other hand, lacks the ability to induce DNA damage directly or indirectly. Because NGTX compounds have different features from GTX compounds, it may be hypothesized that GTX and NGTX induce distinct gene expression profiles which consequently may be used for mechanism-based classifying unknown compounds as GTX or NGTX (8, 9).

Length of exposure has a major influence on gene expression profiles in cells *in vitro*, and this appears to have a higher impact than compound's dose (10, 11). Therefore, it may be hypothesized that the exposure period affects class discrimination based on transcriptomics data.

The current study therefore aimed to investigate whether incorporating time-dependency as a parameter improves mechanism-based classification models to discriminate GTX and NGTX carcinogens. For this, we apply whole genome gene expression analysis to sandwich-cultured primary mouse hepatocytes. Primary mouse hepatocytes are metabolic competent and are of additional advantage because of the availability of the complete sequence of the mouse genome (12) and of transgenic mouse models which allow relevant mechanistic studies. Gene expression profiles generated at four

time points from primary mouse hepatocytes treated with four model compounds, were used in the training phase. From these, a classification model was derived, which was subsequently validated by using a set of two additional compounds. The functionality of the resulting classifiers was analyzed by MetaCore. Because histone H2AX is phosphorylated on serine-139 by ataxia telangiectasia mutated (ATM) kinase in response to DNA damage, particularly double strand breaks (13, 14), we analyzed the formation of phosphorylated H2AX (γ H2AX) foci in order to confirm GTX properties of the compounds.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), Hanks' calcium- and magnesium-free buffer, Alexa fluor 488 goat anti-mouse IgG antibody, insulin and Trizol were obtained from Invitrogen (Breda, The Netherlands). Glucagon, hydrocortisone, collagenase type IV, Benzo(a)pyrene (BaP), Aflatoxin B1 (AFB1), Cyclosporine A (CsA), Dimethylnitrosamine (DMN), Mitomycin C (MitC), Trypan blue, dimethylsulphoxide (DMSO), bovine serum albumin (BSA), 4',6-diamidino-2-phenylindole (DAPI) and Tween-20 were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) from C.N. Schmidt BV (Amsterdam, The Netherlands). Triton X-100, NaCl, Na₂HPO₄·2H₂O and NaH₂PO₄ were obtained from Merck (Darmstadt, Germany) and paraformaldehyde from ICN biomedical (Auroro, Ohio). The anti-phospho-Histone H2A-X (ser139) Clone JWB 301 antibody was purchased by Upstate Biotechnology (Lake Placid, NY). Vectashield® Mounting Medium was supplied by Vector Labs (Burlingame, CA). Collagen Type I Rat Tail was obtained from BD BioSciences (Bedford, MA). The iScript cDNA Synthesis kit en iQ SYBR Green Supermix were purchased from BioRad (Hercules, CA). Primers were designed using Primer Express 2.0 from Applied Biosystems (Foster City, CA) and purchased from Operon (Cologne, Germany). The RNeasy minikit was obtained from Qiagen, Westburg B.V. (Leusden, The Netherlands). The 5x MegaScript T7 Kit was obtained from Ambion (Austin, TX). The GeneChip® Expression 3'-Amplification Two-Cycle cDNA Synthesis Kit and Reagents, the Hybridization, Wash and Stain Kit and the Mouse Genome 430 2.0 Arrays were purchased from Affymetrix (Santa Clara, CA).

Animals and isolation of hepatocytes

Permission for isolating primary hepatocytes from mice was obtained from the Animal Ethical Committee. Adult male C57BL/6 mice (Charles River), weighing 20-25 g, were obtained from Charles River GmbH, Sulzfeld, Germany. This mouse strain was chosen

because it is frequently used in toxicological and pharmacological investigations, and it is a common background for transgenic mouse strains. The animals were housed in macrolon cages with sawdust bedding at 22°C and 50-60% humidity. The light cycle was 12 h light/12 h dark. Feed and tap water were available *ad libitum*.

Hepatocytes were isolated by a two-step collagenase perfusion method according to Seglen (15), with modifications as described before (16).

Cell culture and treatment

Cells with viability >85%, determined by trypan blue exclusion, were cultured in a collagen-collagen sandwich formation as described before (16, 17). Prior to treatment, primary cultures of mouse hepatocytes were allowed to recover for 40-42 h at 37°C in a humidified chamber with 95%/5% air/CO₂ in serum-free culture medium supplemented with insulin 0.5 U/ml, glucagon (7 ng/ml), hydrocortisone (7.5 µg/ml) and 2% penicillin/streptomycin (5000 U/ml penicillin; 5000 µm/ml streptomycin). Culture medium was refreshed every 24 h. After the recovery period, the culture medium was replaced by culture medium containing one of the selected four compounds, or with vehicle control (Table 1). Cells were incubated for 12, 24, 36 or 48 h before being used for detecting double-stranded (ds) DNA breaks or harvested for RNA isolation by adding Trizol reagent. These time points were chosen as they are regularly used in genotoxicity tests and because stability of the cells until 48h has been demonstrated (16). Three independent replicate biological experiments were conducted for each compound.

Detection of dsDNA breaks

Double-stranded DNA breaks were detected by immunostaining of the phosphorylated histone H2AX (γH2AX) as described before (18). Hepatocytes, used for the detection of dsDNA breaks, were cultured in a sandwich configuration on top of a cover slip. After desired exposure times, medium was aspirated and the cells were rinsed with 1x Phosphate-buffered saline (PBS) before fixation in 4% Paraformaldehyde for 10 min at ambient temperature. The cells were washed again with 1xPBS and made permeable with 0.2% Triton X-100 at 4°C for 5 min. Thereafter cells were washed two times with 1xPBS for 5 min and blocked during 1 h at 37°C with blocking buffer (1% BSA+0.5% Tween-20 in 1X PBS). Samples were washed two times with 1xPBS for 5 min and incubated with the first antibody (Mouse monoclonal γH2AX (Ser-139)) 1:1000 in dilution buffer (0.5% BSA + 0.5% Tween-20 in 1X PBS) during 2 h at 37°C. After two washing steps with 1xPBS cells were incubated with the second antibody (Alexa 488 labelled) 1:1000 in dilution buffer during 1 h at 37°C and washed again two times with 1xPBS. Samples were incubated with DAPI for 15 min at room temperature, dehydrated in series of ethanol solutions (70, 90 and 100%) for 3 min and air-dried. Vectashield® Mounting medium was used to stick the cover slips onto microscopic glass slides. Microscopic photographs were taken and samples were scored manually. A damage score,

ranging from 0 to 4, determined by the grade of damage, was given to 100 cells per sample. The damage scores were multiplied by the amount of cells with that score and all together calculated to get a total percentage of damage in each sample. For the statistical analysis the T-Test was used with a significance level of 5%.

RNA isolation

Total RNA was isolated from cultured mouse hepatocytes using Trizol and the RNeasy kit according to the manufacturer's protocol. RNA concentrations were measured by means of a spectrophotometer and the quality of each RNA preparation was determined using a bio-analyzer (Agilent Technologies, The Netherlands). Extracted RNA was stored at -80°C until further analysis.

Whole genome gene expression analysis

Targets were prepared according to the Affymetrix protocol. The cRNA targets were hybridized according to the manufacturer's recommended procedures on high-density oligonucleotide gene chips (Affymetrix Mouse Genome 430 2.0 GeneChip arrays). The gene chips were washed and stained using an Affymetrix fluidics station and scanned by means of an Affymetrix GeneArray scanner.

A total of sixty GeneChips was run. Normalization quality controls, including scaling factors, average intensities, present calls, background intensities, noise, and raw Q values, were within acceptable limits, according to the manufacturer, for all chips. Hybridization controls BioB, BioC, BioD, and CreX, were identified on all chips and yielded the expected increases in intensities.

Data analysis

Selection of differentially expressed probe sets

Sixty datasets were obtained from this experiment. Raw data were imported into ArrayTrack (19) and normalized using Robust Multi-array Average (RMA, integrated into ArrayTrack) (20). Present-Marginal-Absent calls were used to identify and omit probe sets of poor quality (21). Subsequently, the total remaining probe sets for each time point (12 h: 22986; 24 h: 23606; 36 h: 23811; 48 h: 24358; total: 26666), were logarithmically (base 2) transformed and corrected for their DMSO vehicle control. For each time point probe sets were then filtered for those for which expression was in at least one compound up- or downregulated by a minimum of 1.2-fold in at least two out of three experiments with expressions altered in the same direction in all replicate and with a mean fold up- or downregulated of 1.5 (22). The generated list with differentially expressed genes (log 2 ratios) was used for hierarchical clustering (HCA) with the Ward's minimum variance method (23) and prediction analysis of microarray (PAM).

Table 1 Overview for the compounds used in primary mouse hepatocyte exposures and the number of differentially expressed genes for the treatments per time point.

Chemical	Abbreviation	Solvent and dose (v/v %)	Dose	PAM training/test	12h	24h	36h	48h
Differentially expressed genes								
<u>GTX carcinogens</u>								
Benzo(a)pyrene	BaP	DMSO, 0.5 %	30 µM	training	83	1407	1523	2189
Aflatoxin B1	AFB1	DMSO, 0.5 %	15 µM	training	563	2343	1773	1701
Dimethylnitrosamine	DMN	Culture medium	2 mM	test		3073		3679
Mitomycin C	MitC	Ethanol, 0.5 %	5 µM	test		5276		8222
<u>NGTX carcinogens</u>								
cyclosporin A	CsA	DMSO, 0.5 %	1 µM	training	311	857	272	158
2,3,7,8-tetrachlorodibenzo-p-dioxin	TCDD	DMSO, 0.5 %	1 nM	training	376	834	403	551
<u>PAM</u>					1070	3910	3084	3616

Correlation analysis

Genes that correlated with γ H2AX foci formation were identified with the gene expression profile analysis suite (GEPAS 4.0; CIPF, Valencia, Spain). The values for γ H2AX foci formation, corrected by subtracting the values for the DMSO from the values for the exposed samples and log (base 2) transformed, were correlated with the log (base 2) transformed expression changes of the filtered probe sets (24 h: 23606; 48 h: 24358). Spearman correlation coefficients were calculated and correlating genes were selected by $P < 0.05$ for Prediction Analysis of Microarray software (PAM).

Pathway analysis

The complete data sets of genes from all compounds of all experiments (log2 of the ratios for treated-control) were uploaded in T-profiler (24) to identify transcriptional regulation of biochemical pathways and biological processes in the complete data set of genes without any pre-selection of genes. T-profiler uses the T-test to score the difference between the mean expression level of predefined groups of genes and that of all other genes without any pre-selection of modulated genes (24). Significance was determined by generating an E value, a Bonferroni corrected P value. Pathways and processes were significant when E values were below 0.05 and used for hierarchical clustering GenePattern (25) using Pearson correlation with pairwise complete-linkage.

Class discrimination and functional analyses of classifiers

The Prediction Analysis of Microarray software (PAM) was used for identifying genomic classifiers and for discrimination of GTX and NGTX carcinogens (26). PAM uses gene expression data to identify a subset of genes that best characterize each class by using the method of “nearest shrunken centroids” (26).

For this analysis, the gene list with differentially expressed probe sets (Table 1) was used. Ten-fold cross validation was applied in all time points, using each treatment as an independent experiment. For each time point, a set of genes (classifiers) was generated by using the smallest estimated misclassification error rate and a $> 80\%$ test probability. Additionally, two well-known GTX compounds were used to validate the classification. Genes correlating with γ H2AX foci formation at 24 and 48 h were also used for PAM to investigate if this improves the classification. The classifiers from the differentially expressed probe sets from the each time point were further analyzed for functional annotation by MetaCore using the shortest path algorithm (GeneGo, San Diego, CA). Again, data derived at 12 h were omitted. The total list of probe sets, without probe sets of poor quality, were selected for each time point as described before (24 h: 23606; 36 h: 23811; 48 h: 24358) and used as background list in MetaCore. A false discovery rate of 0.2 was used to select significant MetaCore maps. MetaCore was also used to generate biological networks.

Results

Time-dependent gene expression profiles

We selected four compounds in order to examine time-dependent differences in class discrimination performance by mouse hepatocyte gene expression changes which might be induced by GTX or NGTX carcinogens. Only non-cytotoxic doses as determined by the MTT assay (ca 80% viability), were used (Table 1). Only one dose was investigated, as several studies have shown that in *in vitro* models, treatment time has a much larger impact on chemically induced gene expression modifications than dose (10, 11). In total, 60 gene chips were run. No overt outliers appeared between the three replicates, as investigated by hierarchical clustering analysis (HCA). For each compound and time point, the differentially expressed probe sets were retrieved (Table 1).

To visualize variability and time-dependent effects on gene expression profiles from GTX and NGTX compounds, HCA for each time point was done by including only modulated gene expressions from that treatment period (Figure 1). For all compounds, the clustering of replicates improved with treatment time: at 12h it was quite poor while at 48h for all compounds, replicates grouped together. Furthermore, only at 48h the GTX and NGTX classes were completely separated from each other.

Detection of dsDNA breaks

The generation of phosphorylation of γ H2AX (forming of γ H2AX foci), which is a signature for dsDNA damage (27, 14), after 24 and 48 h exposure to the four compounds is shown in Figure 2. Only one dose and two time points were investigated. At 48 h, levels of γ H2AX foci in cells treated with GTX compounds were significantly increased as compared to DMSO controls, while at 24 h only AFB1 had induced a significant increase compared to DMSO controls. Cells treated with the NGTX compounds, on the other hand, showed no changes in levels of γ H2AX foci compared to the controls, except for CsA at 48 h when a significant decrease compared to the DMSO controls was shown. Time had only a significant effect on the cells treated with BaP, where more γ H2AX foci were detected as exposure time elongated.

Figure 1 Hierarchical clustering per time point of treatments using genes differentially expressed in primary mouse hepatocytes after exposure to BaP, AFB1, TCDD or CsA for (A) 12, (B) 24, (C) 36 and (D) 48 h (all in triplicate experiments).

Figure 2 Levels of γ H2AX foci, a measure for dsDNA breaks, in primary mouse hepatocytes exposed to BaP, AFB1, TCDD, CsA or solvent for 24 or 48 h (average with SD of 3 experiments).
* Significant (p -value < 0.05) compared to solvent controls
Significant (p -value < 0.05) over time

Pathway analysis

Pathways and processes significantly altered in hepatocytes in at least two experiments and altered in the same direction in all three replicates, were considered significant. For each compound and each time point significantly altered pathways were selected (in total 471 pathways). Only 5 pathways were significantly altered at 12 h. At 24 h, 215 pathways were significantly altered, 175 at 36 h and 382 pathways at 48 h. More pathways were significantly changed after treatment of hepatocytes with a GTX compound. Hierarchical clustering of these pathways with GenePattern (25) is presented in Figure 3, showing distinct patterns for groups of pathways. Processes related to macromolecules, cellular organisation and regulation are mostly upregulated by all compounds at 24 h and signal transduction is only upregulated by the NGTX compounds at 24 h. Immune responses, extracellular, membrane and cytoplasm processes, lipid-related processes and metabolic and biosynthetic processes are downregulated by GTX compounds and upregulated by NGTX compounds at later time points. P53-related gene groups are upregulated by GTX compounds at 36 h. Cytochrome P450-related pathways were clearly triggered by treatment with BaP and TCDD.

