Prediction of hepatotoxic responses in humans : a toxicogenomics-based parallelogram approach'

ISBN: 978-90-8559-361-4

The studies presented in this thesis are supported by the Netherlands Organisation for Health Research and Development, program Alternatives to Animal Experiments (project number 3170.0049) and the Dutch Ministry of Economic Affairs. Studies are perfomed within the Nutrition and Toxicology Research Institute Maastricht (NUTRIM), which is part of the Graduate School VLAG (Food Technology Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

Prediction of hepatotoxic responses in humans: a toxicogenomics-based parallelogram appraoch

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. mr. G.P.M.F. Mols
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 13 maart 2008 om 16:00 uur.

door

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geboren op 3 september 1979
te Amelo

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General introduction

HEPATOCYTE-BASED *IN VITRO* MODELS

Conventional studies on the assessment of possible toxicity extensively rely on the use of animal systems to examine histopathological changes, changes in serum levels of hepatic enzymes as markers of organ toxicity, and tissue toxin levels. Animal experiments in toxicology and pharmacology are generally time-consuming and expensive. The use of simple, well established *in vitro* models in investigative toxicology and pharmacology, however, might aid in saving resources. *In vitro* models can contribute to identification of potential toxicity in early stages and to the decrease in attrition rates of drugs during lead discovery. In addition, in contrast to the complexity of *in vivo* studies, conditions of *in vitro* models are well defined and enable specific manipulation and control in order to unravel the mode of action in direct relation to the dose level of compound or derived metabolites. Furthermore, as *in vivo* toxicity studies raise animal welfare/ethical concerns, *in vitro* studies have the promise to contribute to the reduction, refinement, and replacement of animal experimentation.

Due to its functional position between the gastro-intestinal tract and the systemic circulation and its biochemical properties, the liver plays an important role in the metabolism of exogenous substances and is therefore an important target organ for toxicity. For this reason, hepatocyte systems have traditionally been used for *in vitro* investigations of toxicity of compounds [1-3]. Well established *in vitro* systems used in investigative pharmacology and toxicology include hepatic cell lines, perfused livers, precision-cut liver slices, and primary hepatocyte cultures [1, 3-5].

The use of primary systems is recommended, because, unlike immortal hepatic cell lines, they retain the specific functions of hepatocytes, e.g. plasma protein synthesis and secretion, and cytochrome P450 (CYP450) induction and biosynthesis [4, 6]. Hepatocyte-based *in vitro* models used for hepatotoxicity studies are summarized in Table 1. In the perfused liver, *in vivo* liver functions are preserved. However, viability of *ex vivo* perused liver is not sustained beyond two to three hours post-isolation, thus only allowing investigation of short time effects. Further, this system is inappropriate for investigation of large numbers of compounds and concentrations at once [5]. Liver slices serve as a suitable *in vitro* model as they maintain the three-dimensional structure and contain all the cell types present in the liver *in vivo*. Viability is maintained for 48 hours and several compounds can be studied at different concentrations with slices from a single liver [5, 7]. However, interassay variability is introduced as not all the cells are preserved similarly per slice [5]. Furthermore, unless thin liver slices are used, compounds might poorly penetrate into the inner cell layers of slices [7].

Cell cultures of freshly isolated hepatocytes are the most commonly used hepatic *in vitro* model to study hepatotoxicity [4]. Primary hepatocytes are considered the most relevant *in vitro* model for advancing the knowledge of liver functions and the mechanisms underlying drug- and compound-induced changes [8]. However, in primary hepatocytes, only one cell type is present and they show a gradual loss of metabolic capacity with prolonged culturing [4, 9]. The

Table 1. Hepatocyte-based *in vitro* systems [1, 4, 5, 8].

System	Advantages	Disadvantages
Perfused liver	Closest resemblance of the *in vivo* situation Maintains three dimensional architecture and bile flow	Maximum viability up to 2-3 hours Inappropriate for the investigation of various experimental conditions at once
Precision-cut liver slices	Maintains three dimensional architecture All cell types are present Several compounds can be tested at different concentrations	Maximum viability up to 48 hours Cells are not similar preserved per slice causing interassay variability Special equipment for preparation is needed
Conventional primary hepatocyte monolayer cultures	Easy to prepare Suitable for cytotoxicity assays	Maximum viability up to a few days Only one cell type present Unsteady appearance over time/ morphological unstable
Primary hepatocyte sandwich cultures	Prolonged longevity compared to monolayer cultures (up to several weeks) Maintainance of hepatocyte-specific functions for several days Steady appearance over time/ morphologically stable Retention of structures resembling bile canaliculi	Only one cell type present Gradual loss of metabolic capacity
Hepatic cell lines	Easy to maintain in culture Promising new developments: HepaRG cells (102, 103)	HepG2 cells: lack of substantial set of liver specific functions HepG2 cells: re-expression of CYPs by transfection does not mimic regulation of gene expression in normal hepatocytes

current status on primary hepatocyte cultures in toxicology is recently extensively reviewed by the leading researchers in the field [8].

The value of primary hepatocytes has been enhanced further in recent years by technical improvements. In particular, culturing hepatocytes in sandwich configuration between two layers of gelled extracellular matrix proteins has dramatically prolonged the longevity of cultures for displaying hepatocyte-specific functions [8, 10]. The most commonly used matrices are collagen type I and matrigel [4, 8]. A schematic representation of hepatocytes cultured in sandwich configuration is presented in Figure 1. Dunn *et al.* [11, 12] were the first to show that collagen mimics the extracellular matrix resulting in matrix-induced polarisation of hepatocytes, which greatly enhances hepatocyte viability and morphology (Figure 2). Furthermore, extracellular matrix overlay and cell-cell contacts in sandwich-culture facilitate the formation of gap junctions and functional bile canalicular networks and gap junctions in hepatocytes [13, 14].

METABOLIZING CAPACITY OF HEPATOCYTE SANDWICH CULTURES

In vitro hepatotoxicity investigations mostly rely on hepatocyte preparations from laboratory animals. Especially, the technique to isolate hepatocytes from rats is simple and well established

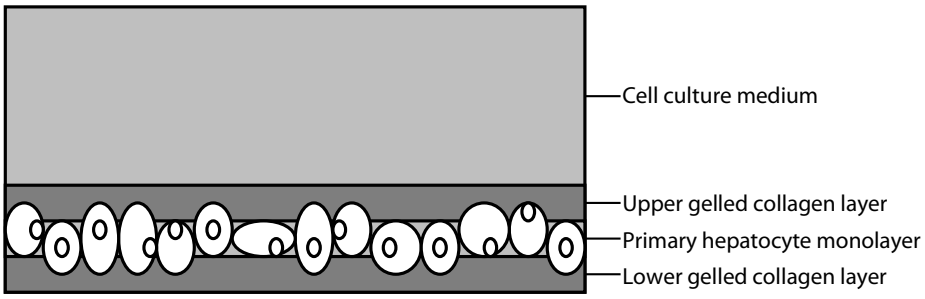

Figure 1. Schematic representation of hepatocytes cultured in sandwich configuration.

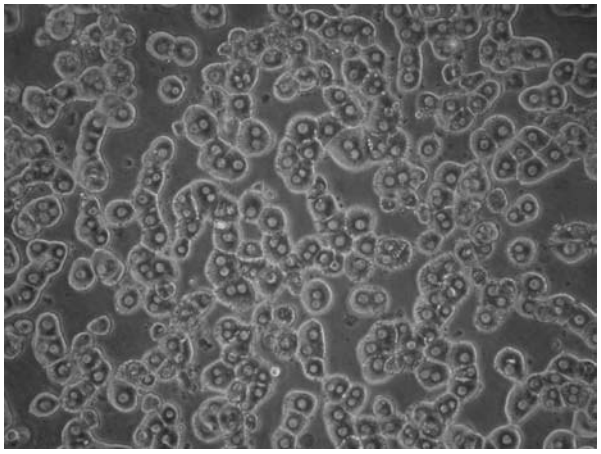

Figure 2. Morphology of rat hepatocytes cultured for up to 72h in sandwich configuration. The picture was taken with a Nikon Coolpix 995 digital camera attached to an Olympus phase-contrast microscope (200 x magnification).

[15-17]. However, the loss of liver specific functions, in particular CYP450 enzyme activity, in rat hepatocyte-based *in vitro* models limits the investigation of compounds for which toxicity depends on metabolism by the CYP450 enzyme system and raises uncertainty about the relevance of findings in cultured cells to the intact liver [18, 19]. Three interrelated factors have shown to be indispensable for optimal expression and maintenance of hepatic structure and function *in vitro*, namely, extracellular matrix, cell-cell contacts, and medium composition [19]. In rat hepatocytes cultured in sandwich configuration these three elements can be sustained. Still, not all of the metabolic enzymes in sandwich-cultured rat hepatocytes are maintained [18].

The functional post-isolation alterations affecting the liver specific functions, particular the CYP450 enzymes, are much less pronounced in cultured human hepatocytes [20]. Comparison of metabolic activity between rat and human hepatocytes in sandwich culture showed that enzyme levels of rat hepatocytes fluctuated significantly after isolation, whereas there was only a slight decrease in the enzyme activities of human hepatocytes, suggesting that human cells might be more suitable for *in vitro* studies [21]. Additionally, due to metabolism-mediated

species differences in response to compounds, human hepatocytes in culture is the system that probably best predicts toxic effects in humans [20-22]. Historically, using human tissue has posed a major problem due to its limited and irregular availability as a source of research material [23]. However, recent advances in isolation and culture techniques enabled obtaining primary human hepatocytes of high quality and viability for long term culture [24-26].

A major complication of using human hepatocytes to investigate hepatotoxicity is variability. For example, resection specimens available for research might show various stages of a variety of diseases, e.g. steatosis (fatty liver), cirrhosis, and carcinogenesis in adjacent tissue [22]. Furthermore, tremendous inter-individual variability in the expression of CYP450 enzymes is caused by various reasons including life style factors [6, 22]. This inter-individual variability observed in human hepatocytes *in vitro* reflects the genuine phenotypic variability in the human population [27]. Therefore, it might be suggested that variability resulting in differences in metabolism represents the real situation opposed to that for hepatocytes from genetically identical rats.

Despite the shortcomings of both primary rat and human hepatocytes, long-term retention of morphology and viability make the sandwich model the preferable *in vitro* alternative system for toxicology and pharmacology at the moment [8, 20]. Its unique features in comparison with other hepatocyte-based *in vitro* models, make the sandwich model the preferred culture format of the Food and Drug Administration (FDA) [8]. Still, advancements in culture techniques, e.g. optimization of medium composition or development of co-cultures of hepatocytes and other cell types, are needed to increase the metabolic capacity of *in vitro* systems including the sandwich model, thereby increasing their relevance to the situation *in vivo*.

CONVENTIONAL ASSAYS FOR HEPATOTOXICITY ASSESSMENT

The number of possible endpoints for obtaining information at the cellular and molecular level to detect and measure potential adverse effects of compounds in mammalian and non-mammalian *in vitro* and *in vivo* systems is extensive [28, 5]. Morphological changes can be observed with light and electron microscopy. Membrane integrity can be determined by the trypan blue exclusion test or enzyme leakage assays, like the lactate dehydrogenase (LDH) assay. The MTT reduction assay is a metabolic assay which measures the ability of mitochondria to reduce tetrazolium salt. Other metabolic assays measure the GSH content, or lipid peroxidation [4, 5]. However, these assays are non-specific and do not detect some types of toxicity, especially if cell viability is unaffected [4]. Conventional methods which measure liver specific endpoints particularly determine the neosynthesis of liver specific plasma proteins (albumin, transferrin, acute-phase proteins, gluconeogenesis, glycolysis, lipoprotein synthesis, and bile acid secretion [5]). Additionally, mechanisms of toxicity can be elucidated by measuring unscheduled DNA synthesis, DNA damage, and protein adducts, and morphological alterations and changes in drug metabolism [4]. As these assays generally measure only one specific biological endpoint

at a time, selection of the appropriate assay needs accurate generation of hypotheses on the mode of action of the compound under investigation. As opposed to these assays, which generally measure few endpoints at a time, new technologies have been developed which measure thousands of endpoints simultaneously, thereby incredibly increasing the amount of information which can be obtained from a single experiment. One of these technologies will be described in the following sections.

TOXICOGENOMICS

The large genome-sequencing projects and the subsequent identification of the complete genome sequences of multiple species made it possible to engineer DNA microarrays which allow for the simultaneous measurement the expression of thousands of genes [29]. This determination of levels of multiple gene transcripts has been termed transcriptomics. The microarray technology uses glass slides (microarrays) on which thousands of different probes (single-strand cDNA molecules or, more recently, oligonucleotides) are printed. Each probe is specific for a single gene. By hybridization of the microarray with a pool of fluorophore labelled mRNAs or cDNAs (generated from mRNAs), each labelled nucleic acid strand will only hybridize with the particular spot on the array containing the complementary DNA molecule for this specific gene [30].

Toxicogenomics is the term used for the application of functional genomics technologies, like transcriptomics and the microarray technology, in toxicology [30-33]. In order to determine the nature of the toxic response of a certain compound, a typical toxicogenomics experiment compares gene expression profiles in samples from exposed animals or cells to the profiles of non-exposed control animals/cells. When using the two-colour microarray technology, Agilent or Operon microarrays (the one-colour method with Affymetrix GeneChips™ will not be explained here: for more information refer to Lockhart *et al.* [34]), mRNAs or cDNAs of the exposed and control animal/cells are normally labelled with a red fluorophore and a green fluorophore, respectively. Both samples are hybridized together on the microarray. Quantification of both fluorescence signals enables the determination of a ratio of expression for each gene in the test sample with respect to the control sample resulting in the relative expression level [30]. For more in-depth information on two-colour arrays refer to Schena *et al.* [29] and DeRisi *et al.* 35. A schematic representation of a typical microarray experiment is shown in Figure 3.

The field of toxicogenomics, through the use of microarrays, has the potential to advance the understanding on the mode-of-action of compounds. Several studies have been performed in which animals or cells were treated with compounds in order to identify compound-specific gene expression profiles reflective of its toxic mechanism of action, in this way contributing to mechanistic toxicology [36-43]. The expression changes corresponding to a specific mode-of-action can be used as a fingerprint to classify compounds with respect to toxicity. A new compound

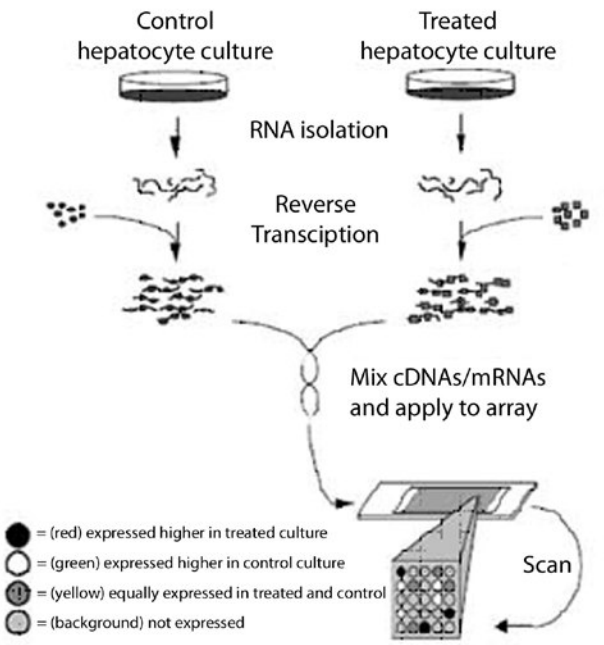

Figure 3. Schematic representation of a typical *in vitro* microarray experiment. Modified from http://dir.niehs.nih.gov/microarray/figures/background.pdf (2007).

can thus be identified as putatively toxic based on the common mechanisms of response at the transcriptional level, contributing to predictive toxicology [30, 40, 42, 44]. These mechanistic and predictive toxicogenomics studies were performed *in vivo* as well as *in vitro*. Although transcriptomic analysis has shown decreased gene expression of *in vitro* models as compared to intact liver [45, 46], which is basically inherent to the *in vitro* model as such, experimental data suggest the suitability of *in vitro* models in toxicogenomics studies [45-50]. Still, as an advantage to *in vitro* as well as *in vivo* toxicogenomics studies, the sensitivity of the microarray technology has enabled identification of potential adverse effects of compounds at lower doses and earlier time points compared to conventional assays of toxicity [51].

Toxicogenomics-based parallelogram approach

The parallelogram approach presented in Figure 4 shows the suitability of toxicogenomics-based studies to make in *vitro*-to-*in vivo* comparisons and interspecies extrapolations. The toxicogenomics approach can aid in the scientific challenge of extrapolating *in vitro* results to the situation *in vivo* by comparing similar endpoints, compound-induced changes in the complete transcriptome. Similar comparisons can be made between species, since the overlap between animal and human genomes is incredibly high (it was estimated that 90% of the rat

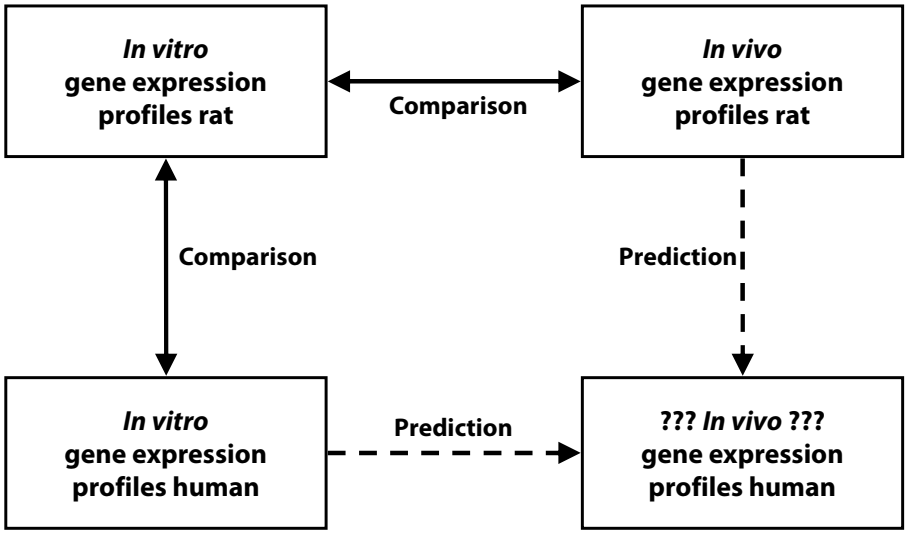

Figure 4. Toxicogenomics-based parallelogram approach showing comparison of gene expression profiles between rat and human, *in vitro* and *in vivo*.

genes possess strict orthologues in the human genome [52]). Completion of the parallelogram might then predict mechanistic changes as they could occur *in vivo* in humans.