Class discrimination

For each time point, the log₂ of the ratio's for the differentially expressed genes upon treatments with BaP, AFB1, TCDD or CsA was uploaded into PAM in order to identify molecular classifiers, e.g. a subset of genes, that can best discriminate GTX from NGTX carcinogens. PAM generates a misclassification error rate curve based on cross-validation reducing the number of genes (data not shown). The misclassification error rate reached < 0.1 for all time points, except for 12 h where the misclassification was always > 0.4. Therefore, data sampled at 12 h, were not further analyzed. For each time point, a set of classifiers was generated by using a threshold with a misclassification error rate of 0 and a probability for classification of >80% for the training set data. This resulted in 15 genes after 24 h of exposure, 16 genes after 36 h and 26 genes after 48 h: scores generated by PAM for all classifiers are presented in Table 2. Classifiers upregulated in GTX compounds were downregulated in NGTX compounds and vice versa.

To investigate whether gene expression changes correlating with γ H2AX foci formation after exposure to BaP, AFB1, CsA or TCDD, are also capable of discriminating GTX from NGTX compounds, Spearman correlation coefficients were calculated and correlating genes were selected at $P < 0.05$. This resulted in 683 correlating genes after 24 h of exposure and 374 genes after 48 h of exposure. These genes were also uploaded into PAM. A misclassification error rate of 0 was reached after exposure periods of 24 and 48 h. Classifiers were again generated by using a threshold with a misclassification error rate of 0 and a classification probability of >80% for the training set data, resulting 19 classifiers after 24 h of exposure and 22 classifiers after 48 h, presented in Table 2.

From these classifiers, 6 genes were common between exposure periods of 24 and 48 h. From the classifiers found after 24 h of exposure 10 were common with classifiers found from the differentially expressed genes. After 48 h of exposure 8 were common with classifiers found from the differentially expressed genes.

For the purpose of validating class discrimination profiles, primary hepatocytes were treated with two additional genotoxic compounds, DMN and MitC, or vehicle control for the apparently optimal exposure periods of 24 or 48 h. A sampling time of 12 h was not included as the classification was not successful at that early time point. Also after 36 h of exposure, no analysis was performed as classifiers found at this particular time point, were barely different from those at 24 and 48 h. The classifiers generated by PAM from the differentially expressed probe sets and from the significantly modulated pathways by the training set, were then applied for classifying these two additional GTX compounds. After both exposure periods, all independent triplicate treatments of both compounds were classified correctly as GTX with a predicted test probability of 100%. Additionally, the list of classifiers generated from the genes correlating with γ H2AX foci formation by PAM was validated by evaluating these new compounds. Only after 48 h of exposure, however, the independent triplicate treatments of both compounds were classified correctly as GTX with, though with a predicted test probability > 90%. After 24 h the predicted test probability was lower but both compounds were again classified correct (data not shown).

Functional analyses of classifiers

Because the classifiers generated by PAM after 36 h of exposure were barely different from those at 24 and 48 h, only classifiers after 24 and 48 h of exposure were used for over-representation analysis in MetaCore maps. Classifiers appeared to have a predominant role in transcription, responses to extracellular stimuli, DNA damage, cell cycle and apoptosis and survival and also in aminoacid, carbohydrate, lipid, nucleotide and steroid metabolism. One classifier, namely Mdm2, was present in a large number of MetaCore maps. A network was generated using shortest path algorithm to visualize possible interactions between the major classifiers based on literature text mining (Figure 4). Again, this network demonstrated the pivotal role of Mdm2. Mdm2 appeared upregulated in GTX, and downregulated in NGTX compounds. FHIT, which was downregulated in GTX compounds, had an inhibiting role on Mdm2, while XPG, which as Figure 4 shows, was indirectly upregulated in GTX, was induced by Mdm2. Esr1, inhibited by Mdm2, had an inhibiting role on C10orf11 and on NCKX3, which were both downregulated in GTX compounds. Esr1 had a stimulating role on C3orf26, Lactoferrin and PDK4, but only C3orf26 was downregulated in GTX compounds. Mdm2 had also an inhibiting role on P53 and indirectly on SERA and MRCK γ . But only SERA appeared downregulated in GTX compounds. This network plays a role in cellular proliferation and tumor suppression.

Figure 3 Hierarchical clustering of all significantly altered pathways after treatment with AFB1, BaP, TCDD or CsA for 12, 24, 36 and 48 h, based on the *t*-values generated by *T*-profiler. Yellow and blue mean up- and downregulation, respectively.

Table 2 Genes selected by PAM for class discrimination and their GTX and NGTX scores.

GENEBANKACC	Genename	Differentially expressed genes						Genes correlating with γ H2AX			
		24		36		48		24		48	
		Score						Score			
		GTX	NGTX	GTX	NGTX	GTX	NGTX	GTX	NGTX	GTX	NGTX
NM_008522	Ltf	0.05	-0.05	0.33	-0.33	0.35	-0.35	0.16	-0.16	0.51	-0.51
AK005731	1700007K13Rik	0.32	-0.32	0.25	-0.25	0.23	-0.23	0.42	-0.42	0.34	-0.34
BI651416	Cdc42bpg	0.25	-0.25	0.16	-0.16	0.14	-0.14	0.37	-0.37	0.28	-0.28
BE956581	Cpt1c			0.16	-0.16	0.10	-0.10			0.26	-0.26
X58876	Mdm2	0.01	-0.01			0.09	-0.09	0.11	-0.11	0.25	-0.25
BB463610	4632434I11Rik							0.02	-0.02	0.18	-0.18
BB043558	9230114K14Rik			0.11	-0.11	0.05	-0.05			0.17	-0.17
AF297615	Ggta1					0.04	-0.04			0.16	-0.16
AK004719	Mdm2									0.11	-0.11
AF335325	Ddit4l									0.11	-0.11
BM198879	Ercc5	0.09	-0.09					0.18	-0.18	0.11	-0.11
BC008105	Polk									0.10	-0.10
BC003267	BC003267									0.10	-0.10
AV327248	Zfp365									0.09	-0.09
AV246296	Eda2r									0.07	-0.07
BC021352	Plod2									0.07	-0.07
AK014608	4632434I11Rik									0.07	-0.07
AF033112	Siva									0.06	-0.06
AK007854	1810053B23Rik									0.06	-0.06
BG797099	Ddit4l									0.02	-0.02
AV273409	Wnt9a									0.00	0.00
NM_030697	Ankrd47	0.19	-0.19	0.16	-0.16			0.29	-0.29		
AV377066	9130221J18Rik	0.11	-0.11					0.17	-0.17		
BM200015	Hsd12	0.05	-0.05					0.14	-0.14		
NM_013743	Pdk4	0.04	-0.04					0.13	-0.13		
BM230508	A030007D23Rik							0.08	-0.08		
NM_011316	Saa4	0.00	0.00					0.07	-0.07		
NM_011388	Slc10a2							0.07	-0.07		
BB414224	Tmprss2							0.05	-0.05		
NM_019976	Psrc1							0.02	-0.02		
BC025198	Rap2a							0.02	-0.02		
AI451920	C530044N13Rik							0.01	-0.01		
AV009441	Ivl							0.00	0.00		
BB821363	Scfd2	-0.18	0.18			0.05	-0.05				
BC026831	Fads1					0.00	0.00				
BC024358	Tpm2					0.00	0.00				
AA561726	Phgdh					-0.01	0.01				
NM_008935	Prom1					-0.02	0.02				
BB454777	4930579E17Rik			-0.01	0.01	-0.03	0.03				
AK003186	Tpm2					-0.03	0.03				
AV213379	Oxct1					-0.03	0.03				
NM_024188	Oxct1					-0.05	0.05				
BB458460	Chchd6					-0.05	0.05				
BB246912	1700112E06Rik					-0.06	0.06				
BC024120	Tat					-0.07	0.07				
AF055573	Fhit			-0.11	0.11	-0.11	0.11				
AI747296	Gmds	-0.04	0.04	-0.24	0.24	-0.14	0.14				
BI247584	Fdps					-0.14	0.14				
NM_053122	Immp2l			-0.16	0.16	-0.16	0.16				
L21027	Phgdh					-0.16	0.16				
AF000969	Cadps2	-0.21	0.21	-0.58	0.58	-0.29	0.29				
BC017615	Slc24a3			-0.11	0.11	-0.35	0.35				
AI596632	Ptprg	-0.24	0.24	-0.16	0.16						
AK010447	Smyd3			-0.04	0.04						
AK019979	2610528E23Rik			-0.10	0.10						
NM_012006	Acot1	0.06	-0.06								
NM_026517	Rpl22l1			0.00	0.00

Figure 4 Biological network generated in MetaCore, to map interactions between the classifiers selected in primary mouse hepatocytes after 24, 36 and 48 h exposure to BaP, AFB1, TCDD or CsA. Those genes that have expression values are labelled with circles/disks of different shades of red or blue, according to the corresponding levels of up- or down-regulation, respectively. A green arrow indicates activation, a red arrow indicates inhibition.

Discussion

With the aim to find alternatives for expensive and long-lasting chronic rodent bioassays, primary mouse hepatocytes were used to evaluate the time-dependence of this *in vitro* assay to classify carcinogens by their modes of action using gene expression profiling.

Evaluation of γ H2AX foci formation confirmed the correct selection of GTX and NGTX compounds. Induction of γ H2AX foci is a measure for the formation of dsDNA breaks in human cell lines and in the liver of mice (13, 14), but this has, to our knowledge, not previously been demonstrated in primary mouse hepatocytes. The two GTX carcinogens appeared clearly capable of inducing γ H2AX foci formation, while the two NGTX carcinogens, on the other hand, were not able to cause γ H2AX foci formation. These results are in line with findings on cell lines done by Zhou et al. (28), who found comparable results in human amnion FL and Chinese hamster CHL cells after 24 h of exposure to BaP and other GTX compounds. NGTX compounds CsA and azathioprine did not show an induction in γ H2AX foci formation in human amnion FL and Chinese hamster CHL cells in the study of Zhou et al. (28).

To discriminate GTX and NGTX compounds based on gene expression profiles, we used a supervised clustering approach, namely PAM. Because length of exposure has been shown to have a major influence on gene expression profiles in cells *in vitro* (10, 11) it may be hypothesized that exposure period affects class discrimination based on transcriptomics data. In the current study class discrimination is indeed dependent on exposure time. At 12 h the two classes could not be discriminated in the training set. After longer exposure times, however, class discrimination was successful. At the 12 h time point, a high misclassification error rate was shown. At the other time points, misclassification errors were 0 and therefore these incubation periods all seem suited for class discrimination.

The lists of classifiers sampled at 24, 36 and 48 h of exposure were compared. Only 1 classifier at 36 h of exposure was unique, all the other classifiers at 36 h were also present in the list of classifiers at 24 or 48 h. Six classifiers were present in the lists for 24 and 48 h. Consequently, transcriptomic response at 36 h of exposure have not been evaluated in the subsequent validation.

The PAM classification models based on classifiers generated at 24 and 48h of exposure were validated in a study with two additional compounds, DMN and MitC. Based on their gene expression profiles, both compounds were correctly classified as GTX, demonstrating that the obtained classifiers following exposure to carcinogens may indeed be able to discriminate between GTX and NGTX classes. At both time points, classification was perfect, although the classifiers lists were quite different. Gene expression modifications correlating with γ H2AX foci formation as induced by the four model compounds, were also used in PAM as a training set. Generated classifiers were vali-

dated again by using the additional compounds and both compounds were classified correct only at 48 h, but with lower test probabilities as compared to class discrimination with all differentially expressed probe sets. This suggests that DNA damage-related genes are an important, but not the only contributor in discriminating GTX from NGTX. The applicability of class discrimination for carcinogens by gene expression profiling was shown in several mouse and rat *in vivo* studies (6, 29-31). Furthermore the reliability of classification methods similar to the one we used, were previously successfully demonstrated in *in vivo* and *in vitro* (HepG2 cells) systems to discriminate GTX from NGTX carcinogens (6, 7, 32, 33). Most of these studies did not focus on time-dependent discrimination, but evaluated changes at only one time point. The study from Uehara et al. showed time-dependent increase in discrimination scores by PAM in rats treated with NGTX (33). The *in vivo* study from Ellinger-Ziegelbauer et al. demonstrated that more differences between GTX and NGTX compounds were observed after treatment for 7 day compared to 1 or 3 days (34).

Since it is known that DNA damage is induced by GTX compounds, it is not surprising that many of the classifiers for GTX are involved in cell cycle checkpoints, repair mechanism and response to DNA damage. The induction of γ H2AX foci formation confirms that also in these primary hepatocytes cultures the GTX compounds induce DNA damage. Predominantly, the formation of these foci is a result of double-strand DNA damage, which can be caused by repair mechanisms deleting DNA adducts (27, 28).

Analysis by MetaCore demonstrated that the murine double minute (Mdm2) oncogene is the most prominent classifier is, which is a regulator of p53, but also has some p53-independent activities (35). The Mdm2 oncoprotein binds to p53 and thereby blocks its ability to inhibit cellular proliferation or to induce cell death (36). Mdm2 mediates the ubiquitination of p53 and promotes the degradation of p53 by the proteasome (37). Overexpression of Mdm2 is related to the increase of tumorigenic potential and can overcome the suppression of growth activity of p53 (38, 39, 40). This p53 is related to DNA damage responses as levels of p53 are increasing vary fast in cells sustaining DNA damage (41). Mdm2 and P53 form a feedback loop which plays a role in cell cycle arrest and apoptosis (42). GTX compounds might therefore be able to induce overexpression of this Mdm2 oncogene. In our study treatment with GTX compounds clearly increases the Mdm2 gene expression.

Furthermore, the gene expression of the Mdm2 oncogene is suppressed by FHIT, which is a tumor suppressor gene (40). Treatment with GTX compounds caused a downregulation of this FHIT gene in our study, leading to stimulation of Mdm2. Other classifiers linked to Mdm2 gene in the network from MetaCore are involved in response to DNA damage and metabolism. Furthermore, Mdm2 was previously detected as a classifier that could discriminate between GTX and NGTX in rat livers (33). Although the other classifiers found in that study are different from our classifiers, the affected

responses to DNA damage and cell cycle regulation-related processes, are comparable. More *in vivo* class discrimination studies found classifiers involved in metabolism, cell cycle-related and DNA damage responses (31, 29). An HepG2 *in vitro* study detected classifiers mainly involved in apoptosis-related pathways; however, also some p53-related DNA damage responses were detected (7).