Analysis of toxicogenomics data

The amount of gene-expression data generated in a toxicogenomics study is vast. One in *vitro* toxicogenomics experiment conducted in triplicate investigating one compound and its effects on gene expression profiles in three dose groups at two time-points already requires twenty-four microarrays and the attendant measurement of as many as 20,000 or more transcripts per array. The careful collection, management, and integration of these data is essential for interpreting toxicological outcomes [33]. The challenge of toxicogenomics studies is to turn the large raw data sets with relatively high amounts of noise and without obvious biological meaning into relevant conclusions [30, 53]. Accepted methods applied for this purpose first normalize the data to adjust for differences between arrays in labeling and detection efficiencies for the fluorescent labels and for differences in the quantity of initial RNA from the two samples examined in the assay. After normalization, data are typically given as 'expression ratios' and offer the opportunity to look at 'differentially expressed genes'. These genes are selected using a post-normalization cut-off, e.g. a twofold increase or decrease in measured level [54, 55]. More sophisticated methods have been developed in which computed confidence intervals are used to determine differentially expressed genes [56]. The next sections will describe some higher-order computational tools and procedures which are applied to assist with the interpretation and visualization of complex multivariate microarray gene expression data [54, 55].

Data-driven analysis methods Examining a dataset on a gene-by-gene basis is time consuming and difficult to carry out across an entire dataset. One way of accelerating data interpretation is to approach the data from a higher level of organization. This can be done using data-driven methods, such as hierarchical clustering [57], k-means clustering [58], Self-Organizing Maps (SOMs) [59, 60], and Principal Component Analysis (PCA) [61], which identify groups of genes with similar expression patterns. These cluster methods are referred to as unsupervised methods. Supervised methods represent a powerful alternative for identifying patterns of gene expression and can be applied if one has some previous information about which genes are expected to cluster together. Support Vector Machines (SVMs) are an example of such supervised methods and have been successfully applied to discriminate different classes of toxicants [43]. Different data-driven and statistical methods to analyze microarray data are extensively reviewed [55, 62].

Biochemical pathway and biological process analysis A complementary approach to data-driven analysis methods is partitionate genes at the level of known biochemical pathways and biological processes [63]. Investigation of the interrelationship between genes in a biological context may significantly increase the biological information that can be retrieved from a single toxicogenomics experiment [64]. Several initiatives have contributed to the development of tools which enable investigation of the underlying pathways and processes affected by a certain compound.

The Gene Ontology (GO) consortium [65] took the initiative to develop an ontology for genes that describes the biological process that refers to a biological objective to which the gene or gene product contributes, the molecular function defined as the biochemical activity of a gene product, and the cellular component, the intracellular target of the gene product [66]. A particular gene can contribute to one or more biological processes, can perform one or more molecular functions, and can be contained by one or more cellular components. GO terms were assigned to genes irrespective of the organism the gene was derived from. Therefore, the GO concept enables, in a flexible and dynamic way, the annotation of homologous gene and protein sequences in multiple organisms using a common vocabulary [66].

Although GO analysis results in lists of genes that share biology, function, and/or location within the cell, it lacks the level of biological information which provides the interrelation between the genes, the "ordered assemblies of molecular functions" that collaborate to produce a biological function [64]. Therefore, other approaches are available which aim to describe the inter-connection between genes and proteins in pathway format, and implement these into the analysis: databases were created, based on classical and biochemical investigations, containing a variety of metabolic, regulatory, and signaling pathways. Examples are GenMAPP [67], KEGG [68], Biocarta [69], and Reactome [70].

MAPPFinder is the pathway analysis tool that integrates the different initiatives which developed databases containing biochemical pathways and biological processes [63]. Generally, the user defines a subset of genes, e.g. genes with similar expression patterns resulting from

cluster analysis or statistical pre-selection resulting in subsets of differentially expressed genes, for which MAPPFinder produces a ranked list of pathways and processes over-represented compared to the complete data set of genes. GenMAPP can be used to visualize up- and down-regulation, and/or significance of genes within the over-represented biochemical pathways, depending on the user-defined settings [71]. Commercial pathway analysis tools include PathwayAssist™ [72], ePathArt [73], Ingenuity Pathway Analysis Tool [74], and MetaCore™ [75]. The content of these applications is often pathways and networks derived from text-mining, describing inter-relationships between biological molecules from literature, with selected networks converted towards pathway mapps, curated by domain specific experts.

Recently, pathway analysis methods have been developed for which no user-defined subset of genes needs to be selected prior to analysis. For example, T-profiler is a pathway analysis tool which analyzes distribution of gene expression within a predefined category, either GO terms, pathways, or other gene-sets [76]. Predominance of biological processes or pathways is determined using a *T* test to compare mean expression levels of genes within the processes or pathways with the expression levels of all other genes on the microarray. A group can be scored as significantly induced or repressed even if the expression of none of its individual member genes changes significantly [76]. Another pathway analysis tool which does not require pre-selection and takes into account existing pathways and entire biomolecular network knowledge is Gene Set Enrichment Analysis (GSEA). This tool uses a non-parametric Mann-Whitney statistical test to calculate the *P* value indicating the significance of the enrichment score [77].

Pathway analysis has already shown to be particularly useful in inter-platform comparison of microarray data, as pathway analysis offers translation of genes which sometimes have different annotations across array platforms to a common and therefore easy comparable vocabulary [78]. In the same way, pathway analysis has the advantage to contribute to interspecies comparison of adverse effects of compounds as annotation of similar genes is sometimes (slightly) different between species whereas annotation of biochemical pathways and biological processes is highly homologous. On the other hand, the inter-relation between pathways complicates statistics. Furthermore, pathway analysis methods can result in bias in discovery, since ranking of pathways is based upon the initial biological content.

Phenotypic anchoring

Since the introduction of the microarray technology a decade ago, the technology has established itself in clinical and regulatory settings. Recently, the Microarray Quality Control (MAQC) showed that microarray data generated from different platforms (Agilent, Affymetrix) and at different test sites resulted in similar biological interpretations [78-80]. Although much of the available toxicogenomic data published to date has been limited to a description of alterations in gene expression data, primarily in the format of listings of differentially expressed genes, these new approaches to assess potential adverse affects of drugs and chemicals should not exclude conventional assays of toxicity. Combining the compound-induced gene sets resulting

from toxicogenomics studies with suitable conventional assays (e.g. histopathology and clinical chemistry assays *in vivo*; MTT and LDH assays *in vitro*) helps to distinguish the true toxicological effect from other gene expression changes unrelated to toxicity [81]. This phenotypic anchoring may yield better interspecies extrapolation, better comparison between systems (*in vitro* versus *in vivo*; various hepatocyte-based *in vitro* systems), and, of course, better insights into pathways of toxicity and disease processes. Gene expression profiling has been successfully characterized for its ability to reflect the results derived from conventional toxicology assays through phenotypic anchoring in a study in which toxicogenomic results of subtoxic and toxic doses of acetaminophen [51] were linked to GSH content in the liver, nitrosine protein adducts, and 8-OH-dG DNA lesions [82].

MODEL COMPOUNDS UNDER INVESTIGATION

As has been mentioned above, toxicogenomics has promise to facilitate *in vitro*-to-*in vivo* and interspecies extrapolation by generation of easily comparable compound-induced gene expression profiles in biological model systems. Phenotypic anchoring as well as the use of well documented model compounds contributes to the identification of the true toxicological responses in these systems. The compounds under investigation in the studies described in this thesis are coumarin, thioacetamide, and acetaminophen, all well-known liver toxicants causing centrilobular necrosis, thus providing excellent opportunities for phenotype anchoring of gene expression profiles as generated from hepatocytes *in vitro*. Documented toxicological properties of these compounds will be described in the following sections.