Pathway analysis was used to visualize compound- and time-related effects. At 12 h of treatment hardly any effects are observed on pathways. This is in agreement with the effects on genes, indicating that this time point is not suitable for further transcriptomics experiments. The biggest effects on pathways, however, are observed at 48 h of treatment. At 24 h of treatment an upregulation of most gene sets related to macromolecules, cellular organisation and regulation by all compounds is observed. These processes might be naturally occurring in primary hepatocytes in culture as it was shown before in untreated hepatocytes that cellular organisation and macromolecule related gene groups are mainly upregulated during culture (16). Besides these common processes triggered by all compounds, differences between the GTX and NGTX compounds are observed. Signal transduction gene groups are only upregulated by the NGTX compounds at 24 h. Gene groups involved in immune responses, extracellular, membrane and cytoplasm processes, lipid-related processes and metabolic and biosynthetic processes are mainly downregulated by GTX compounds and upregulated by NGTX compounds at later time points. The few P53-related gene groups are mainly upregulated by GTX compounds, especially at 36 h. Treatment of primary mouse hepatocytes with CsA triggered only a small number of pathways in these cells and more pathways were significantly altered at 48 h of treatment. CsA is a well-known human carcinogen, but showed no tumor development before in wild-type male C57BL/6 mice (43); this might explain the observed minor effects on pathways in this mouse hepatocyte model. Also treatment of primary mouse hepatocytes with TCDD led to a small number of significantly modulated pathways, with most pathways modulated at 48 h of treatment, showing that both NGTX compounds have less pronounced effects on primary mouse hepatocytes and their effects are observed at a later stage as compared to GTX compounds. The major differences between GTX and NGTX compounds at the level of pathways were detected in P53-related processes and immune responses. Furthermore cytochromeP450-related gene groups are mostly upregulated by BaP and TCDD at 36 and 48 h. Most likely this is due to the activation of the Aryl hydrocarbon receptor by these compounds (44, 45).

In summary, the present study demonstrates that in primary mouse hepatocytes, exposure length is of major importance for classifying GTX and NGTX compounds based on gene expression profiling. 12h of exposure appeared too short, and a treatment period of 48 h seems optimal.

These findings may be used to further develop an *in vitro* assay of mouse primary hepatocytes using gene expression profiling to predict possible carcinogens, and thus that the use of mouse primary hepatocytes is a specific and promising *in vitro* model.

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Chapter 6

Gene Expression Profiling in Primary Mouse Hepatocytes Discriminates Between True and False Genotoxic Compounds

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Abstract

Since well-established *in vitro* methods for testing the genotoxic potency of chemicals – such as the Ames/Salmonella test, the mouse lymphoma assay, the micronucleus test and the chromosomal aberration test – show an extremely high false positive rate in that they wrongly predict *in vivo* genotoxicity and carcinogenicity, there is an urgent need for more reliable assays. Within this context, we investigated whether gene expression profiling in metabolically competent primary mouse hepatocyte cultures is capable of discriminating true genotoxic compounds (GTX) from false positive genotoxic compounds (FP GTX). Sandwich-cultured primary hepatocytes from male C57BL/6 mice were treated for 24 and 48h with five true GTX and five false GTX compounds. Immunostaining of γ H2AX foci was used to verify the induction of DNA damage by these compounds. Whole genome gene expression modifications were analyzed by means of Affymetrix mouse genome 430 2.0 microarrays. Selected differentially expressed genes were subjected to hierarchical clustering and class prediction methods. The γ H2AX assay revealed significant induction of DNA damage by treatment with all true GTX compounds, whereas false GTX compounds were not able to increase γ H2AX formation, except for 4-Nitro-o-phenylenediamine (4-NP). Only after 48 h, replicates for each compound cluster perfectly together. Class prediction resulted in a correct classification for 9 out of 10 compounds at both time points; only 2-Acetylaminofluorene (2-AAF) was misclassified. The 76 classifiers at 24 h of exposure and 97 classifiers at 48 h of exposure were mostly involved in several metabolic and biosynthetic processes, immune responses and apoptosis. The present study shows that gene expression profiling in primary mouse hepatocytes is capable of discriminating between true GTX compounds and false positive GTX compounds.

Introduction

Well-established *in vitro* systems frequently used to identify the genotoxic potency of chemicals are the bacterial Ames test, the mouse lymphoma assay, the micronucleus test and the chromosomal aberration test (1). These classic *in vitro* genotoxicity tests, however, have been shown to generate an extremely high false positive rate in terms of erroneously predicting carcinogenicity *in vivo* (1). The specificity of the bacterial Ames test, for example, is 73.9%, while the mouse lymphoma assay, the micronucleus test and the chromosomal aberration test have a specificity even below 45%. However, a positive outcome from the *in vitro* test battery requires subsequent *in vivo* testing for genotoxicity (2). In case of a false positive outcome, this implies that without reason animals are subject to such additional *in vivo* testing. This leads to costs and animal burden, based on the wrong arguments. Therefore, novel *in vitro* systems need to be developed which are capable of reliably discriminating genotoxins and non-genotoxins. Chemical compounds, which are capable of causing gene mutations or chromosomal damage *in vitro* are defined as genotoxins (GTX). In most cases this is by covalent binding with DNA (3, 4). Formed damage may be not or incorrectly repaired which leads to mutations accumulating in time and - in a worst case - ultimately inducing the formation of tumors (3, 4).

The liver is the principal organ involved in the biotransformation of xenobiotic substances, with its capacity to convert hydrophobic compounds into water-soluble products that can be readily secreted from the body. This transformation first involves activation to intermediates which are often highly reactive and can bind to DNA, causing DNA damage (5). The liver also represents a major target organ for chemical carcinogens *in vivo* (6, 7). The use of *in vitro* liver systems is therefore relevant to screen potential carcinogenic compounds.

Additionally, the application of global gene expression analysis has the potential of providing new biomarkers for predicting the toxic risks of chemicals for humans. Simultaneously to identifying a characteristic set of genes from a database of reference profiles in order to predict a compound's unknown toxicity, these methodologies may enhance our understanding of the mechanisms underlying the compound's toxic effects (8, 3). Gene expression profiling analysis is shown to be a valuable tool for investigating mechanisms of toxicity in several *in vitro* liver systems and *in vivo* (9-13).

In this context, the current study focuses on the application of chemically-induced altered gene expression profiling to primary mouse hepatocytes as an *in vitro* system for the purpose of discriminating GTX compounds from false positive GTX compounds. The false positive GTX compounds are defined as non-carcinogens, demonstrating only genotoxicity in *in vitro* tests, but not *in vivo* in rodent bioassays..

Primary mouse hepatocytes are applied in this study as an *in vitro* system to discriminate false positive GTX compounds from true GTX compounds, because (A) they are

metabolic competent, (B) the complete sequence of the mouse genome is available (14), and (C) transgenic mouse models may allow relevant mechanistic follow-up studies. Gene expression profiles of primary mouse hepatocytes, were challenged with five true GTX compounds and five false positive GTX compounds, and were compared in order to generate a list of classifiers which may be able to discriminate these two classes. The formation of phosphorylated H2AX (γ H2AX) foci, which particularly monitor double strand breaks as histone H2AX becomes phosphorylated on serine-139 by ataxia telangiectasia mutated (ATM) kinase and accumulates at the breaks (15, 16), was assessed in order to phenotypically discriminate these two classes.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), Hanks' calcium- and magnesium-free buffer, Alexa fluor 488 goat anti-mouse IgG antibody, insulin and Trizol were obtained from Invitrogen (Breda, The Netherlands). Glucagon, hydrocortisone, collagenase type IV, Benzo(a)pyrene (BaP), Aflatoxin B1 (AFB1), 2-Acetylaminofluorene (2-AAF), Dimethylnitrosamine (DMN), Mitomycin C (MitC), o-Anthranilic acid (ANAC), 2-(Chloromethyl)pyridine.HCl (2-CP), 4-Nitro-o-phenylenediamine (4-NP), Quercetin (Q), 8-Hydroxyquinoline (8-HQ), Trypan blue, dimethylsulphoxide (DMSO), bovine serum albumin (BSA), 4',6-diamidino-2-phenylindole (DAPI) and Tween-20 were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Triton X-100, NaCl, Na₂HPO₄·2H₂O and NaH₂PO₄ were obtained from Merck (Darmstadt, Germany) and paraformaldehyde from ICN biomedicals (Auroro, Ohio). The anti-phospho-Histone H2A-X (ser139) Clone JBW 301 antibody was purchased by Upstate Biotechnology (Lake Placid, NY). Vectashield® Mounting Medium was supplied by Vector Labs (Burlingame, CA). Collagen Type I Rat Tail was obtained from BD BioSciences (Bedford, MA). The RNeasy minikit was obtained from Qiagen, Westburg B.V. (Leusden, The Netherlands). The 5x MegaScript T7 Kit was obtained from Ambion (Austin, TX). The GeneChip® Expression 3'-Amplification Two-Cycle cDNA Synthesis Kit and Reagents, the Hybridization, Wash and Stain Kit and the Mouse Genome 430 2.0 Arrays were purchased from Affymetrix (Santa Clara, CA).

Animals

Permission for performing animal studies was obtained from the Animal Ethical Committee. Adult male C57BL/6 mice (Charles River), weighing 20-25 g, were obtained from Charles River GmbH, Sulzfeld, Germany. This mouse strain was chosen because it is frequently used in toxicological and pharmacological investigations, and it is a com-

mon background for transgenic mouse strains. The animals were housed in macrolon cages with sawdust bedding at 22°C and 50-60% humidity. The light cycle was 12 h light/12 h dark. Feed and tap water were available *ad libitum*.

Isolation of hepatocytes

Hepatocytes were isolated from adult male C57BL/6 mice by a two-step collagenase perfusion method according to Seglen and Casciano (17, 18), with modifications as described before (19). Cell viability and yield were determined by trypan blue exclusion.

Cell culturing and treatments

Cells with viability >85%, were cultured in a collagen-collagen sandwich formation as described before (19, 20, 21). Prior to treatment, primary cultures of mouse hepatocytes were allowed to recover for 40-42 h at 37°C in a humidified chamber with 95%/5% air/CO₂ in serum-free culture medium supplemented with insulin 0.5U/ml, glucagon (7 ng/ml), hydrocortisone (7.5 µg/ml) and 2% penicillin/streptomycin (5000 U/ml penicillin; 5000 µm/ml streptomycin). Culture medium was refreshed every 24 h. After the recovery period, the culture medium was replaced by culture medium containing one of the selected ten compounds, or with vehicle control. Only non-cytotoxic doses were used for each compound, which were determined by the MTT assay (ca 80% viability after 24 h incubation) and are presented in Table 1. Cells were incubated for 24 or 48 h before being used for detecting double-stranded (ds) DNA breaks or harvested for RNA isolation by adding Trizol reagent. Three independent replicate biological experiments with hepatocytes from different mice were conducted for each compound.

Detection of dsDNA breaks

Double-stranded DNA breaks were detected by immunostaining of the phosphorylated histone H2AX (γH2AX) as described before (22, Chapter 5). Samples were corrected for their vehicle control and for the statistical analysis of dose-dependent effects the t-test was used (p-value < 0.05).

RNA isolation

Total RNA was isolated from cultured mouse hepatocytes using Trizol and by means of the RNeasy kit according to the manufacturer's protocol. RNA concentrations were measured by means of a spectrophotometer and the quality of each RNA preparation was determined by means of a bio-analyzer (Agilent Technologies, The Netherlands). Only samples with a good quality (clear 18S and 28S peaks and RIN>6) were used for hybridization. Extracted RNA was stored at -80°C until further analysis.

Whole genome gene expression analysis

Targets were prepared according to the Affymetrix protocol. cRNA targets were hybridized according to the manufacturer's recommended procedures on high-density oligonucleotide gene chips (Affymetrix Mouse Genome 430 2.0 GeneChip arrays). The gene chips were washed and stained using an Affymetrix fluidics station and scanned by means of an Affymetrix GeneArray scanner.

A total of eighty-two GeneChips was run (ArrayExpress nummers volgen nog). Normalization quality controls, including scaling factors, average intensities, present calls, background intensities, noise, and raw Q values, were within acceptable limits for all chips. Hybridization controls BioB, BioC, BioD, and CreX, were identified on all chips and yielded the expected increases in intensities.

Data analysis

Selection of differentially expressed probe sets

Eighty-two datasets were obtained from this experiment. Raw data were imported into ArrayTrack (23, 24) and normalized using Robust Multi-array Average (RMA, integrated into ArrayTrack) (25).

Present-Marginal-Absent calls were used to identify and omit probe sets of poor quality (26). Subsequently, the remaining probe sets were logarithmically (base 2) transformed, corrected for vehicle control, and subjected to all further analyses (24 h: 26100; 48 h: 26690; total: 27363). Probe sets for which expression was up- or down-regulated by at least one compound at a minimum of 1.2-fold in at least two out of three experiments with expressions altered in the same direction in all replicates and with a mean fold up- or down-regulation of 1.5 were selected (27). This was repeated for each time point. The generated list with differentially expressed probe sets (log 2 ratios) was used for hierarchical clustering (HCA) and prediction analysis of microarray (PAM) (10776 probe sets at 24 h and 12180 probe sets at 48 h).

Correlation analysis

Genes that correlated with γ H2AX foci formation were identified using the gene expression profile analysis suite (GEPAS 4.0; CIPF, Valencia, Spain). The values for γ H2AX foci formation, corrected by subtracting the values for DMSO control from the log (base 2) transformed values for the exposed samples, were correlated with the expression changes of differentially expressed probe sets. Spearman correlation coefficients were calculated and correlating genes ($P < 0.05$) were selected for PAM.

Pathway analysis

The complete data sets of genes from all compounds of all experiments (log2 of the ratios for treated-control) were uploaded in T-profiler (28) to identify transcriptional regulation of biochemical pathways and biological processes in the complete data set of genes without any pre-selection of genes. T-profiler uses the T-test to score the differ-

ence between the mean expression level of predefined groups of genes and that of all other genes without any pre-selection of modulated genes (28). Significance was determined by generating an E value, a Bonferroni corrected P value. Pathways and processes were significant when E values were below 0.05 and used for hierarchical clustering GenePattern (29) using Pearson correlation with pairwise complete-linkage.

Class prediction and functional analysis of classifiers

The software tool “prediction analysis of microarray” (PAM) was used for discriminating true GTX compounds from false positive GTX compounds (30). PAM uses gene expression data to calculate the shrunken centroid for each class and identifies the specific genes that determine the centroid most. Based on the nearest shrunken centroid, PAM is also capable of predicting to which class an unknown sample belongs (30). Class prediction was performed after 24 h and 48 h of exposure.

For this analysis, the gene list with differentially expressed probe sets was used. For each exposure period, 3 sets of genes (classifiers) were generated by PAM, using all ten treatments, based on the smallest estimated misclassification error rate (generated by 10-fold cross-validation) and a $> 80\%$ predicted test probability. This was done by using 2 experiments as training set and the third experiment for validation. This was done for all 3 possible combinations, each time leaving out another experiment. For each time point, the classifiers that were in common between the three training sets, were set as the final classifier set for that time point and were further analyzed for functional annotation by MetaCore using the shortest path algorithm (GeneGo, San Diego, CA). In total, 76 classifiers from 24 h and 97 classifiers from 48 h were analyzed by MetaCore. The total list of probe sets, without probe sets of poor quality, were selected for each time point as described before (24 h: 26100; 48 h: 26690; total: 27363) and used as background list in MetaCore. A false discovery rate of 0.3 was used to select significant MetaCore maps.

Table 1 Overview for the compounds used in primary mouse hepatocyte exposure.

Chemical	Abbreviation	CAS nr.	Solvent and dose (v/v %)	Dose	GTX in vitro	GTX in vivo	Carcinogen
<u>GTX carcinogens</u>							
Benzo(a)pyrene	BaP	50-32-8	DMSO, 0.5 %	30 µM	+	+	+
Aflatoxin B1	AFB1	1162-65-8	DMSO, 0.5 %	15 µM	+	+	+
2-Acetylaminofluorene	2-AAF	53-96-3	DMSO, 0.5 %	125 µM	+	+	+
Dimethylnitrosamine	DMN	62-75-9		2 mM	+	+	+
Mitomycin C	MitC	50-07-7	Ethanol, 0.5 %	5 µM	+	+	+
<u>False positive GTX</u>							
o-Anthranilic acid	ANAC	118-92-3	DMSO, 0.5 %	2 mM	+	-	-
2-(Chloromethyl)pyridine.HCl	2-CP	6959-47-3	DMSO, 0.5 %	125 µM	+	-	-
4-Nitro-o-phenylenediamine	4-NP	99-56-9	DMSO, 0.5 %	2 mM	+	-	-
Quercetin	Q	117-39-5	DMSO, 0.5 %	200 µM	+	-	-
8-Hydroxyquinoline	8-HQ	148-24-3	Ethanol, 0.5 %	150 µM	+	-	-

Results

Ten compounds were selected to examine the usefulness of sandwich-cultured primary mouse hepatocytes for discriminating true GTX compounds from their false positive GTX compounds. All true GTX compounds, chosen for this study, were shown to be GTX *in vitro* and *in vivo* and to be carcinogenic in mouse liver. The false positive GTX compounds are all GTX in at least 3 *in vitro* tests, non-GTX in *in vivo* tests and they are non-carcinogens (1). Non-cytotoxic doses were used for all compounds, determined by the MTT assay (ca 80% viability after 24 h incubation) (Table 1).

Detection of dsDNA breaks

The formation of phosphorylated γ H2AX foci, which is a signature for dsDNA breaks (31, 16, 32), in relation to exposure to ten compounds in comparison to vehicle controls for 24 and 48 h is shown in Figure 1. dsDNA break frequencies in cells treated with true GTX compounds were significantly increased. Cells treated with false positive GTX compounds, with exception of 4-NP, showed no significant increase in γ H2AX foci formation. Time had only a significant effect following treatment with BaP. A significant increase in γ H2AX foci formation was detected in these cells as exposure time elongated. The results for Q-treated cells, however, are less reliable as non-specific staining of the collagen layer was detected in these samples (data not shown).

Figure 1 Levels of dsDNA breaks (γ H2AX foci) in primary mouse hepatocytes exposed to GTX and FP GTX compounds for 24 or 48 h (average with SD of 3 experiments and corrected for solvent control)

* Significant (p -value < 0.05) compared to solvent controls

Significant (p -value < 0.05) over time

Hierarchical clustering analyses

To visualize possible similarities and differences between gene expression profiles from all compounds, HCA was performed by including only differentially expressed genes in samples from the 24 h and 48 h, respectively (10776 probe sets at 24 h and 12180 probe sets at 48 h) (Figure 2). Only after 48 h of exposure, replicates from each compound cluster perfectly together, not at 24 h. At none of the time points a clear clustering occurs of true or false positive GTX compounds. At 48 h MitC and DMN cluster separately from all the other compounds. All false positive GTX compounds cluster together, with the exception of 4-NP which is clustered separately from all compounds.

Figure 2 Hierarchical clustering of treatments with differentially expressed genes in primary mouse hepatocytes after exposure to GTX and FP GTX compounds at (A) 24 h and (b) 48 h.

Pathway analysis

Pathways and processes significantly altered in hepatocytes in at least two experiments and changed in the same direction in all three replicates, were considered affected. For each compound and each time point significantly altered pathways were selected (in total 1422 pathways). Hierarchical clustering of these pathways with GenePattern (29) is presented in Figure 3, showing distinct patterns for groups of pathways. Differences between the two time points are especially observed after treatment with 2-CP or 8-HQ. The effects on pathways after treatment with 2-AAF are clearly different from the other true GTX compounds, especially in pathways related to metabolic and biosynthetic processes, immune responses, glycolysis and gluconeogenesis, mitochondrial and cell energy related processes and ribosomal and translation processes.

Class prediction

In order to identify classifier genes whose expression changes best discriminate true GTX compounds from the false positive GTX compounds, the class prediction software tool PAM was used. For each time point, initially 3 lists of classifiers were generated, based on analyses by PAM of the data from 2 experiments and leaving out the third. The experiment left out (consisting of 10 treatments), was used for validation. Ultimately, the classifiers from each time point which were in common between these three training sets were selected. This resulted in 76 classifiers after 24 h of exposure and 97 classifiers after 48 h, presented in Table 2. 34 classifiers were in common between exposure periods of 24 and 48 h.

The results for each validation are presented in Table 3. Each validation test resulted in a perfect classification of 9 out of the 10 compounds. Only 2-AAF was misclassified in all validation tests and for all experiments at both time points.

In an attempt to improve class discrimination, genes correlating with γ H2AX foci formation were also fed into PAM; this resulted in a similar outcome (data not shown).

Functional analyses of classifiers

The classifiers as selected by PAM from the differentially expressed probe sets from the each time point were used for over-representation analysis in MetaCore maps. Classifiers appeared to have a predominant role in several metabolic processes, immune and damage responses and apoptosis at both time points.

Figure 3 Hierarchical clustering of all significantly altered pathways after treatment with true GTX and false positive GTX compounds for 24 and 48 h, based on the t-values generated by T-profiler. Yellow and blue mean up- and downregulation, respectively.

Table 2 Genes selected by PAM, which were common in all validation sets, for class discrimination and their true GTX compound and FP GTX compound scores.

GENEBANKACC	Gene Symbol	24		48	
		Score		Score	
		GTX	NGTX	GTX	NGTX
AA561726	382244			0.05	-0.05
AF000969	375800	-0.05	0.05	0.19	-0.19
AF022072	365836	0.00	0.00	0.08	-0.08
AF033112	376872			-0.10	0.10
AF055573	366751			0.21	-0.21
AF065917	357372	0.05	-0.05		
AF069954	356166			-0.02	0.02
AF204959	364872	0.03	-0.03		
AF297615	358267	0.05	-0.05	-0.14	0.14
AF335325	376288			0.00	0.00
AI594683	387405	0.09	-0.09	-0.17	0.17
AI596632	383599	-0.06	0.06		
AI747296	383239			0.01	-0.01
AI987929	374725	-0.03	0.03		
AK004419	378573	-0.02	0.02		
AK005731	372301	0.19	-0.19	-0.39	0.39
AK007766	378303	-0.03	0.03		
AK007854	378379	0.11	-0.11	-0.02	0.02
AK009736	374461	-0.03	0.03		
AK010447	378452	-0.13	0.13	0.13	-0.13
AK010738	378971			0.00	0.00
AK014608	374420	0.04	-0.04	-0.14	0.14
AK017734	371811			-0.03	0.03
AK018383	353878			-0.04	0.04
AK019979	371961	-0.11	0.11	0.02	-0.02
AV095209	386153	-0.11	0.11	0.02	-0.02
AV141095	382253	-0.06	0.06	0.01	-0.01
AV173571	384913	-0.02	0.02		
AV173869	384058			0.07	-0.07
AV216768	390175			0.02	-0.02
AV219418	383896	0.03	-0.03	-0.01	0.01
AV221299	392995			0.03	-0.03
AV223474	390168	-0.03	0.03		
AV246296	394849			-0.06	0.06
AV251625	393625			-0.01	0.01
AV254764	386766			-0.03	0.03
AV298304	384191	0.04	-0.04		
AV327248	382034	0.03	-0.03	-0.17	0.17
AV377066	365590	0.00	0.00		
AW240761	360689	-0.01	0.01		
AW543460	384409	-0.03	0.03		
AW543723	396735	0.04	-0.04		
AW986246	372263	-0.06	0.06		

BB009155	389978			-0.08	0.08
BB027848	392880	0.03	-0.03	-0.03	0.03
BB043558	386143	0.12	-0.12	-0.29	0.29
BB046347	370831			-0.01	0.01
BB140436	387651			0.00	0.00
BB204486	385734			0.04	-0.04
BB223872	389580			-0.15	0.15
BB246912	376663			0.15	-0.15
BB275142	390167	0.16	-0.16	-0.07	0.07
BB308836	390965	-0.01	0.01		
BB318221	392210	-0.12	0.12		
BB463610	383399	0.10	-0.10		
BB458460	392250			0.02	-0.02
BB463610	383399			-0.19	0.19
BB526605	386240	-0.04	0.04		
BB730977	386916			0.05	-0.05
BB743970	388064			-0.11	0.11
BB770528	377524	0.06	-0.06	-0.02	0.02
BB781615	390898			-0.09	0.09
BB821363	382138	-0.07	0.07		
BC003284	364436			-0.09	0.09
BC004827	374892	-0.01	0.01	0.06	-0.06
BC005552	375001			0.01	-0.01
BC006713	358440			-0.08	0.08
BC008626	363008	0.17	-0.17		
BC010318	366153			0.04	-0.04
BC010462	364901	0.11	-0.11		
BC012247	353835	0.06	-0.06	-0.13	0.13
BC012260	363632	0.04	-0.04		
BC012955	367082			0.05	-0.05
BC013893	376296			-0.09	0.09
BC015270	359187	0.02	-0.02		
BC016073	362815	-0.07	0.07		
BC016265	363619	-0.03	0.03		
BC017615	363489			0.15	-0.15
BC019379	371844			0.08	-0.08
BC019882	363791	0.10	-0.10	-0.19	0.19
BC021352	354685			-0.03	0.03
BC022148	369161			-0.20	0.20
BC025169	375604			0.04	-0.04
BC025501	375599	0.01	-0.01		
BC025837	375175	0.00	0.00		
BC025915	376116	-0.01	0.01		
BC026131	357954			0.04	-0.04
BC026422	375638	0.01	-0.01		
BC027026	355021			-0.14	0.14
BC027121	363139			-0.11	0.11
BC027185	364867			-0.10	0.10
BE956581	385458			-0.16	0.16
BF719766	392437	0.00	0.00	-0.11	0.11
BG065754	372817	0.01	-0.01	-0.09	0.09

Discrimination for True and False GTX Compounds

BG066491	385964			0.21	-0.21
BG066903	373044	-0.01	0.01		
BG069873	396155	0.03	-0.03		
BG070887	383449	-0.04	0.04		
BG076151	383144	0.00	0.00	-0.02	0.02
BG076333	353474			0.02	-0.02
BG261907	356202	-0.11	0.11		
BG797099	361167			-0.11	0.11
BG967046	386449			-0.08	0.08
BI651416	387321	0.18	-0.18	-0.36	0.36
BI660702	397278	0.07	-0.07		
BI966443	362070			-0.02	0.02
BM198879	374683	0.03	-0.03	-0.04	0.04
BM200015	368650			-0.15	0.15
BM230508	389864	0.07	-0.07	-0.17	0.17
BM936480	385793			-0.05	0.05
BQ174991	383555			0.03	-0.03
L21027	368225			0.04	-0.04
NM_007556	374349			0.06	-0.06
NM_007980	358144			-0.01	0.01
NM_007987	393266	0.07	-0.07	-0.27	0.27
NM_008522	372809	0.13	-0.13	-0.31	0.31
NM_008576	357701	-0.04	0.04		
NM_008935	354314			0.00	0.00
NM_009897	355477			-0.02	0.02
NM_011075	359047			-0.08	0.08
NM_011176	357469	0.09	-0.09	-0.16	0.16
NM_011316	353539	0.06	-0.06	-0.01	0.01
NM_012006	370922	0.15	-0.15	-0.14	0.14
NM_013642	370453			0.03	-0.03
NM_013738	371675			-0.06	0.06
NM_013866	359040	0.05	-0.05		
NM_013929	358083			-0.02	0.02
NM_018770	356065	0.00	0.00		
NM_018881	360649			-0.05	0.05
NM_019738	354279			0.01	-0.01
NM_021451	357674	0.01	-0.01		
NM_025770	353067	-0.01	0.01		
NM_028803	356267	-0.06	0.06		
NM_030697	370462	0.03	-0.03	-0.07	0.07
NM_053122	353952			0.21	-0.21
NM_053168	354166	0.02	-0.02		
NM_134147	354301			0.06	-0.06
NM_134188	360821	0.01	-0.01	0.00	0.00
Y15910	370060	-0.11	0.11		
X62701	377843			0.01	-0.01

Table 3 Class prediction by PAM of GTX and FP GTX compounds at 24 and 48 h in primary mouse hepatocytes.

Data from 2 experiments were used for training, followed by validation of the classifiers with data from the remaining experiment.

Compound	Carcinogenic class	24h validation			48h validation		
		exp 1	exp 2	exp 3	exp 1	exp 2	exp 3
AFB1	GTX	GTX	GTX	GTX	GTX	GTX	GTX
BaP	GTX	GTX	GTX	GTX	GTX	GTX	GTX
2-AAF	GTX	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX
DMN	GTX	GTX	GTX	GTX	GTX	GTX	GTX
MitC	GTX	GTX	GTX	GTX	GTX	GTX	GTX
2-CP	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX
4-NP	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX
ANAC	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX
Q	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX	FP GTX
8Q	FP GTX	FP GTX	FP GTX*	FP GTX	FP GTX	FP GTX	FP GTX

Discussion

The well-established *in vitro* test battery for genotoxicity has an extremely high false positive rate for genotoxicity and carcinogenicity (1). The predictive value of *in vitro* experiments is poor and this implies that a large number of chemicals are unnecessarily subjected to subsequent *in vivo* testing, thereby increasing the amount of animal studies. Therefore, there is a need for novel *in vitro* systems for discriminating genotoxic from non-genotoxic compounds, which demonstrates a relative low false positive rate, thereby generating a realistic and not longer over-estimated demand for genotoxicity and carcinogenicity testing in long-term rodent bioassays.

Therefore, in the current study, the application of gene expression profiles from primary mouse hepatocytes as an *in vitro* tool for discriminating false positive GTX compounds from true GTX compounds was assessed.