Coumarin

Coumarin (1,2-benzopyrone) is a natural product found in many plants and essential oils. Coumarin has a pleasant odor and is used as a fragrance ingredient in perfumes, cosmetics, soaps and detergents [83]. Coumarin has a clinical value in the treatment of high protein lymphoedema [84] and metastatic renal carcinoma [85], and as a precursor of several anticoagulants, notably warfarin. Coumarin was banned in the USA in 1954 based on reports of hepatotoxicity in rats, causing severe centrilobular necrosis, and was recommended for withdrawal from use in the UK in 1965 [84, 86, 87]. Consecutive research revealed that coumarin is metabolized quite differently in man compared to the rat and other species [86]. The severe hepatotoxicity caused by coumarin in rats depends on conversion of coumarin to the toxic metabolite coumarin 3,4-epoxide (CE) by CYP450 enzymes of the 1A and 2E subfamily [88, 89]. CE rearranges spontaneously to the more stable *o*-hydroxyphenylacetaldehyde (*o*-HPA). Both CE and *o*-HPA are assumed to contribute to the hepatotoxicity of coumarin in rats as they conjugate with critical cellular macromolecules [83, 90-94].

In humans, coumarin 7-hydroxylation (7-HC), catalyzed by hepatic CYP2A6, is the major route of coumarin biotransformation in most subjects [86-88, 95, 96]. 7-Hydroxycoumarin and its glucuronide and sulfate conjugates are nontoxic and water soluble, as a cause, they are readily excreted in the urine following an oral dose in most subjects [84, 89, 95]. Reports of adverse effects in humans resulting from coumarin administration are therefore rare [83, 84, 87]. Still, genetic polymorphisms in CYP2A6 can result in deficiency of the 7-HC pathway [87] and minor routes comprising biotransformation via human CYP1A and 2E subfamilies can still result in formation of the toxic metabolites CE and *o*-HPA [97].

Thioacetamide

Thioacetamide (CH3-C(S)NH2) was first used to control the decay of oranges and then as a fungicide. Now it is being used in leather, textile, and paper industries as an accelerator in the vulcanization of buna rubber and as a stabilizer for motor fuels. Metabolism via CYP2E1 appears to be the primary mechanism underlying thioacetamide-induced liver injury [98]. Studies have shown that thioacetamide is S-oxidized at the thioamide group to thioacetamide sulfoxide (CH3-C(SO)NH2) and subsequently to di-S-oxide (CH3-C(SO2)NH2). The reactive intermediate(s) in this pathway covalently bind to hepatic macromolecules and eventually cause centrilobular necrosis [99, 100]. For this adverse affect, thioacetamide has been used in several toxicogenomic studies in order to identify classes of compounds [37, 41]. Problems might be expected when using thioacetamide for investigation of the relevance of *in vitro* systems for the situation *in vivo*, as it has been reported that although thioacetamide is a very potent hepatotoxicant *in vivo*, the toxic potency is less in conventional hepatocyte-based *in vitro* systems [101].

Acetaminophen

Acetaminophen or paracetamol (N-acetyl-para-aminophenol) is a widely used over-the-counter analgesic and antipyretic drug. The drug is safe at therapeutic doses but causes liver failure, centrilobular necrosis, when overdosed. Although several CYP450s have been reported to metabolize acetaminophen among which CYP1A2 [102], CYP3A4 [103, 104], and CYP2A6 [105], metabolic activation of acetaminophen by CYP2E1 to N-acetyl-*p*-benzoquinone imine (NAPQI) is the most critical step in acetaminophen toxicity [106, 107]. NAPQI reacts with hepatic glutathione (GSH) leading to its depletion by as much as 90% [108]. Once, when glutathione-conjugation capacity is insufficient, NAPQI covalently binds to cellular proteins including proteins of the plasma membrane and mitochondria [109, 110]. Although correspondence has been observed between conventional rat hepatocyte *in vitro* systems and rats *in vivo* using the proteomics approach [49, 111], the difference in concentrations applied *in vitro* and plasma concentrations reached *in vivo* are more than two-fold [110]. Therefore, the relevance of *in vitro* results to the situation *in vivo* of the available publications on acetaminophen remains questionable [110]. Considering the species differences in response to acetaminophen, rats have been reported to be relatively immune to acetaminophen, since they convert most of the compound to other metabolites than NAPQI [109].

AIMS AND OUTLINE OF THE THESIS

Objective of the thesis

As the liver is the most important target organ of toxicity, conventional toxicology studies have a major focus on compound-induced endpoints in livers of laboratory animals or hepatocyte-based *in vitro* systems. The number of endpoints that can be measured at once has been incredibly enhanced since the introduction of toxicogenomics. In particular, the microarray technology provides thousands of endpoints, namely, gene expression profiles reflective of hepatotoxicity. Furthermore, the endpoints measured using the toxicogenomics approach are similar in *in vivo* models as well as hepatocyte-based *in vitro* systems. Especially herein lies the promise of toxicogenomics to improve the classical parallelogram approach as these similar endpoints, gene expression profiles, differentially expressed genes or biological responses given as toxicant-induced pathways and processes, may be compared *in vitro*-to-*in vivo* and between species, enabling extrapolation of the observed responses to the situation in humans *in vivo*.

The objective of this thesis is more reliably predict toxic responses which would occur in livers of humans upon exposure to well-known hepatotoxicants using a toxicogenomics-based parallelogram approach. Hypothetically, the reliable extrapolation of hepatotoxicant-induced gene expression profiles identified in well-established rat hepatocyte-based *in vitro* systems to the situation in rats *in vivo* as well as to responses in primary human hepatocytes, might result in the identification of responses relevant for human exposure, ultimately contributing to the reduction, refinement, and replacement of animal experimentation.

Outline of the thesis

In this thesis, the toxicogenomics-based parallelogram approach was applied to well-established *in vitro* systems, to sandwich-cultured primary rat and human hepatocytes, and to rats *in vivo* exposed to well-known hepatotoxic compounds in order to yield gene expression profiles reflective of hepatotoxicity. Verification by phenotypic anchoring and by documented toxic properties of the well-known compounds was performed to aid in identification of true hepatotoxic responses.

In the first experiments conducted as part of this thesis and described in **Chapter II**, the rat *in vitro* system in which hepatocytes are cultured in sandwich configuration is optimized in order to increase the basal metabolic competence of the system. Therefore, a mixture of well-known CYP450 inducers, phenobarbital, dexamethasone, and β-naphthoflavone is added to culture medium of sandwich-cultured primary rat hepatocytes. In order to evaluate the value of this improved hepatocyte system, gene expression profiles and CYP450 enzyme activities are compared between the modified model containing enzyme inducers and the standard model, without inducers, both in relation to the gene expression patterns in liver cells.

In **Chapter III**, the toxicogenomics approach is applied to evaluate the relevance of toxicant-induced gene expression profiles in the optimized rat *in vitro* system, the modified model, for the situation in rats *in vivo*. Therefore, sandwich-cultured primary rat hepatocytes either cultured in standard or in modified medium and rats *in vivo* are exposed to the model compound coumarin. Coumarin-induced cytotoxicity data and gene expression profiles generated in both *in vitro* models are compared to toxicity and gene expression profiles in rat livers in the *in vivo* study. The expected differences of bioactivation of coumarin in the standard model versus the modified model are verified by measurement of coumarin and one of its metabolites in medium. In the experiments described in **Chapter IV**, the toxicogenomics-based parallelogram approach is used to identify coumarin-induced responses, toxic or non-toxic, relevant for the situation in humans. Therefore, gene expression in terms of biochemical pathways and biological processes in primary human hepatocytes cultured in sandwich configuration are compared to results obtained in **Chapter III**.

The mode of action of the model compound thioacetamide is investigated using the toxicogenomics approach in livers of rats exposed *in vivo* in the experiments described in **Chapter V**. MAPPFinder analysis is performed to translate thioacetamide-induced gene expression profiles to biochemical pathways and biological processes. Phenotypic anchoring of traditional toxicity measures is used to distinguish true toxic responses. In **Chapter VI**, it is investigated whether analysis of pathways and processes as deduced from gene expression profiles in sandwich-cultured primary rat and human hepatocytes following treatment with thioacetamide can improve interspecies comparison of the toxicogenomics response. MAPPFinder is used to determine over-representation of pathways and processes in the subset of significantly expressed genes; T-profiler is performed to determine enrichment of pathways and processes in the complete data set of genes without pre-selection of a subset. Both approaches are compared for their suitability.

The final study described in **Chapter VII** aims to compare acetaminophen-induced gene expression profiles from rat liver cells *in vitro* with *in vivo* and between rat and human primary hepatocytes in a parallelogram design in order to predict human risk of liver injury upon acetaminophen intake. To this purpose, acetaminophen-induced gene expression profiles, MAPPFinder and T-profiler results in sandwich-cultured primary human and rat hepatocytes are compared to results of a rat *in vivo* study publicly available on the internet (GEO database, Heinloth *et al*., 2006).

Chapter VIII contains the general discussion on *in vitro*-to-*in vivo* comparisons and interspecies extrapolation investigated using this toxicogenomics approach.

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Chapter II

Gene expression profiling of sandwich-cultured rat hepatocytes with increased metabolic competence

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ABSTRACT

A rapid decline of cytochrome P450 (CYP450) enzyme activities remains a drawback of rat hepatocyte-based *in vitro* cultures. Consequently, judgment of the toxic potential of compounds that need bioactivation by CYP450s may not be adequate using this model. In the present study, an improved hepatocyte-based *in vitro* system was developed with special focus on metabolic competence. Therefore, a mixture of CYP450 inducers, phenobarbital, dexamethasone and β-naphthoflavone, was added to culture medium of sandwich-cultured rat hepatocytes. The resulting modified model was evaluated by comparing its genome-wide expression profiles with liver and a standard model without the inducer mixture. Metabolic capacity for CYP450 enzymes showed that the modified model resembled more closely the *in vivo* situation. Gene expression results revealed large differences between *in vivo* and both *in vitro* models. The slight differences between the two sandwich models were predominantly represented by gene expression changes in CYP450s. Importantly, in the modified model, expression ratios of the phase I and the majority of phase II genes more closely resembled liver *in vivo*. The CYP450 enzyme activities corresponded with gene expression data. In conclusion, for toxicological applications using sandwich-cultured hepatocytes, the modified model may be preferred.

INTRODUCTION

Toxicological research extensively relies on data obtained from *in vivo* studies. As *in vivo* biological complexity can not be effectively modeled *in vitro* yet, replacement of these *in vivo* studies is likely to be a long-term prospect [1]. Nevertheless, *in vitro* assays represent a valuable tool for investigating mechanisms of toxicology [2]. Traditionally, as the liver is the main organ for metabolism of many compounds, hepatocyte systems are frequently used for *in vitro* investigations [2, 3].

Evident shortcomings of rat liver *in vitro* systems are represented by the rapid decline of liver specific functions, in particular of cytochrome P450 (CYP450) enzyme activities [4, 5]. Therefore, the *in vitro* models have limited relevance for the *in vivo* situation with respect to chemicals for which metabolism depends on the CYP450 enzyme system. Many options to maintain liver specific functions in hepatocyte-based *in vitro* systems have been investigated. Three interrelated elements of hepatic *in vitro* systems, namely, extracellular matrix, cell-cell contacts and medium composition, are indispensable for optimal expression of hepatic structure and function [6]. In hepatocytes cultured in sandwich configuration, between two layers of collagen, these three factors can be sustained. Collagen mimics the extracellular matrix resulting in matrix-induced polarization of hepatocytes which greatly enhances hepatocyte viability and morphology, as was first shown by Dunn *et al*. [7]. Furthermore, extracellular matrix overlay and cell-cell contacts in sandwich-culture facilitate the formation of functional bile canalicular networks and gap junctions in hepatocytes [7].

A recent study on *in vitro* hepatotoxicity of several known *in vivo* liver toxicants showed that the collagen sandwich model appeared to be a good model to study toxicity of direct acting compounds. Due to loss of metabolic enzyme activity, however, compounds that need to be metabolically activated by the CYP450 enzyme system were poorly converted to the toxic metabolites [8]. Further, a genomics approach was used to investigate the relevance of various hepatic *in vitro* models for the *in vivo* situation [4]. Gene expression profiles of primary hepatocytes either cultured conventionally or in a collagen sandwich-culture, liver slices and immortal cell lines of liver origin were compared with gene expression profiles of the *in vivo* liver. No gene expression profiles of any of these *in vitro* systems appeared to be comparable to the *in vivo* situation [4]. Thus, even though extracellular matrix and cell-cell contact are preserved as much as possible in sandwich-cultured hepatocytes, this system does not fully represent the *in vivo* situation and as a consequence can not represent toxicities of compounds that require activation.

In the aim to develop an improved hepatocyte-based *in vitro* system with special focus on metabolic competence, in the present study a mixture of the known CYP450 inducers phenobarbital (PB), dexamethasone (DEX) and β-naphthoflavone (β-NF) was added to culture medium of sandwich-cultured rat hepatocytes. These inducers are known to induce Cyp2b1/2, Cyp3a

and Cyp1a1/2, respectively. Both PB and DEX have already been used as medium supplements by others, separately or in combination [6, 9, 10], however, the combination with β-NF is unique.

In order to evaluate the value of this improved hepatocyte system, gene expression profiles and CYP450 enzyme activities were compared between the modified model containing enzyme inducers and the standard model, without inducers, both in relation to the gene expression patterns in liver cells.

METHODS

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), gentamycin, phosphate buffered saline (PBS) and TRIzol™ were obtained from Invitrogen (Breda, The Netherlands). Glucagon, hydrocortisone hemisuccinate, PB, DEX, β-NF, Krebs Henseleit buffer (KHB) and testosterone were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Collagenase type B was purchased from Roche (Mannheim, Germany). The RNeasy minikit was obtained from Qiagen, Westburg B.V. (Leusden, The Netherlands). Cyanine 5-CTP and cyanine 3-CTP were purchased from Perkin Elmer (Boston, MA). Agilent's low RNA input fluorescent linear amplification kit and the hybridization solution were obtained from Agilent Technologies (Palo Alto, CA). Testosterone metabolites were purchased from Steraloids (Newport, RI). All other chemicals were of analytical grade.

Animals

Male Wistar rats (Crl: (WI) WU BR), 9-12 weeks of age, 180-250 g, were obtained from Charles River GmbH, Sulzfeld, Germany. During the acclimatization period and until sacrifice, the animals were housed individually in macrolon cages with wire tops and sawdust bedding at 22 °C and 50-60% humidity. The light cycle was 12h light/12h dark. Feed and tap water were available ad libitum.

Culture of rat hepatocytes

Hepatocytes were isolated from three individual rats according to a two-step collagenase perfusion technique as described by Seglen [11, 12] with minor modifications [13]. Hepatocyte preparations with viability greater than 85% as determined by Trypan Blue exclusion were used and cultured on collagen gel 14 pre-coated 6-well plates at a density of 1.25×10^6 cells per well in sandwich configuration. Sandwich-cultures were essentially prepared by the method of Beken *et al.* [15]. Hepatocytes were allowed to attach for 4h in DMEM supplemented with 10% FCS, insulin (0.5 U/ml), glucagon (7 ng/ml), gentamycin (50 µg/ml). After attachment, dead cells were removed by washing and the upper collagen layer was applied. Cells were kept in serum-free culture medium. For the standard system, standard culture medium consisted of DMEM

supplemented with insulin (0.5 U/ml), glucagon (7 ng/ml), hydrocortisone hemisuccinate (7.5 µg/ml) and gentamycin (50 µg/ml). In the modified system, sandwich-cultured hepatocytes were maintained in standard culture medium enriched with a mixture of inducers consisting of 1 mM PB, 10 µM DEX and 5 µM β-NF. These concentrations were selected as they are classically used for specific CYP450 induction [16]. The mixture showed no effects on cytotoxicity as measured by the MTT reduction and LDH leakage assay (data not shown). PB was added as a concentrated stock solution in PBS. DEX and β-NF were added in dimethylsulphoxide (DMSO). The final DMSO concentration was equalized in all culture media to 0.2% (v/v). Cultures were incubated at 37 °C in a humidified incubator gassed with 5% CO_2. Medium was changed on a daily basis during a period of 72h.

Microarray analysis

Hybridization design Gene expression profiling was conducted on hepatocytes cultured in triplicate at 72h of culture. Additionally, gene expression profiling was performed on whole liver samples obtained from the livers of three rats of the same strain and same age as the rats used for hepatocyte isolation. For microarray studies, all samples (liver and cultured hepatocytes) were hybridized against a reference RNA set consisting of a pool of three liver samples, resulting in a total of nine microarrays.

Total RNA extraction RNA was isolated using TRIzol™ according to the manufacturer's protocol. Pieces of liver were directly homogenized into TRIzol™. For cultured hepatocytes, TRIzol™ was added onto the upper collagen layer and cells were collected. RNA was purified using the RNeasy minikit including an additional DNA digestion step. The RNA concentration was determined spectrophotometrically by UV-absorbance at 260 nm. RNA quality was determined using the Bioanalyzer (Agilent Technologies, Palo Alto, CA). All samples contained intact total RNA with a 28S/18S rRNA ratio>1.5.

Labeling RNA samples from individual liver samples and hepatocyte cultures either maintained in standard or modified culture medium were labeled with cyanine 5-CTP, whereas the common reference consisting of pooled liver RNA was labeled with cyanine 3-CTP. Labeling was performed using Agilent's low RNA input fluorescent linear amplification kit following manufacturer's instruction. Briefly, double-stranded cDNA was synthesized using MMLV-RT with T7 promoter primer, starting with 1.5 µg of total RNA. Cyanine-labeled cRNA targets were transcribed using T7 RNA Polymerase. The amplified cRNA was purified using RNeasy mini spin columns. Synthesized cRNA products were quantified spectrophotometrically.