For the purpose of phenotypically discriminating true GTX from false positive GTX compounds, we evaluated differences in γ H2AX foci formation. Some true GTX carcinogens, like BaP, 2-AAF and MitC, are shown before to be able to induce γ H2AX foci formation in different human cells (33-35). It is therefore to be expected that this γ H2AX foci formation can also be detected in primary mouse hepatocytes treated with true GTX compounds. All true GTX compounds are significantly capable of inducing the formation of γ H2AX foci in both time points, while the false positive GTX, with the exception of 4-NP, do no. This demonstrates the metabolic competence of the primary mouse hepatocytes, as all true GTX compounds are not directly active but need to be transformed to their ultimate carcinogenic metabolite. Only one false positive GTX compound, namely 4-NP, a very powerful direct-acting *in vitro* mutagen, is able to induce γ H2AX foci formation. This indicates that 4-NP might in fact be a true GTX

agent, but it may also confirm our test for γ H2AX foci as it is also *in vitro*. Only in cells treated with BaP, a significant influence of exposure duration on γ H2AX foci formation has been detected, indicating that both time points might be optimal for the phenotypical discrimination of true GTX compounds from the false positive GTX compounds.

Gene expression data were analyzed with PAM as a method for class prediction. PAM is a supervised clustering tool designed for class prediction analyses based on microarray data. The training sets generated a list of genes, called classifiers, that appeared to be able to discriminate between classes, and these were validated by using the test sets. By using PAM, only 2-AAF is misclassified as a false positive GTX compound, in all experiments and at both time points, while all the other compounds are always correctly classified. The obtained classifiers therefore seem to reliably discriminate between true and false positive GTX compounds both at 24 and 48 h of exposure, showing for this data set a specificity of 90 %. This high percentage proves the principle that gene expression profiles in primary mouse hepatocytes can classify compounds according to their GTX potential, with the compounds used in this study. Both 24 h and 48 h treatment seem to be fit for this purpose. Comparable results were found in some *in vivo* and *in vitro* studies on gene expression profiles, but these studies were based on discriminating GTX from NGTX carcinogens, DNA damaging from non-DNA damaging GTX compounds, or discriminating compounds according to their carcinogenic potential (13, 36-39, 40, 41). For the well-established *in vitro* test battery specificity appears to be extremely low. The specificity of the Ames test was within a reasonable 73.9%, but all mammalian cell tests had very low specificity, even below 45%. The current study can, therefore, be the basis for developing a new predictive screen, which can, as alternatives to current rodent genotoxicity testing models, be applied in chemical safety procedures. The next step for that, would be to include more chemicals.

The classifiers generated by PAM from the each time point were used for overrepresentation analysis in MetaCore maps. It is not surprising that many of these classifiers are involved in apoptosis-related processes at both time points. It is well documented that the DNA damage responses involves induction of apoptosis (42). Many *in vivo* and *in vitro* studies on gene expression profiles of GTX compounds have found also genes involved in apoptosis, but these were however not the same genes (13, 36-39, 40, 41). Further, groups of classifiers from our study appear involved in metabolism and biosynthesis. Many similar metabolism-related genes were previously found in rat *in vivo* studies on gene expression modifications by GTX and NGTX compounds, but also these were not the same genes (37, 38). This overlap between our *in vitro* model with the standard rodent bioassays indicates that our classifiers are specific and reliable and thus both exposure periods are suitable for discrimination of true and false positive GTX compounds.

After evaluating classification at the single gene level, we also analyzed data at a more generic level. Pathway analysis was used to visualize time-related effects. Time appears

to have some effects on pathways, especially after treatment with 2-CP and 8-HQ. Most striking, however, is that the effects on pathways after treatment with 2-AAF are clearly different from the other true GTX compounds.

Thus treatments with 2-AAF, a well-known true GTX compound that causes liver tumors in C57BL/6 male mice and has proven to be genotoxic in the mouse in several *in vivo* and *in vitro* studies (43, 44), are misclassified by PAM using gene expression changes. The level of γ H2AX foci formation induced *in vitro* by 2-AAF, however, is comparable to that from other true GTX compounds BaP and AFB1. Apparently, in mice hepatocytes, differences between 2-AAF and the other true GTX are only pronounced at the gene expression level. Because it is known that DNA damage induced by GTX compounds, leads to stimulation of apoptosis and suppression of the cell cycle, we tried to find an explanation for the misclassification of 2-AAF by particularly evaluating genes related to apoptosis and cell cycle (45, 46). Around 16% of the differentially expressed probe sets and almost one third of the classifiers generated by PAM appeared to be reversely expressed by treatment with 2-AAF compared to the other GTX compounds, especially genes involved in apoptosis (Ahr, Bax, Serpinb2 and several Caspases) and cell cycle (Cdkn1a). Because 2-AAF has this reversed effect on genes involved in apoptosis and cell cycle, and because precisely these genes are important for the classification of GTX compounds, 2-AAF is misclassified by using gene expression profiles. But because 2-AAF is able to introduce DNA damage, it is correctly classified as a true GTX compound by the evaluation of γ H2AX foci formation.

In summary, this study demonstrates that true GTX compounds can be reliably discriminated from false positive GTX compounds in primary mouse hepatocytes by gene expression profiling, when using PAM. We provide proof of the principle that primary mouse hepatocytes may represent a novel *in vitro* system with the capacity to screen compounds for their true genotoxic potential.

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Chapter 7

Summary and General Discussion

The research described in this thesis focused on a toxicogenomics approach toward developing alternatives for animal testing with regard to genotoxicity testing. The main goal was to identify a set of gene-expression effect markers that is able to screen compounds for their true genotoxic potential in an relevant *in vitro* model. At the same time the gene-expression data was used to study the underlying molecular mechanisms associated with chemical carcinogenesis.

Hepatic *in vitro* models are used frequently in toxicological studies because the liver plays a major role in the metabolism of many compounds and also represents an important target organ in systemic toxicity (1, 2). Hepatic cell lines, primary hepatocyte cultures and precision-cut liver slices from various species are well established *in vitro* systems for these studies (1, 2, 3). As precision-cut liver slices have a very short live span and immortalized cell lines have lost many liver specific functions, primary cultures of hepatocytes are preferentially used for *in vitro* studies on liver toxicity (3, 4, 5). Primary human and rat hepatocyte cultures are well established alternative *in vitro* systems used in toxicological studies. Mouse primary hepatocyte cultures are less well described, however, while mouse models gain importance in toxicology as transgenic mouse models provide an opportunity for studying mechanisms of toxicity. Furthermore, liver cancer in the mouse is the most frequently observed cancer in this species. For these reasons, primary mouse hepatocytes were explored in this research.

In the strategy to identify possible carcinogenic properties of chemical compounds, a tiered approach is used. First *in vitro* and if deemed necessary *in vivo* genotoxicity tests are conducted, followed by animal carcinogenicity tests. The classic 2 year rodent bioassay used to assess chemical compounds for their carcinogenic potential, is very expensive and time-consuming as it requires the use of many animals and large quantities of the test compound and also has several ethical drawbacks. Furthermore the reliability, relevance and effectiveness of these *in vivo* assays are subjected to many uncertainties with regard to the extrapolation from animal to human risk. Therefore the use of *in vitro* models for predicting *in vivo* carcinogenicity in humans is increasingly considered (6). Earlier *in vitro* systems used for the identification of the genotoxic and thus possible carcinogenic potency of chemicals are the bacterial Ames test, the mouse lymphoma assay, the micronucleus test and the chromosomal aberration test (7). These systems have the benefit of being relatively simple and cheap. An important disadvantage of these *in vitro* genotoxicity tests, however, is the generation of an extremely high false positive rate when compared with *in vivo* genotoxicity and carcinogenicity results (7). This calls for better *in vitro* assays for predicting genotoxicity and carcinogenicity, generating less false positives.

Genomics-based technologies enable to investigate the function of the complete genome by expression analyses at the level of transcription (transcriptomics), translation (proteomics) and metabolism (metabonomics). Combining these technologies with the re-

quired bio-informatics tools will cause a revolution in current hazard and risk assessment practices.

Hypothetically, toxicogenomics will lead to the identification of “fingerprints”, a set of gene-expression effect markers which able to test compounds for their carcinogenic potential, in that gene-expression fingerprints should be positive for chemical carcinogens while being able to discriminate between GTX and NGTX carcinogens and between true and false positive GTX compounds, and should be negative for non-carcinogens. These “fingerprints” should be highly similar between a laboratory animal model and man.

Eventually, this may lead to the development of a predictive high-throughput screen which can be used for chemical safety procedures as an alternative to current rodent testing models which is more specific, dedicated, cheaper and socially more acceptable than currently available animal models.

The objective of this thesis is to evaluate and to optimize the use of *in vitro* primary mouse hepatocyte cultures to identify sets of gene-expression effect markers that are able to discriminate between true genotoxic and false positive genotoxic carcinogens.

HepG2 study

In order to determine the optimal time points for gene expression profiling in cells *in vitro*, first time-dependent changes in the cellular responses upon exposure to a well-known carcinogen were investigated with the intention to further apply this in primary mouse hepatocytes (8, 9). In our lab it was shown before that gene expression profiling could be used to discriminate genotoxic from non-genotoxic carcinogens (10). Time-dependent effects, however, were not investigated and therefore first experiments were conducted in human hepatoma (HepG2) cells, described in **Chapter 2**. The changes in gene expression profiles of HepG2 cells caused by the model carcinogen Benzo(a)pyrene (BaP) were investigated at 12 time points after exposure, in relation to DNA adduct levels and cell cycle distribution.

In this detailed time series study, numerous temporal changes in clusters of genes upon exposure of cells to the carcinogen BaP have been observed, thereby providing an unprecedented insight in time-dependent interactions of the affected processes and pathways.

The most interesting effects were observed with regard to DNA repair. Both base excision repair and global genomic repair correlated with DNA adducts and were induced from 9/12h until 60h, when adducts were still present.

Furthermore, the results from this study also suggest that when the levels of cells in the G1-phase are low and DNA adduct levels are high, the metabolism of the cells is mainly directed towards that of nucleotides, while metabolism of amino acids and lipids is

downregulated. Once cell cycle distribution has normalized and most DNA adducts have been repaired, metabolic processes return to control levels. Furthermore, several reaction pathways in gene expression, transcription and translation are upregulated in sequential periods.

All these observations indicate that the cellular transcriptome is strictly regulated and adapts to the changing needs of cells following acute exposure to a carcinogen. Time-dependent effects of BaP on gene expression profiling is observed indicating that more time points should be evaluated for gene expression profiling in next studies with primary mouse hepatocytes.

Mouse hepatocyte-based *in vitro* model

So far, primary mouse hepatocytes have not been used frequently in toxicology. Therefore, the potential usefulness of a mouse hepatocyte-based *in vitro* model for toxicology was assessed by investigating time-dependent competence for xenobiotic metabolism and stability of gene expression profiles in **Chapter 3**. Gene expression profiles turned out to be very different between the three time points (0, 42 and 90 h) analyzed. These time-dependent changes can be attributed to the adaptation of the primary hepatocytes to their *in vitro* environment. The gene expression profiles of the replicates, however, turned out to be quite similar per time point, despite that larger inter-animal variations were observed immediately after the isolation of the cells. This observation might be caused by variation in initial stress induced by the enzymatic separation and isolation of hepatocytes (11). This inter-animal variability is less clear in the later time points, indicating that the stress responses are fading.

Pathway analysis, used to identify biochemical pathways and biological processes that were differentially expressed, revealed initial effects at the level of external signalling pathways and energy production gene sets. Later effects were observed for gene sets related to transcription, translation, membranes and cell cycle related. The overall picture based on these pathway analyses, was that liver-specific functions were continuously decreasing and that gross cellular organizations were continuously upregulated.

To investigate the metabolic competence of sandwich-cultured mouse hepatocytes in more detail, gene expression profiles from phase I and phase II biotransformation enzymes were evaluated during the cultivation period. In general the expression values of all phase I biotransformation enzymes are decreasing over the whole cultivation period. Also enzyme activities of most Cyp450 enzymes are decreasing over the whole time period, only Cyp2B9-10/13/19/23 showed an increase in activity and expression levels. After 90 h in culture, all Cyp450 enzymes were still active in the primary hepatocytes. Most Cyp450s in conventional sandwich-cultured rat hepatocytes appeared to be decreasing very rapidly, even within the short time period of 24 h after isolation (12, 13).

While in our study gene expression levels showed a slower decrease over time indicating that mouse hepatocytes remain more stable with respect to Cyp450 gene expression as compared to rat hepatocytes but not to human hepatocytes.

The decrease for phase II enzyme gene expression was only to 92 % of the original levels in the end. This is in line with several rat studies who suggest that phase II enzyme activities are better preserved in culture than phase I enzyme activities (14, 15).

An overall comparison of the gene expression levels from phase I and phase II enzymes from rat and mouse hepatocytes demonstrated that mouse hepatocytes exhibit a more stable gene expression pattern than primary rat hepatocytes.

All these results indicate that the sandwich-cultured primary mouse hepatocyte *in vitro* system has little or no inter-animal variations, demonstrating its reproducibility. It was shown to be robust and it appears to maintain its metabolic competence better as compared to rat hepatocytes. Based on our gene expression data, we recommend applying a 42 h recovery period before using the mouse hepatocytes to reduce stress responses caused by the perfusion method.

Next, in **Chapter 4**, the primary mouse hepatocyte model was further characterised by evaluating gene expression profiles after exposure to a model carcinogen, namely BaP. Because we observed in **Chapter 2**, that, in HepG2 cells, time has a major influence on gene expression profiles after exposure to BaP, time-dependent effects on DNA damage and gene expression profile changes caused by BaP in primary mouse hepatocytes were investigated. Also dose-dependent effects were investigated in this model. Therefore the hepatocytes were, after a recovery period of 42 h, as recommended in **Chapter 3**, treated for several time points with various non-toxic concentrations of BaP.

Gene expression analysis revealed that primary mouse hepatocytes show clear time- and dose-dependent effects induced by exposure to BaP. Time-dependent effects of BaP seemed to be more important compared to dose-dependent effects. These time effects, however, are very different from those found in **Chapter 2**, as a continuous up- or down-regulation was observed in the primary mouse hepatocytes, while more variation was observed in HepG2 cells. As BaP is known to cause cell cycle arrest in dividing cells in order to allow for damaged DNA to be repaired, it is not surprising that effects of BaP differ between proliferating cells such as HepG2 cells, and non proliferating-cells such as primary mouse hepatocytes (8, 16, 17, 18).

To further determine the relevance of the primary mouse *in vitro* system, gene expression results were compared to an *in vivo* mouse study which evaluated genomics responses to BaP in the liver. Some similarities between these two systems were revealed. However both more differentially expressed probe sets and significantly modulated gene groups were found in primary hepatocytes. However, it has to be noted that the used doses and dosing regimes in both models, are not comparable, with one high dose *in vitro* and multiple low doses *in vivo*.