Hybridization For microarray hybridization, Cy5-sample cRNA and Cy3-common reference cRNA were combined. cRNAs were fragmented at 60 °C for 30 min with fragmentation solution followed by hybridization on Agilent 22K format 60-mer oligo microarrays (G4130A from Agilent

Technologies, Palo Alto, CA) for 17h at 60 ºC with Agilent hybridization solution. Arrays were washed according to manufacturer's instruction. Microarrays were scanned using a Packard Scanarray Express confocal laser scanner (PerkinElmer, Boston, MA). Resulting TIFF images were loaded into Imagene 5.0 (Biodiscovery Inc. El Segundo, CA) to further process and collect the gene expression data.

Data analysis Data were transferred to GeneSight 4.1 (Biodiscovery Inc. El Segundo, CA). Flagged spots, consisting of poor quality spots, negative and positive controls, were excluded. For each spot, median local background intensity was subtracted from the median spot intensity and spots from low expression genes (with a net intensity of <20 in both channels), were excluded from further analysis. These background-corrected median intensities were log transformed by base 2. Data were normalized using the Lowess algorithm [17]. All genes that passed these criteria in the standard model, the modified model and liver were used for principal component analysis (PCA) (provided by GeneSight 4.1). Additionally, gene expression ratios were loaded into Excel (Microsoft Corporation, Redmond, WA). For further analysis, at least two values per gene had to be available for a gene to be selected. A paired two-sided *T* test (with random variance model) was performed on the resulting dataset using BRB-Array Tools Version 3.4.0 Beta_1 [18] to select a subset of genes that changed significantly ($P<0.05$ and a difference in log 2 expression ratio between the standard model and the modified model of at least 0.5) between the standard model and the modified model. Gene Ontology (GO) mapping was performed on the complete data set with a filtering on the subset of genes significantly changed between the standard model and the modified model to determine significantly changed GOs between the standard model and the modified model using GenMAPP (version 2.0, Gladstone Institutes 2000-2004). GO terms were developed within the GO project and provide a controlled vocabulary to describe gene and gene product attributes in any organism [19]. The three organizing principles of GO are molecular function, biological process and cellular component. A gene product may have one or more molecular functions, is used in one or more biological processes and might be associated with one or more cellular components.

Real-time PCR

Genes of particular interest (*Cyp1a1* and *Cyp2b1/2*) which were not present on the array or which were filtered out due to low expression values were measured using real-time PCR. Double-stranded cDNA which was synthesized in the labeling step during the microarray procedure was diluted 1:20 with MQ. For real-time PCR, 10 µl of this cDNA solution was used. Primers were designed using the Beacon Designer V4 software. Standard curves for *Cyp1a1*, *Cyp2b1/2* and β-actin were generated on serial dilutions of control PCR products containing the cDNA of interest (calculated in copy numbers). For efficient real-time PCR assays the slope of the standard curves should be between -3.1 and -3.5 and the correlation coefficient should be at least 0.980. In addition, blank controls should not show any fluorescent signal. Melting

Table 1. Primers and probes used for rat *Cyp1a1*, *Cyp2b2* and β-actin in Taqman assay.

Gene Symbol	Primers and Probes	Sequence 5'to 3'
Cyp1a1	Forward primer	ACAGACCTCAGCTGCCCTATCT
	Reverse primer	TGAATGGGACAAAGGATGAATG
	Probe	5'-FAM- AGGCCTTCATCCTGGAGACCTTCCG-TAMRA-3'
Cyp2b1/2	Forward primer	GGTGGAGGAACTGCGGAAAT
	Reverse primer	TGATGCACTGGAAGAGGAAGGT
	Probe	5'-FAM-CCAGGGAGCCCCACTGGATCC-BHQ1-3'
β-actin	Forward primer	TTCAACACCCCAGCCATGT
	Reverse primer	GTGGTACGACCAGAGGCATACA

FAM, 6-carboxyfluorescein fluorescent reporter dye; TAMRA, 6-carboxytetramethylrhodamine quencher dye; BHQ1, black hole quencher 1.

curves should show only one specific melting peak. Copy numbers of *Cyp1a1*, *Cyp2b1/2* and β-actin were determined in all samples using real-time PCR assays in an iCycler iQ (Biorad, Veenendaal, The Netherlands). Real-time PCR assays for *Cyp1a1* and *Cyp2b1/2* were performed using TaqMan probes. For β-actine a SYBR Green assay was used. Probe and primer sequences are shown in Table 1. In short, in a 96-wells plate calibration samples were mixed with a real-time PCR solution containing forward and reverse PCR primers, Taq polymerase, dNTP's and SYBR Green (for β-actin) or a TaqMan probe (for *Cyp1a1* and *Cyp2b1/2*). Real-time PCR reactions using SYBR Green were run using the following protocol: an initial activation step of at 95 °C for 3 minutes was followed by 45 cycles of 15 seconds at 95 °C, 63 °C for 30 seconds and 72 °C for 20 seconds. Fluorescence was monitored during the 63 °C step. Subsequently, a melting curve was generated by increasing the set point temperature from 53 °C to 94 °C by 0.5 °C and measuring the fluorescence. Real-time PCR reactions using a TaqMan probe were run using the following protocol: an initial activation step of at 95 °C for 10 minutes was followed by 45 cycles of 15 seconds at 95 °C and 1 minute at 60 °C where fluorescence was monitored. *Cyp1a1*/β-actine and *Cyp2b1/2*/β-actine ratios were calculated to normalize *Cyp1a1* and *Cyp2b1/2* mRNA expression. Similar to microarray results, ratios were base 2 log transformed and adjusted for values measured in liver.

Testosterone hydroxylation

Enzyme activities were determined in freshly isolated hepatocytes and in hepatocytes cultured for 72h in either the standard or modified medium. Determination of testosterone hydroxylation activity was as previously described [20]. In brief, freshly isolated hepatocytes were diluted to a concentration of 6.25 x 10^5 cells/ml in non-supplemented FCS-free DMEM. Hepatocytes were divided into portions of 2 ml each in 5 ml flasks. After 72h of culture in sandwich configuration with standard or modified medium, the medium was replaced by KHB (9.6 g/L KHB, $CaCl_2 \cdot 2H_2O$ (2.54 mM), $NaHCO_3$ (25 mM), pH 7.4). A concentrated stock solution of testosterone in methanol was added to obtain a concentration of 125 μM (final methanol concentration in medium was 0.6%). Incubation mixtures for freshly isolated hepatocytes were oxygenized (95% O_2; 5% CO_2) and kept at 37 °C in a shaking water bath. Testosterone incubations with hepatocytes

in sandwich configuration were performed in a humidified atmosphere of air (95%) and CO_2 (5%) at 37 ºC. After one hour incubation, testosterone and its metabolites were extracted from the media with dichloromethane and subsequently analyzed using a HPLC-UV method on a reversed-phase Symmetry® C18, 3.5 µm, 150 x 2.1 mm column (Waters Chromatography B.V., WAT106005, Etten-Leur, The Netherlands), detection wavelength 242 nm. From 0 to 45 minutes, the column was eluted with an aqueous solution of methanol and acetonitrile in a linear gradient, with the methanol and acetonitrile concentration increasing from 29% to 48% and 0.6% to 3.5%, respectively. After 50 minutes, the mobile phase consisted of 52% methanol and 4.2% acetonitrile, where after it remained isocratic during the next 5 minutes. Elution proceeded at a temperature of 48 ºC and 0.3 ml/min.

Additionally, HPLC analysis was performed on known concentrations of authentic standards (15β-OH-, 6β-OH-, 19-OH-, 7α-OH-, 16α-OH-, 16β-OH-, 2α-OH-testosterone and androstenedione). Concentrations of metabolites were quantified by comparing the ratio (metabolite/internal standard) with the ratios resulting from the calibration curves of the authentic standards, using TotalChrom™ Chromatography Software, version 6.2.0 (Perkin Elmer Boston, MA).