The effects of BaP on DNA damage in primary mouse hepatocytes, as marker of genotoxic effects, were also studied in **Chapter 4**. The staining of γ H2AX foci was used to detect double-strand DNA (dsDNA) breaks, and DNA was isolated to determine BaP-DNA adducts. Both DNA adducts and dsDNA breaks appeared to increase time- and dose-dependently. This time- and dose-dependent influence was also observed upon gene expression analysis. The results in **Chapter 4** show that primary mouse hepatocytes are able to sensitively respond to exposure to BaP: they are able to metabolize BaP, which causes DNA adducts and well-characterized changes in gene expression profiles.

Class discrimination

Because research described in the previous chapters demonstrated primary mouse hepatocytes to be robust, to maintain its metabolic competence during the culturing period and to respond as expected after exposure to a well-known genotoxic carcinogen, in **Chapter 5**, we investigated the competence of primary mouse hepatocytes to discriminate genotoxic (GTX) carcinogens from non-genotoxic (NGTX) carcinogens. As duration of exposure has a major influence on gene expression profiles, shown in **Chapter 2** and **Chapter 4**, it was hypothesized that exposure period also affects class discrimination based on transcriptomics data. Primary mouse hepatocytes were therefore analyzed at several time points during exposure to two GTX and two NGTX carcinogens. Discrimination of GTX and NGTX compounds based on gene expression profiles, was performed by the Prediction Analysis of Microarray software (PAM), which represents a supervised clustering approach. In the study described in **Chapter 5**, class discrimination indeed appears dependent on exposure time. The use of PAM resulted in successful class discrimination only in the later time points, while after 12 h of treatment the compounds were misclassified. The classifiers generated by PAM were compared and those generated after 36 h of exposure turned out to show much overlap with those at 24 or 48 h. Therefore only these two time points were investigated in a validation study with two extra GTX compounds. Based on their gene expression profiles, both compounds were correctly classified as GTX, thus demonstrating that the obtained classifiers following exposure to carcinogens may indeed be able to discriminate between GTX and NGTX classes.

Immunostaining of γ H2AX foci was again used to phenotypically verify DNA damage. The γ H2AX assay confirmed significant induction of damage by treatment with GTX compounds, whereas NGTX compounds were not able to induce that.

The study described in **Chapter 5** showed that gene expression profiling in primary mouse hepatocytes has the potential of discriminating between GTX and NGTX compounds and that this classification improved with increasing treatment time.

In **Chapter 6** we thereupon investigated whether a primary mouse hepatocyte *in vitro* system is capable of discriminating GTX compounds from false-positive GTX compounds by applying the toxicogenomics approach. The false-positive GTX compounds, chosen for this study, are considered to be non-carcinogens, showing only genotoxicity in *in vitro* tests while not inducing genotoxicity *in vivo*.

Because in **Chapter 5** we found that 24 and 48 h are the optimal time points for class discrimination, primary mouse hepatocytes were treated for 24 and 48h with five true GTX and five false-positive GTX compounds. Clustering of true and false-positive GTX compound-induced gene expression changes by HCA revealed a much better clustering at 48 h compared to 24 h, demonstrating that 48 h is more optimal for classification of true and false-positive GTX compounds.

Upon applying PAM for each time point analyzed for gene expression changes, gene classifiers could be selected resulting in a correct classification for 9 compounds at both time points. Only 2-Acetylaminofluorene (2-AAF) was misclassified as a false-positive GTX compound. This might be explained by the particular gene expression profiles of genes involved in apoptosis and cell cycle. These genes are normally stimulated by DNA damage induced by GTX compounds (19, 20) and are reversely expressed after treatment with 2-AAF compared to the other true GTX compounds. And because these genes are involved in the classification of GTX compounds, 2-AAF might misclassified by using gene expression profiles.

The obtained classifiers from PAM seem to reliably discriminate true from false-positive GTX compounds both at 24 and 48 h. Both time points are in this case optimal for classification of true and false-positive GTX compounds.

In an attempt to phenotypically discriminate true GTX from false-positive GTX compounds, the differences in γ H2AX foci formation were also evaluated in **Chapter 6**. All true GTX carcinogens were significantly capable of inducing the formation of γ H2AX foci in both time points and only one false-positive GTX compound, namely 4-NP, seemed to be able to induce γ H2AX foci formation.

Only in cells treated with BaP a significant influence of time was detected, indicating again that both time points might be optimal for the discrimination of true GTX compounds from the false-positive GTX compounds.

The study described in **Chapter 6** demonstrates that true GTX carcinogens can be reasonably well discriminated from false-positive GTX compounds in primary mouse hepatocytes by gene expression profiling, depending on the method used. The most successful discrimination was generated by using PAM. Although the discrimination is not perfect, gene expression profiling in primary mouse hepatocyte can be new *in vitro* system with the capacity to discriminate true GTX from false positive GTX compounds. The applicability of class discrimination for GTX compounds by gene expression profiling was previously shown in several *in vivo* and *in vitro* studies (7, 10, 21-37). Compared to the frequently used chronic rodent bioassays the use of primary mouse hepato-

cytes for the screening of the genotoxic potential of chemical compounds is clearly an improvement as it requires much less animals and it is less time-consuming. Compared to some well-established *in vitro* systems, like the Ames test, the mouse lymphoma assay, the micronucleus test and the chromosomal aberration test, the primary mouse hepatocyte model might generate a lower false positive rate as the false-positive GTX compounds used in this research all showed genotoxicity in some well-established *in vitro* systems frequently used to identify the carcinogenic potency, but were clearly classified separately from the true GTX carcinogens in primary mouse hepatocytes. Therefore this model can be the basis for developing predictive screens, which can, as alternatives to current rodent genotoxicity testing models, be applied in chemical safety procedures. In combination with phenotypic anchoring this model can lead to the use of a specific *in vitro* system that predict specific *in vivo* toxicity.

Limitations and recommendations

The primary mouse *in vitro* model used in this research has some limitations regarding to its use for the evaluation of the genotoxic potential of chemical compounds.

The isolation of primary mouse hepatocytes by the two-step collagenase perfusion is a technically elaborated procedure and requires some practice. The hepatocytes need to be freshly derived from the animal in order to retain their liver-specific functions. Long-term cultures of functional hepatocytes are difficult to establish, but in a sandwich configuration, hepatocytes can be cultured for a longer time period (38, 39).

Furthermore, the mouse hepatocyte model used in this research only relies on one liver cell type, namely hepatocytes, whereas other liver cells are absent. Comparison with the *in vivo* situation can also be limited because in the mouse hepatocyte model exchanges between cells and their environment are less well present because of the absence of the blood flow.

Therefore the use of primary human hepatocytes can be appropriate in order to predict *in vivo* toxicity in humans, but is, however, hampered by the large variability between the donors and the limited availability of donor material (3, 4, 5). Rat hepatocytes are an alternative to human hepatocytes, also frequently used but, a rapid decline in liver specific functions, in particular cytochrome P450 (Cyp450) enzyme activity is observed. Therefore a mouse *in vitro* hepatocyte system might still be the best alternative for the human situation. In addition, since the complete sequence of the mouse genome is known (40) and transgenic mouse models are widely available, primary mouse hepatocytes are better fit for the purpose of performing mechanistic investigations of liver toxicity.

Class prediction described in this research was based on the use of some model compounds and turned out to be time-dependent. Therefore, it is recommended to study

class prediction based on gene expression profiles at more than one time point. Based on gene expression profiles 11 out of 12 (92 %) compounds were classified correct. In order to increase the reliability of the system and to decrease the false positive rate, the research described in this research should be expanded by using much more compounds.

Class discrimination also turned out to be dependent on the classification methods used. Not all methods result in a correct classification and therefore several methods should be applied in order to generate the optimal class discrimination.

Conclusion

In conclusion, the research described in this thesis, indicates that the sandwich-cultured primary mouse hepatocyte *in vitro* model has little or no inter-animal variations, it is relatively stable and maintains its metabolic competence longer than rat hepatocytes, while being almost similar in stability to that of human hepatocytes. Based on our gene expression data, we recommend applying a 42 h recovery period before exposing the mouse hepatocytes. Primary mouse hepatocytes appear also able to metabolize BaP, which cause DNA adducts and changes in gene expression profiles.

This research also shows that gene expression profiles derived from primary mouse hepatocytes exposed to classes of genotoxins can be applied for class discrimination. A successful classification of GTX and NGTX compounds may be generated, with an increased performance at longer exposure time to the chemical compounds. Also true GTX carcinogens may be successfully discriminated from false-positive GTX compounds by gene expression profiling. Although this discrimination is not perfect, primary mouse hepatocytes may represent a novel *in vitro* system with the capacity of profiling compounds for their genotoxic potential.

This research has thus laid a fundament for further developing an *in vitro* assay of mouse primary hepatocytes by using gene expression profiling for the ultimate purpose of predicting possible carcinogens. This model may be applied in chemical safety procedures as it has the potential of providing detailed understanding of the mode of action of toxicants. By linking this information to the parameters studied in routine toxicity tests (phenotypic anchoring) this may eventually lead to the use of specific *in vitro* systems that predict specific *in vivo* toxicity.

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Nederlandse Samenvatting

Het onderzoek, beschreven in deze thesis, is voornamelijk gericht op het ontwikkelen van alternatieven voor dierproeven op gebied van genotoxiciteitstesten met behulp van toxicogenomics. Het voornaamste doel was het identificeren van een set merkers die een effect hebben op de genexpressie en die in staat zijn verbindingen te toetsen op hun genotoxisch vermogen in een relevant *in vitro* model. Daarnaast werd de genexpressie data gebruikt om de onderliggende moleculaire mechanismen, die gepaard gaan met chemische carcinogenese, te bestuderen.

In vitro levermodellen worden frequent gebruikt in toxicologische studies omdat de lever een belangrijke rol speelt in het metabolisme van vele verbindingen en omdat, in systemische toxiciteit, de lever een belangrijk doel-orgaan is (1, 2). Levercellijnen, primaire hepatocyten culturen en lever coupes van verschillende diersoorten zijn goed ingeburgerd als *in vitro* systemen voor dergelijke studies (1, 2, 3). Aangezien lever coupes een zeer korte levensduur hebben en cellijnen een groot deel van de leverspecifieke functies verloren hebben worden primaire hepatocyt culturen bij voorkeur gebruikt voor *in vitro* studies op gebied van lever toxiciteit (3, 4, 5). Primaire hepatocyten culturen van de mens en rat zijn veelgebruikte *in vitro* systemen in toxicologische studies. Primaire hepatocyten culturen van de muis zijn echter minder goed beschreven, maar muizen modellen worden steeds belangrijker in de toxicologie omdat transgene muizen modellen de mogelijkheid geven om de mechanismen van toxiciteit te bestuderen. Bovendien is leverkanker de meest voorkomende kanker in muizen. Om deze redenen werden, in dit onderzoek, primaire muizen hepatocyten onderzocht.

Als strategie om de mogelijke kankerverwekkende eigenschappen van chemische verbindingen te identificeren werd een gedifferentieerde aanpak gebruikt. Ten eerste werden *in vitro* en, indien noodzakelijk, *in vivo* genotoxiciteitstesten uitgevoerd, gevolgd door carcinogeniciteitstesten in dieren. De klassieke 2 jaar durende knaagdier bioassay, gebruikt om chemische stoffen te beoordelen op hun carcinogeen vermogen, is erg duur en tijdrovend omwille van het gebruik van een groot aantal dieren en grote hoeveelheden van de teststof en heeft daarnaast ook een aantal ethische bezwaren. Bovendien wordt de betrouwbaarheid, de relevantie en de doeltreffendheid van deze *in vivo* testen onderworpen aan tal van onzekerheden met betrekking tot de risico- extrapolatie van dier naar mens. Daarom wordt het gebruik van *in vitro* modellen voor het voorspellen van de *in vivo* carcinogeniciteit bij de mens steeds meer overwogen (6). Eerdere *in vitro* systemen die gebruikt werden voor de identificatie van het genotoxische, en dus mogelijk kankerverwekkend vermogen van chemische stoffen, zijn de bacteriële Ames test, de muislymfom test, de micronucleus test en de chromosoom aberratie test (7). Deze systemen hebben het voordeel relatief eenvoudig en goedkoop te zijn. Een belangrijk nadeel van deze *in vitro* genotoxiciteitstesten zijn echter de van extreem veel vals-positieven die uit deze test komen ten opzichte van de *in vivo* genotoxiciteit en carci-

nogeniteit resultaten (7). Dit vraagt om betere *in vitro* testen voor het voorspellen van genotoxiciteit en carcinogeniteit die minder valse positieven voortbrengen.

Genomics-technologieën zijn in staat de functie van het complete genoom te onderzoeken met behulp van expressie analyses op het niveau van transcriptie (transcriptomics), translatie (proteomics) en metabolisme (metabonomics). De combinatie van deze technologieën met de benodigde bio-informatica hulpmiddelen, zal leiden tot een revolutie in de huidige risico- en gevaren-evaluatie.

Hypothetisch gezien zal de toxicogenomica leiden tot de identificatie van zogeheten “fingerprints”, een reeks van genexpressie-effect merkers die in staat zijn om verbindingen te testen op hun carcinogeen vermogen. Deze genexpressie “fingerprints” zouden negatief moeten zijn voor niet-kankerverwekkende stoffen en positief moeten zijn voor de chemische kankerverwekkende stoffen, terwijl ze in staat moeten zijn een onderscheid te maken tussen de GTX en NGTX carcinogenen en tussen echte en valse positieve GTX verbindingen. Bovendien zouden ze zeer vergelijkbaar moeten zijn tussen het proefdier model en de mens.

Uiteindelijk zou dit kunnen leiden tot de ontwikkeling van een voorspellende high-throughput test die meer specifiek, toegewijd, goedkoper en sociaal meer aanvaardbaar is dan de momenteel beschikbare diersmodellen en die, bovendien, gebruikt kan worden in procedures voor chemische veiligheid als een alternatief voor de huidige testen in knaagdieren.

Het doel van dit proefschrift is het evalueren en optimaliseren van het gebruik van *in vitro* primaire hepatocyt culturen van de muis om sets van genexpressie-effect merkers te identificeren die in staat zijn onderscheid te maken tussen echte en valse positieve genotoxische kankerverwekkende stoffen.

HepG2 studie

Om de optimale tijdstippen voor profilering van genexpressie in cellen *in vitro* te bepalen werden eerst tijdsafhankelijke veranderingen in de cellulaire respons na blootstelling aan een bekend carcinogeen onderzocht, met de bedoeling om dit verder toe te passen in de primaire muizen hepatocyten (8, 9). Het is reeds in ons laboratorium aangetoond dat genexpressie-profielen gebruikt kunnen worden om genotoxische van niet-genotoxische carcinogenen te dicrimineren (10). Tijdsafhankelijke effecten werden echter nog niet onderzocht en daarom werden de eerste experimenten uitgevoerd in de menselijke hepaatoom (HepG2) cellen, beschreven in **Hoofdstuk 2**. De veranderingen in de genexpressie-profielen van HepG2-cellen, veroorzaakt door het model carcinogeen Benzo(a)pyreen (BaP), werden, met betrekking tot DNA-adduct niveaus en celcyclus distributie, onderzocht op 12 tijdstippen na de blootstelling.