RESULTS

Complete set of genes

To investigate which *in vitro* system most closely resembled the *in vivo* situation in terms of its gene expression profile, PCA was performed on the complete set of genes (Fig. 1). The

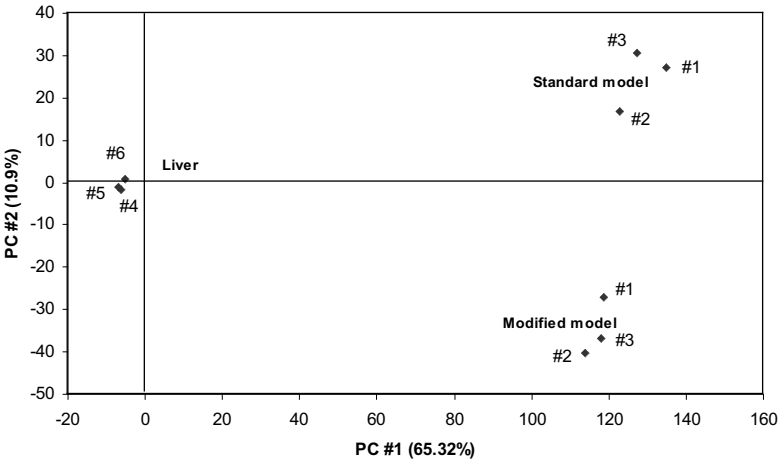

Figure 1. Principal component analysis of a combined dataset of log 2 gene expression data from both *in vitro* models (rat #1, #2 and #3) and rat liver (rat #4, #5 and #6). Each symbol represents an independent experiment. Two principal components were generated and plotted for each system and dose group.

expression profiles of the two *in vitro* models appeared to differ much less from each other than from the liver *in vivo*. The standard model and the modified model were discriminated by principal component (PC) #2, which contains a small proportion of the total variance in the dataset (10.9%), while PC #1, containing most of the variance (65.32%), separates the *in vivo* gene expression profiles from those *in vitro*.

The data set prepared for GO mapping using GenMAPP contained 7437 genes with expression ratios in the standard model, the modified model and liver *in vivo*. Of these genes, 1321 were significantly differentially expressed between the standard model and the modified model ($P<0.05$, log 2 expression difference>0.5). Twelve GO terms, covering molecular functions (F) and biological processes (P), were significantly changed between the standard model and the modified model (Z score>1.96; number of genes changed>5), as presented in Table 2. Thereafter, it was investigated whether expression of genes within the GO terms was closer to liver *in vivo* in either the standard model or the modified model. For this purpose, for both the standard and the modified model, the proportion of genes with expression values that more closely resembled those in liver were calculated. For ten out of the twelve GO terms, the expression of the majority of gene in the modified model more closely resembled liver expression levels, as opposed to the standard model. Within two GO terms, "unspecific monooxygenase activity" and "heme binding", even more than 90% of the genes in the modified model better resembled liver. All changed genes that appeared closer to liver in the modified model in these two molecular functions were CYP450s.

Table 2. Significantly changed Gene Ontologies between the standard model and the modified model.

GO Name	GO type $	*Z* score	Total number measured genes	Total number changed genes	% genes closer to liver	
					Standard model	Modified model
Unspecific monooxygenase activity	F	3.08	16	8	0	100
Heme binding	F	2.12	32	11	9	91
Iron ion binding	F	3.72	70	25	20	80
Inflammatory response	P	2.65	12	7	29	71
Lyase activity	F	3.05	30	12	33	67
Oxidoreductase activity	F	3.00	130	37	35	65
Apoptosis	P	2.22	31	8	38	63
Calcium ion binding	F	2.00	107	29	41	59
Metal ion binding	F	2.59	199	60	45	55
Immune response	P	2.47	29	11	45	55
Cell adhesion	P	2.62	28	11	55	45
Protein binding	F	2.03	171	45	60	40

$ GO terms covering molecular functions (F) and biological processes (P).

Phase I and phase II biotransformation enzymes

Expression ratios of all phase I and phase II biotransformation genes were selected from the complete gene expression dataset and are presented as means ± SD in Table 3 and Table 4, respectively. No expression values were obtained from the toxicologically relevant CYP450s *Cyp1a1* and *Cyp2b1/2* on the microarray. Therefore, these genes were additionally analysed using real-time PCR. Log 2 expression ratios of the real-time PCR results were included in Table 3. Compared to the expression values for the standard model, the expression of most CYP450s in the modified model was closer to that in liver (Table 2). The expression values of many CYP450s were significantly different between the standard model and the modified

Table 3. Log 2 expression ratios of mRNA levels for phase I enzymes in the standard model, the modified model and liver.

Genbank Accession Number	Gene Symbol	Liver	Standard Model	Modified Model
NM_012541	Cyp1a2*	-0.63 ± 0.74	-2.35 ± 0.22	0.91 ± 0.10
NM_012692	Cyp2a1**	-0.15 ± 0.13	-1.70 ± 0.05	0.05 ± 0.09
NM_012693	Cyp2a2*	-0.30 ± 0.09	-2.46 ± 0.35	-1.38 ± 0.12
NM_198733	Cyp2b21	0.22 ± 0.03	-2.53 ± 0.61	-2.08 ± 0.24
NM_019184	Cyp2c	0.12 ± 0.15	-3.31 ± 0.84	-2.57 ± 0.44
NM_017158	Cyp2c7	0.07 ± 0.25	-2.90 ± 0.71	-2.39 ± 0.37
NM_031839	Cyp2c23	0.14 ± 0.20	-2.42 ± 0.65	-2.45 ± 0.54
NM_031839	Cyp2c23	0.14 ± 0.09	-1.65 ± 0.37	-1.59 ± 0.27
NM_031572	Cyp2c40*	-0.01 ± 0.05	-3.66 ± 1.14	-2.48 ± 0.37
NM_173304	Cyp2d10	-0.29 ± 0.12	-1.41 ± 0.33	-1.56 ± 0.40
NM_138515	Cyp2d22	0.01 ± 0.13	-0.31 ± 0.11	-0.51 ± 0.12
NM_012730	Cyp2d26	-0.45 ± 0.30	-2.13 ± 0.23	-1.68 ± 0.34
NM_031543	Cyp2e1*	-0.17 ± 0.43	-3.05 ± 0.59	-2.28 ± 0.37
NM_019303	Cyp2f2**	-0.08 ± 0.07	-1.45 ± 0.30	-0.39 ± 0.36
NM_023025	Cyp2j4	0.11 ± 0.28	-0.51 ± 0.14	-0.41 ± 0.06
NM_175766	Cyp2j9*	0.22 ± 0.10	-2.40 ± 0.35	-1.68 ± 0.09
NM_134369	Cyp2t1	-0.05 ± 0.18	-1.10 ± 0.26	-1.11 ± 0.10
NM_013105	Cyp3a3**	0.11 ± 0.25	-3.59 ± 1.05	-0.16 ± 0.47
NM_153312	Cyp3a11	-0.02 ± 0.28	-4.04 ± 1.71	-2.68 ± 0.51
NM_147206	Cyp3a13*	-0.20 ± 0.92	-3.22 ± 0.83	-2.12 ± 0.29
NM_145782	Cyp3a18*	-0.16 ± 0.52	-3.19 ± 0.78	-2.28 ± 0.29
NM_031605	Cyp4a12	-0.21 ± 0.33	-1.52 ± 0.19	-1.45 ± 0.20
NM_016999	Cyp4b1**	-0.05 ± 0.27	2.42 ± 0.29	0.29 ± 0.26
NM_019623	Cyp4f2	-0.04 ± 0.14	-1.08 ± 0.19	-1.21 ± 0.20
NM_173123	Cyp4f4*	-0.15 ± 0.12	-2.58 ± 0.40	-2.06 ± 0.31
NM_031241	Cyp8b1	-0.23 ± 0.31	-2.70 ± 0.53	-2.41 ± 0.44
NM_017286	Cyp11a1	0.14 ± 0.08	0.00 ± 0.05	0.17 ± 0.08
NM_199401	Cyp20a1	0.06 ± 0.08	0.89 ± 0.22	0.88 ± 0.24
Real-time PCR	Cyp1a1*	0.00 ± 0.36	5.21 ± 2.65	11.35 ± 0.13
Real-time PCR	Cyp2b1/2**	0.00 ± 1.80	-6.32 ± 0.19	-1.02 ± 1.21

*Expression is significantly different ($P<0.05$) between the standard model and the modified model; **Similar expression values in the modified system and *in vivo*. Log 2 expression values for *Cyp1a1* and *Cyp2b1/2* were measured by real-time PCR analysis.

Table 4. Log 2 expression ratios of mRNA levels for phase II enzymes in the standard model, the modified model and liver.