In deze gedetailleerde tijdsreeks studie werden, na blootstelling van de cellen aan de kankerverwekkende stof BaP, talrijke tijdelijke veranderingen in genenclusters waargenomen. Dit levert daarmee een ongekend inzicht in de tijdsafhankelijke interacties van de betrokken processen en pathways. De meest interessante effecten werden waargenomen met betrekking tot DNA-herstel. Zowel base excisie herstel als globaal genomisch herstel correleerde met DNA-adducten en werden veroorzaakt vanaf 9/12 u tot 60 u, wanneer de adducten nog steeds aanwezig zijn.

De resultaten van deze studie suggereren bovendien ook dat wanneer de celniveaus in de G1-fase laag zijn en DNA adduct niveaus hoog zijn, het metabolisme van de cellen voornamelijk gericht is op die van de nucleotiden, terwijl het metabolisme van aminozuren en lipiden omlaag gereguleerd is. Zodra de celcyclus distributie weer normaal is en de meeste DNA-adducten hersteld zijn, keren de metabole processen terug tot het controle niveau. Bovendien zijn verscheidene reactie pathways in genexpressie, transcriptie en translatie in opeenvolgende perioden omhoog gereguleerd.

Al deze waarnemingen wijzen erop dat de cellulaire transcriptoom strikt gereguleerd is en dat het zich aanpast aan de veranderende behoeften van de cellen na acute blootstelling aan een carcinogeen. Tijdsafhankelijke effecten van BaP op genexpressie-profielen werden waargenomen, waaruit blijkt dat er verschillende tijdstippen moeten worden geëvalueerd op genexpressie-profilering in de volgende studies met primaire muis hepatocyten.

Hepatocyt-gebaseerd muizen *in vitro* model

Tot nu toe werden de primaire muizen hepatocyten niet vaak gebruikt in de toxicologie. Daarom werd het potentiële nut van een *in vitro* muizen hepatocyten model voor toxicologie beoordeeld door de tijdsafhankelijke bevoegdheid tot het metabolisme van xenobiotica en de stabiliteit van genexpressie-profielen te onderzoeken in **Hoofdstuk 3**. Genexpressie-profielen bleken zeer verschillend zijn tussen de drie geanalyseerde tijdstippen (0, 42 en 90 uur). Deze tijdsafhankelijke veranderingen kunnen worden toegeschreven aan de aanpassing van de primaire hepatocyten aan hun *in vitro* omgeving. Maar de genexpressie-profielen van de replica's echter, bleken zeer vergelijkbaar te zijn per tijdstip, ondanks dat er, onmiddellijk na de isolatie van de cellen, grotere verschillen tussen de dieren werden waargenomen. Dit kan worden veroorzaakt door variatie in de initiële stress veroorzaakt door de enzymatische scheiding en isolatie van hepatocyten (11). Deze inter-dier variabiliteit is minder duidelijk in de latere tijdstippen, wat aangeeft dat de stress responsen vervagen.

Pathway analyse, gebruikt voor de identificatie van de biochemische pathways en biologische processen die differentieel tot expressie werden gebracht, onthulde initiële effecten op het niveau van de externe signalerings pathways en energieproductie genen-

sets. Latere effecten werden waargenomen voor genensets die verband houden met transcriptie, translatie en membraan- en celcyclus-gerelateerde effecten. Het algemene beeld op basis van deze pathway analyses, was dat de leverspecifieke functies continu afnamen en dat, in het algemeen, cellulaire organisaties voortdurend omhoog gereguleerd werden.

Om de metabole bevoegdheid van sandwich-gekweekte muizen hepatocyten meer in detail te onderzoeken, werden genexpressie-profielen van fase I en fase II biotransformatie-enzymen geëvalueerd tijdens de kweekperiode. In het algemeen nemen de expressiewaarden van alle fase I biotransformatie-enzymen af gedurende de gehele kweekperiode. Ook de enzym-activiteiten van de meeste Cyp450 enzymen nemen af gedurende de gehele tijdsperiode, alleen Cyp2B9-10/13/19/23 vertoonde een toename van de activiteit en expressie niveaus. Na 90 uur in kweek bleken alle Cyp450 enzymen nog steeds actief in de primaire hepatocyten. De meeste Cyp450s bleken in conventionele sandwich-gekweekte ratten hepatocyten zeer snel te verminderen, zelfs binnen de korte periode van 24 uur na isolatie (12, 13). Terwijl in onze studie de genexpressie-niveaus een tragere afname in de tijd vertoonde, wat aangeeft dat de muizen hepatocyten meer stabiel blijven op gebied van Cyp450 genexpressie ten opzichte van ratten hepatocyten, maar niet ten opzichte van menselijke hepatocyten.

De genexpressie van fase II enzymen was, op het einde, slechts afgenomen tot 92% van de oorspronkelijke niveaus. Dit is in overeenstemming met verschillende studies bij ratten die suggereren dat, in kweek, fase II enzym-activiteiten beter bewaard worden dan fase I enzym-activiteiten (14, 15).

Een globale vergelijking van de genexpressie-niveaus van fase I en fase II enzymen van hepatocyten van ratten en muizen heeft aangetoond dat de muizen hepatocyten een stabiel genexpressie-patroon vertonen dan de primaire ratten hepatocyten.

Al deze resultaten geven aan dat het in sandwich-gekweekte primaire muizen hepatocyten *in vitro* systeem weinig of geen inter-dier variaties heeft, wat de reproduceerbaarheid aantoont. Er werd aangetoond dat het systeem robuust is en dat het de metabole bevoegdheid beter lijkt te behouden ten opzichte van ratten hepatocyten. Op basis van onze genexpressie gegevens, adviseren wij een herstel periode van 42 u toe te passen alvorens de muizen hepatocyten te gebruiken om stress responsen, veroorzaakt door de perfusie-methode, te verminderen.

Vervolgens wordt in **Hoofdstuk 4** het primaire muizen hepatocyt model verder gekarakteriseerd door de evaluatie van genexpressie-profielen na blootstelling aan een model carcinogeen, namelijk BaP. Omdat we, in **Hoofdstuk 2**, waargenomen hebben dat de tijd een grote invloed heeft op genexpressie-profielen in HepG2 cellen na blootstelling aan BaP, werden tijdsafhankelijke effecten op DNA schade en verandering in genexpressie-profielen ten gevolge van BaP in primaire muizen hepatocyten onderzocht. Ook de dosis-afhankelijke effecten werden onderzocht in dit model. Daarvoor werden de hepatocyten, na een herstel periode van 42 uur, zoals aanbevolen in **Hoofdstuk 3**, gedu-

rende verschillende tijdsperioden behandeld met verschillende niet-toxische concentraties van BaP.

Uit genexpressie analyse bleek dat de primaire muizen hepatocyten duidelijke tijds- en dosis-afhankelijke effecten, geïnduceerd door blootstelling aan BaP, vertonen. Tijdsafhankelijke effecten van BaP bleken belangrijker te zijn vergeleken met de dosisafhankelijke effecten. Deze tijdseffecten, echter, waren zeer verschillend van die beschreven in **Hoofdstuk 2**, aangezien een continue omhoog-of omlaag-regulatie werd waargenomen in de primaire muizen hepatocyten, terwijl meer variatie werd waargenomen in HepG2 cellen. Aangezien BaP gekend is voor het veroorzaken van een halt in de celcyclus in delende cellen, om beschadigd DNA te repareren, is het niet verwonderlijk dat de effecten van BaP verschillen tussen delende cellen, zoals HepG2 cellen, en niet-delende cellen, zoals primaire muizen hepatocyten (8, 16, 17, 18).

Om de relevantie van het primaire muizen *in vitro* systeem verder te bepalen, werden genexpressie resultaten vergeleken met een muizen *in vivo* studie die genomische responsen aan BaP in de lever beoordeelde. Enkele gelijkenissen tussen deze twee systemen werden aangetoond. Maar, in de primaire hepatocyten, echter, werden meer genen differentieel tot expressie gebracht en werden meer gemoduleerde groepen genen gevonden. Toch moet worden opgemerkt dat de gebruikte doses en dosering regimes in beide modellen, niet vergelijkbaar zijn, aangezien enkel één hoge dosis *in vitro* en meerdere lage dosissen *in vivo* gebruikt werden.

Ook de effecten van BaP op DNA-schade, als marker van genotoxische effecten, werden in primaire muizen hepatocyten bestudeerd in **Hoofdstuk 4**. De kleuring van γ H2AX foci werd gebruikt om dubbel streng DNA (dsDNA) breuken op te sporen en DNA werd geïsoleerd om BaP-DNA-adducten te bepalen. Zowel DNA-adducten en dsDNA breuken bleken tijds- en dosis-afhankelijk toe te nemen. Deze tijds- en dosis-afhankelijke invloed werd ook waargenomen in de analyse van genexpressie. De resultaten in **Hoofdstuk 4** tonen aan dat de primaire muizen hepatocyten in staat zijn om gevoelig te reageren op blootstelling aan BaP: ze zijn in staat om BaP te metaboliseren, wat DNA-adducten en goed gekarakteriseerde veranderingen in genexpressie-profielen veroorzaakt.

Klasse discriminatie

Omdat het onderzoek beschreven in de vorige hoofdstukken aangetoond heeft dat primaire muizen hepatocyten robuust zijn, in staat zijn de metabole bevoegdheid te handhaven gedurende de hele kweekperiode en in staat zijn te reageren zoals verwacht na blootstelling aan een bekende genotoxische kankerverwekkende stof, onderzochten we, in **Hoofdstuk 5**, de bevoegdheid van de primaire muizen hepatocyten om genotoxische (GTX) kankerverwekkende stoffen van niet-genotoxische (NGTX) carcinogenen te

onderscheiden. Omdat de duur van blootstelling een grote invloed heeft op genexpressie-profielen, zoals aangetoond in **Hoofdstuk 2** en **Hoofdstuk 4**, werd verondersteld dat de blootstellingsperiode ook van invloed zou kunnen zijn op klasse discriminatie gebaseerd op transcriptomics gegevens. Primaire muizen hepatocyten werden derhalve geanalyseerd op verschillende tijdstippen na blootstelling aan twee GTX en twee NGTX kankerverwekkende stoffen. Discriminatie van GTX en NGTX verbindingen op basis van genexpressie-profielen, werd uitgevoerd door de 'Prediction Analysis of Microarray' software (PAM), waarbij een 'supervised clustering' methode toegepast wordt. In de studie beschreven in **Hoofdstuk 5** blijkt klasse discriminatie inderdaad afhankelijk te zijn van de blootstellingstijd. Het gebruik van PAM resulteerde enkel in een succesvolle klasse discriminatie op de latere tijdstippen, terwijl na 12 uur van de behandeling de verbindingen onjuist geclassificeerd werden. De zogenaamde 'classifiers', gegenereerd door PAM, werden vergeleken met elkaar en de 'classifiers' die na 36 uur van de blootstelling verkregen werden bleken veel overlap te vertonen met die van 24 of 48 uur. Daarom werden alleen deze twee tijdstippen onderzocht in een validatie studie met twee extra GTX verbindingen. Op basis van hun genexpressie-profielen werden beide verbindingen correct geclassificeerd als GTX, waaruit blijkt dat de verkregen 'classifiers' na blootstelling aan kankerverwekkende stoffen inderdaad in staat zijn onderscheid te maken tussen de GTX en NGTX klassen.

Immunokleuring van γ H2AX foci werd gebruikt om fenotypisch DNA-schade te controleren. De γ H2AX test bevestigde significante inductie van schade door de behandeling met GTX verbindingen, terwijl NGTX verbindingen niet in staat waren om schade te induceren.

De studie beschreven in **Hoofdstuk 5** toonde aan dat genexpressie-profilering in de primaire muizen hepatocyten het potentieel heeft om de GTX van de NGTX verbindingen te onderscheiden en dat de classificatie verbeterde met toenemende behandelings-tijd.

In **Hoofdstuk 6** hebben we vervolgens onderzocht of een primaire muizen hepatocyten *in vitro* systeem in staat is onderscheid te maken tussen GTX verbindingen en vals-positieve GTX verbindingen met behulp van een toxicogenomics aanpak. De vals-positieve GTX verbindingen, gekozen voor deze studie, worden beschouwd als niet-kankerverwekkende stoffen die slechts genotoxiciteit vertonen in *in vitro* testen, maar niet *in vivo*.

Omdat we in **Hoofdstuk 5** vonden dat 24 en 48 uur de optimale tijdstippen zijn voor klasse discriminatie werden primaire muizen hepatocyten gedurende 24 en 48 uur behandeld met vijf echte GTX en vijf vals-positieve GTX verbindingen. Clustering van veranderingen in genexpressie, veroorzaakt door echte en vals-positieve GTX verbindingen, met behulp van HCA, bleek een veel betere clustering op te leveren na 48 uur vergeleken met 24 uur, waaruit blijkt dat 48 uur meer optimaal is voor de indeling van waar en vals-positieve GTX verbindingen.

Na de toepassing van PAM voor elk geanalyseerd tijdstip voor de veranderingen in genexpressie, konden ‘classifiers’ geselecteerd worden wat resulteerde in een juiste indeling voor 9 verbindingen op beide tijdstippen. Enkel 2-Acetylaminofluorene (2-AAF) werd ten onrechte aangezien voor een vals-positieve GTX compound. Dit kan worden verklaard door de specifieke genexpressie-profielen van genen die betrokken zijn bij apoptose en celcyclus. Deze genen worden gewoonlijk gestimuleerd door DNA-schade veroorzaakt door GTX verbindingen (19, 20) en worden omgekeerd tot expressie gebracht na behandeling met 2-AAF in vergelijking met de andere echte GTX verbindingen. En omdat deze genen betrokken zijn bij de indeling van de GTX verbindingen, wordt 2-AAF wellicht onjuist geclassificeerd op basis van genexpressie-profielen.

De ‘classifiers’ verkregen uit PAM lijken een betrouwbaar onderscheid te maken tussen echte en vals-positieve GTX verbindingen, zowel op 24 en 48 uur. Beide tijdstippen zijn in dit geval optimaal voor de indeling van echte en vals-positieve GTX verbindingen.

In een poging om fenotypisch een onderscheid te maken tussen echte en vals-positieve GTX verbindingen werden de verschillen in γ H2AX foci vorming ook geëvalueerd in **Hoofdstuk 6**. Alle echte GTX verbindingen waren significant in staat om γ H2AX foci te vormen op beide tijdstippen en enkel één vals-positieve GTX verbinding, namelijk 4-NP, leek γ H2AX foci te kunnen vormen.

Alleen in de cellen die behandeld werden met BaP werd een significante invloed van de tijd waargenomen, waaruit nogmaals blijkt dat beide tijdstippen optimaal kunnen zijn voor de discriminatie van de echte GTX verbindingen van de vals-positieve GTX verbindingen.

De studie beschreven in **Hoofdstuk 6** laat zien dat, afhankelijk van de gebruikte methode, echte GTX carcinogenen redelijk goed onderscheiden kunnen worden van vals-positieve GTX verbindingen in primaire muizen hepatocyten door genexpressie-profilering. De meest succesvolle discriminatie werd met behulp van PAM verkregen. Hoewel de discriminatie niet perfect is, kan genexpressie-profilering in primaire muizen hepatocyten dienen als een nieuw *in vitro* systeem met de capaciteit om echte GTX van vals-positieve GTX verbindingen te onderscheiden.

De toepassing van klasse discriminatie van GTX verbindingen met genexpressie-profilering werd al eerder aangetoond in verschillende *in vivo* en *in vitro* studies (7, 10, 21-37). Vergeleken met de vaak gebruikte chronische knaagdier bioassays, is het gebruik van primaire muizen hepatocyten voor de screening van de genotoxische effecten van chemische verbindingen duidelijk een verbetering omdat het veel minder dieren vergt en minder tijdrovend is. Vergeleken met een aantal gerenommeerde *in vitro* systemen, zoals de Ames-test, de muis lymfoomtest, de micronucleus test en de chromosoom aberratie test, kan het primaire muizen hepatocyten model een lager percentage vals positieve teweegbrengen aangezien alle vals-positieve GTX verbindingen, gebruikt in dit onderzoek, genotoxiciteit vertoonde in een aantal goed gevestigde *in vitro* syste-

men die vaak gebruikt worden om het kankerverwekkende potentieel van verbindingen te identificeren, maar in primaire muizen hepatocyten duidelijk apart van de echte GTX kankerverwekkende stoffen ingedeeld werden. Daarom kan dit model de basis vormen voor de ontwikkeling van een voorspellende test, die als alternatief voor de huidige knaagdier genotoxiciteit testen kan worden toegepast in chemische veiligheidstesten. In combinatie met fenotypische verankering kan dit model leiden tot het gebruik van een specifiek *in vitro* systeem dat specifieke *in vivo* toxiciteit kan voorspellen.

Beperkingen en aanbevelingen

Het primaire muizen *in vitro* model dat gebruikt werd in dit onderzoek heeft een aantal beperkingen met betrekking tot het gebruik ervan voor de beoordeling van de genotoxische effecten van chemische verbindingen.

De isolatie van de primaire muizen hepatocyten met behulp van de twee-stap collagenase perfusie is een technisch ingewikkelde procedure en vereist enige oefening. De hepatocyten moeten vers uit het dier verkregen worden om hun leverspecifieke functies te behouden. Lange-termijn culturen van functionele hepatocyten zijn moeilijk te behouden, maar in een sandwich-configuratie kunnen hepatocyten voor een langere periode gekweekt worden (38, 39).

Bovendien is het muizen hepatocyt model dat gebruikt werd in dit onderzoek slechts berust op één lever-cel type, namelijk hepatocyten, terwijl andere lever cellen afwezig zijn. Vergelijking met de *in vivo* situatie kan ook beperkt zijn, omdat in het muizen hepatocyten model uitwisselingen tussen cellen en hun omgeving minder goed aanwezig zijn vanwege de afwezigheid van de bloedstroom.

Daarom wordt het gebruik van primaire humane hepatocyten als meer geschikt geacht om *in vivo* toxiciteit te voorspellen bij de mens, maar wordt echter gehinderd door de grote variabiliteit tussen de donoren en de beperkte beschikbaarheid van donormateriaal (3, 4, 5). Ratten hepatocyten zijn een alternatief voor de menselijke hepatocyten en worden ook vaak gebruikt, maar een snelle daling in de leverspecifieke functies, met name cytochroom P450 (Cyp450) enzym activiteit, wordt waargenomen. Daarom kan een muizen *in vitro* hepatocyten systeem nog het beste alternatief zijn voor de menselijke situatie. Daarnaast zijn, aangezien de volledige sequentie van het muizen genoom bekend is (40) en transgene muizen modellen op grote schaal beschikbaar zijn, primaire muizen hepatocyten zijn beter geschikt zijn voor het verrichten van mechanistische onderzoeken van de lever toxiciteit.

Klasse voorspelling, beschreven in dit onderzoek, was gebaseerd op het gebruik van bepaalde model verbindingen en bleek tijdsafhankelijk te zijn. Daarom wordt aanbevolen om klasse voorspelling op basis van gen-expressie profielen op meer dan een tijdstip te bestuderen. Op basis van genexpressie-profielen werden 11 van de 12 (92%) verbin-

dingen juist ingedeeld. Om het systeem meer betrouwbaar te maken en om het aantal vals positieven te verminderen zou het onderzoek, beschreven in deze thesis, uitgebreid moeten worden door veel meer van deze verbindingen te gebruiken.

Klasse discriminatie bleek ook afhankelijk te zijn van de gebruikte classificatie methoden. Niet alle methoden resulteren in een juiste classificatie en dus moeten verschillende methoden worden toegepast om een optimale klasse discriminatie te verkrijgen.

Conclusie

Het onderzoek beschreven in dit proefschrift geeft aan dat het sandwich-gekweekte primaire muizen hepatocyten *in vitro* model weinig of geen inter-dier variatie heeft, relatief stabiel is en haar metabole bevoegdheid langer handhaaft dan ratten hepatocyten, terwijl het bijna gelijk in stabiliteit is met die van de humane hepatocyten. Op basis van onze genexpressie gegevens adviseren wij de toepassing van een herstel periode van 42 u alvorens de muizen hepatocyten bloot te stellen. Primaire muizen hepatocyten lijken ook in staat om BaP te metaboliseren, wat vorming van DNA-adducten en veranderingen in genexpressie-profielen veroorzaakt.

Dit onderzoek toont ook aan dat genexpressie-profielen, afkomstig van primaire muizen hepatocyten blootgesteld aan klassen van genotoxines, gebruikt kunnen worden voor klasse discriminatie. Een succesvolle indeling van de GTX en NGTX verbindingen kan verkregen worden met een verbetering in classificatie op langere blootstellingstermijn. Ook echte GTX carcinogenen kunnen met succes worden gediscrimineerd van vals-positieve GTX verbindingen door genexpressie-profilering. Hoewel deze discriminatie niet perfect is, kunnen de primaire muizen hepatocyten een nieuw *in vitro* systeem vertegenwoordigen dat de capaciteit heeft om verbindingen te profileren voor hun genotoxisch potentieel.

Dit onderzoek heeft dus een fundament gelegd voor de verdere ontwikkeling van een *in vitro* assay met primaire muizen hepatocyten door genexpressie-profilering te gebruiken om, als uiteindelijke doel, mogelijke kankerverwekkende stoffen te voorspellen. Dit model kan toegepast worden in de chemische veiligheid procedures aangezien zij de mogelijkheden hebben om gedetailleerde inzichten in het werkingsmechanisme van toxische stoffen te verstrekken. Door deze informatie te koppelen aan de parameters die in routine toxiciteit onderzocht worden (fenotypisch ankers), kan dit uiteindelijk leiden tot het gebruik van specifieke *in vitro* systemen die specifieke *in vivo* toxiciteit voorspellen.

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Merci iedereen!

Karen

Curriculum Vitae

Karen Mathijs was born on April 6th 1981 in Neerpelt, Belgium. After finishing secondary school in the discipline ‘Mathematics & Sciences’ at the WICO Campus Salvator in Hamont-Achel, she started in 1999 to study ‘Biomedical Sciences’ at the Limburg University Centre (currently University Hasselt) in Diepenbeek. After her first two years, she continued her study at the transnational University Limburg (tUL; collaboration between Diepenbeek and Maastricht), where she obtained in 2003 her diploma of ‘Licentiate in the Biomedical Sciences / Master of Health Sciences’. During her study she ran an internship at the Biomedical Research Institute (BIOMED) in Diepenbeek, conducting proteomics research on primary rat oligodendrocytes in multiple sclerosis. Thereafter she started in 2003 to study the specialisation in ‘Molecular Medical Biotechnology’ at the University of Ghent, where she obtained in 2004 her academic degree. During this study she ran an internship at the Flemish Institute for Biotechnology (VIB) in Zwijnaarde, conducting *in vivo* research on the role of matrix-metalloproteinases in TNF-related pathologies and tumor development. Subsequently, from November 2004 until March 2009, she worked as a PhD student at the department of Health Risk Analysis & Toxicology (GRAT) at the Maastricht University. The research conducted during these years and presented in this thesis, occurred under supervision of Prof. Dr. Jos CS Kleinjans and Dr. Joost HM van Delft. Next to conducting research, she attended several courses on handling laboratory animals (according to art.9 of the Dutch law on experimental animals), and for several modules as part of the Postgraduate Education in Toxicology in favour of the registration as a toxicologist.

After obtaining her PhD title Karen, likes to become a teacher in sciences.

Karen Mathijs werd geboren op 6 april 1981 te Neerpelt, België. Na het behalen van haar secundair diploma in Wiskunde-wetenschappen aan de WICO Campus Salvator the Hamont-Achel, startte ze in 1999 aan de studie 'Biomedische Wetenschappen' aan het Limburgs Universitair Centrum (tegenwoordig Universiteit Hasselt) te Diepenbeek. Na de kandidatuurjaren, zette zij haar studie verder aan de transnationale Universiteit Limburg (tUL; samenwerking tussen Diepenbeek en Maastricht), waar zij in 2003 haar diploma 'Licentiaat in de Biomedische Wetenschappen / Doctorandus in de Gezondheidswetenschappen' behaalde. Tijdens deze studie deed ze een stageonderzoek in het Biomedisch Onderzoeksinstituut (BIOMED), waar ze een proteoomstudie deed op primaire ratten oligodendrocyten in relatie tot multiple sclerose.

Vervolgens startte ze in 2003 de specialisatiestudie in 'Moleculaire Medische Biotechnologie' aan de Universiteit van Gent, waar ze in 2004 haar diploma behaalde. Tijdens deze studie deed ze een stageonderzoek aan het Vlaams Instituut voor Biotechnologie (VIB) te Zwijnaarde, waar ze, in een *in vivo* studie, onderzoek deed naar de rol van matrix-metalloproteinasen in TNF-gerelateerde pathologieën en tumor ontwikkeling.

Daarna was Karen, van november 2004 tot en met maart 2009, werkzaam als promovenda bij de capaciteitsgroep GezondheidsRisico Analyse en Toxicology (GRAT) aan de Universiteit Maastricht

Haar promotie onderzoek, dat beschreven is in dit proefschrift, gebeurde onder begeleiding van Prof. Dr. Jos CS Kleinjans and Dr. Joost HM van Delft. Naast het uitvoeren van onderzoek, heeft ze ook verscheidene cursussen gevolgd; waaronder 'Proefdierkunde op grond van art. 9 van de Wet van Dierproeven' en verschillende modules van de 'Postdoctorale Opleiding Toxicologie' ten behoeve van registratie als toxicoloog.

Na het behalen van haar doctors titel wil Karen Mathijs aan de slag in het onderwijs.

List of Publications

FULL PAPERS

Mathijs K., Kienhuis A.S., Brauers K.J.J., Jennen D.G.J., Lahoz A., Kleinjans J.C.S., van Delft J.H.M.: Assessing the metabolic competence of sandwich-cultured Mouse primary hepatocytes. *Drug Metab Dispos*, 2009. **38**: p. 1305-11.

Mathijs K., Brauers K.J.J., Jennen D.G.J., Boorsma A., van Herwijnen M.H.M., Gottschalk R.W.H., Kleinjans J.C.S., van Delft J.H.M.: Discrimination for genotoxic and non-genotoxic carcinogens by gene expression profiling in primary mouse hepatocytes improves with exposure time. Submitted in *Toxicol Sci*.

Mathijs K., Jennen D.G.J., Boorsma A., van Herwijnen M.H.M., Gottschalk R.W.H., van Steeg H., Breit T.M., Luijten M., Kleinjans J.C.S., van Delft J.H.M.: "Omics"-based validation of a primary mouse hepatocyte model for the purpose of genotoxicity profiling using Benzo(a)pyrene as a reference. In preparation.

Mathijs K., Brauers K.J.J., Jennen D.G.J., Kleinjans J.C.S., van Delft J.H.M.: Gene expression profiling in primary mouse hepatocytes discriminates between true and false genotoxic compounds. In preparation.

Hockley S.L., Mathijs K., Staal Y.C.M., Brewer D., Giddings I., van Delft J.H.M., Phillips D.H.: Interlaboratory and Interplatform Comparison of Microarray Gene Expression Analysis of HepG2 Cells Exposed to Benzo(a)pyrene. *Omics*, 2009. **13**(2): p. 115-25.

Van Delft J.H.M., Mathijs K., Staal Y.C.M., van Herwijnen M.H.M., Brauers K.J.J., Boorsma A., Kleinjans J.C.S.: Time Series Analysis of Benzo[A]Pyrene-Induced Transcriptome Changes Reveals Temporary Effects on DNA Repair, Metabolism and Cell Cycle. In preparation.

ABSTRACTS

Mathijs K., Van Delft J.H.M., Kleinjans J.C.S.: Successive gene expression changes in HepG2 cells after B[a]P exposure. *Annual PhD student meeting of the Netherlands Society of Toxicology (NVT), Wageningen, The Netherlands, June 2006.*

Mathijs K., Kienhuis A.S., Brauers K.J., Jennen D.G., Kleinjans J.C.S., van Delft J.H.M.: Sandwich-cultured mouse hepatocytes: Stability, metabolic competence and limitations. *Annual PhD student meeting of the Netherlands Society of Toxicology (NVT), Wageningen, The Netherlands, June 2008.*

Mathijs K., van Herwijnen M.H.M., van Delft J.H.M.: Time- and concentration-dependent changes in gene expression induced by B(a)P in primary mouse hepatocytes. *Gordon Research Conference Toxicogenomics, New London, United States, June 2007.*

Mathijs K., van Herwijnen M.H.M., van Delft J.H.M.: Time- and concentration-dependent changes in gene expression induced by B(a)P in primary mouse hepatocytes. *Annual PhD student meeting of the Netherlands Society of Toxicology (NVT), Wageningen, The Netherlands, June 2007.*

Mathijs K., van Herwijnen M.H.M., van Delft J.H.M.: Time- and concentration-dependent changes in gene expression induced by B(a)P in primary mouse hepatocytes. *Work in Progress Meeting of the Netherlands Toxicogenomics Centre (NTC), Wageningen, The Netherlands, June 2007.*

Mathijs K., van Herwijnen M.H.M., van Delft J.H.M.: Time- and concentration-dependent changes in gene expression induced by B(a)P in primary mouse hepatocytes. *ECNIS Annual Meeting, Maastricht, The Netherlands, February 2007.*