Genbank Accession Number	Gene Symbol	Liver	Standard model	Modified model
NM_012844	Ephx1*	-0.50 ± 0.28	1.56 ± 0.42	0.53 ± 0.40
NM_022936	Ephx2	-0.42 ± 1.66	-0.15 ± 0.16	0.29 ± 0.02
NM_012792	Fmo1*	-0.35 ± 0.21	-3.33 ± 0.89	-2.42 ± 0.45
NM_053433	Fmo3	0.49 ± 0.33	-0.99 ± 1.53	-0.63 ± 0.16
NM_017013	Gsta2*	0.02 ± 0.30	-2.04 ± 0.30	0.93 ± 0.24
NM_031509	Gsta5	-0.11 ± 0.22	-0.77 ± 0.25	-0.69 ± 0.18
NM_017014	Gstm1	-0.19 ± 0.20	1.49 ± 0.22	1.94 ± 0.33
NM_031154	Gstm3	-0.13 ± 0.20	-2.11 ± 0.25	-1.63 ± 0.28
XM_342062	Gsto1	-0.17 ± 0.07	-0.51 ± 0.11	-0.95 ± 0.30
NM_053293	Gstt1	-0.31 ± 0.30	-0.88 ± 0.15	-0.49 ± 0.25
NM_134349	Mgst1	-0.11 ± 0.07	-1.28 ± 0.12	-1.07 ± 0.12
NM_053853	Nat1	0.04 ± 0.12	-1.34 ± 0.15	-1.31 ± 0.19
NM_012818	Nat4	0.01 ± 0.05	-0.25 ± 0.11	-0.16 ± 0.09
XM_342534	Nat5	0.14 ± 0.15	0.49 ± 0.19	0.45 ± 0.19
NM_022635	Nat8*	-0.05 ± 0.57	-2.93 ± 0.54	-1.52 ± 0.40
NM_031834	Sult1a1*	0.08 ± 0.16	-2.15 ± 0.29	-1.44 ± 0.28
NM_031732	Sult1a2*	0.13 ± 0.14	-3.37 ± 0.81	-2.63 ± 0.55
NM_022513	Sult1b1	0.06 ± 0.17	-1.72 ± 0.17	-1.64 ± 0.31
NM_133547	Sult1c2	0.11 ± 0.30	0.41 ± 0.38	-0.04 ± 0.41
NM_130407	Ugt1a7*	-0.50 ± 0.01	0.20 ± 0.22	1.40 ± 0.27
NM_173295	Ugt2b1*	-0.14 ± 0.24	-2.29 ± 0.23	1.32 ± 0.34

*Expression is significantly different ($P<0.05$) between the standard model and the modified model.

model (genes marked with an asterisk). For a subset of these genes (*Cyp2b1/2*, *Cyp4b1*, *Cyp3a3*, *Cyp2f2* and *Cyp2a1*, annotated in bold), the expression was not significantly different anymore between liver and the modified system. Gene expression of *CYP1a1* however, was increased in the modified system to a level less similar to liver compared to the standard model. For the phase II genes fewer differences existed between the standard and modified model. Also here, however, several genes were significantly differentially expressed between both *in vitro* models, and in most of these cases (except for *Ugt1a7*) the values for the modified model were closer to those for the liver *in vivo* (Table 3).

Testosterone hydroxylation

Phase I enzyme activity levels were measured in freshly isolated hepatocytes and in hepatocytes cultured in standard and modified medium using the testosterone hydroxylation assay. CYP enzyme activities are presented in Fig. 2. These were highest in freshly isolated hepatocytes. Activities of Cyp2c13 and Cyp2c12 (15α-hydroxylase), Cyp3a1 and Cyp1a1/2 (6β-hydroxylase) and Cyp2a1 (7α-hydroxylase) in the modified model more closely resembled activities in freshly isolated hepatocytes. However, in the modified model, activities of CYP450s generating the 16α-, 16β-, 2α- and androstenedione metabolites are equally different or more different from activities in freshly isolated hepatocytes compared to hepatocytes cultured in the standard model.

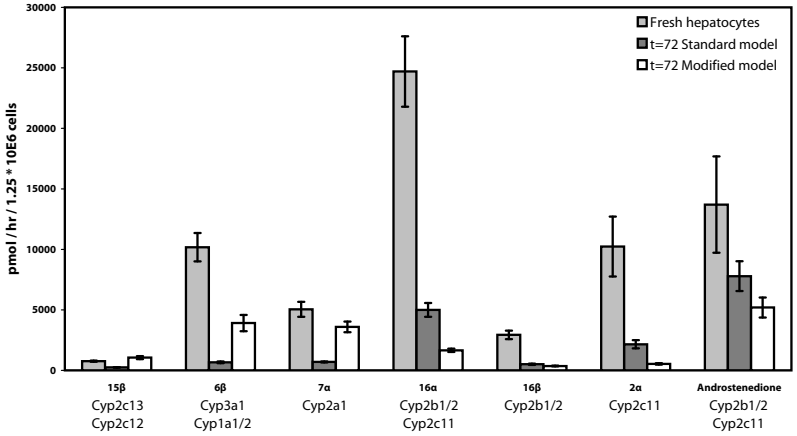

Figure 2. CYP450 activities in the standard and modified model after 72h of culture, and in freshly isolated hepatocytes, as measured by the testosterone hydroxylation assay. The X-axis indicates the testosterone metabolites and the CYP450s by which they are formed [33,34]. The Y-axis represents the amount of metabolite formed per 1.25 x 10^6 cells per hour. Data represent means ± SD (n=3).

DISCUSSION

Hepatocyte *in vitro* systems represent a valuable tool for investigating mechanisms of toxicity and pharmacology as well as for serving as screening tools in the development of new pharmaceuticals [2]. However, the rapid decline in CYP450 enzyme activities constitutes a serious drawback of these *in vitro* models [4, 5]. Many options have been investigated to overcome this situation; one of these is the use of primary hepatocytes cultured in sandwich configuration. In the present study, an improved hepatic *in vitro* system is presented with special focus on metabolic competence.

To evaluate the relevance of this system for the *in vivo* situation in more detail, gene expression profiles were compared between liver and hepatocytes cultured in sandwich configuration in either standard or modified culture medium. The modified medium consisted of standard culture medium [15, 21, 22] supplemented with a mixture of classical concentrations of the CYP450 inducers PB, DEX and β-NF to increase metabolic competence. Both PB and DEX have already been used as medium supplements by others, separately or in combination [6, 9, 10], however, the combination with β-NF is unique. In addition, this report is the first to characterize the effects of a mixture of enzyme inducers on sandwich-cultured hepatocyte *in vitro*, using microarray based gene expression profiling, and by doing so, provides additional information on a multitude of metabolic pathways. PB predominantly induces enzyme activity levels and mRNA expression of Cyp2b1 and Cyp2b2 in rat liver, while DEX is a known inducer of Cyp3a. Both inducers have shown to result in enhancement of the functional longevity of primary cultures of rat

hepatocytes [6, 9, 10]. β-NF was added to the mixture to enlarge the range of induced CYP450s by a supplementary induction of Cyp1a1 and Cyp1a2.

PCA was employed to discover changes at the level of the total gene expression profile; it revealed that gene expression profiles are very different between rat liver *in vivo* and both hepatocyte systems *in vitro*, which can be attributed to adaptation of cultured hepatocytes to the *in vitro* environment. Compared to the *in vivo* situation, gene expression changes between both *in vitro* systems were much smaller. GO mapping, performed to discover changes in biological processes, molecular functions and cellular localization on the complete dataset of genes, revealed that the majority of the significant changes between the two *in vitro* models include a higher proportion of genes for which gene expression in the modified model is more similar to liver than gene expression in the standard model (Table 1). In some of these pathways, expression of almost all genes was closer to liver in the modified system. The genes in these pathways encode predominantly for phase I metabolism enzymes (CYP450s). For this reason, the expression of phase I genes and phase II genes was studied in greater detail. This again demonstrated that for most phase I genes and many phase II genes, the expression in the modified model was more close to liver than the expression in the standard model (Tables 2 and 3).

In the present study, it was shown that expression of the Cyp3a3 gene was sustained to a level equivalent to liver *in vivo* by the inducer mixture, whereas in the standard model it was highly down-regulated. This *Cyp3a3* gene, alias *Cyp3a23*, encodes for the Cyp3a1 protein, which is known to be inducible by PB and DEX [23]. It has been shown before that Cyp3a1 protein expression can be increased to levels equivalent to those in fresh hepatocytes by the addition of 10 μM DEX to the culture medium [23, 24]. The phenobarbital inducible *Cyp2b1/2*, which was not present on the microarray and therefore measured with real-time PCR, showed similar results. The *Cyp2b1/2* expression level was comparable to liver in the modified model, but down-regulated in the standard model. Additionally, the inducer mixture resulted in up-regulation of *Cyp1a2* expression following treatment with the inducer mixture (most likely due to β-NF), whereas compared to liver this gene was down-regulated in hepatocytes cultured in standard culture medium. *Cyp1a1* expression was up-regulated to levels not comparable to liver anymore. It has been described that both DEX and β-NF synergistically activate the *Cyp1a1* gene [25, 26], probably resulting in its overexpression.

Many studies suggest that phase II enzyme activities are better preserved in culture than phase I enzyme activities [6, 27, 28]. Still, the present study shows that expression of phase II genes in *in vitro* models deviates from expression in liver. In the present study, addition of the inducer mixture resulted in retention of expression levels of the phase II genes *Ugt2b1* (known as the PB-inducible form), *Sult1a1*, *Gsta2* and *Ephx1* to levels more comparable to liver. This is consistent with findings in other studies which report that the constituents of the inducer mixture, PB, DEX and β-NF, induce phase II enzymes [6, 29-32].

To corroborate the gene expression findings, enzyme activities were compared between both hepatocyte *in vitro* models and freshly isolated hepatocytes. It is known that enzyme activities in freshly isolated rat hepatocytes do not differ from enzyme activities in rat liver [20]. Furthermore, mRNA levels of CYP450s were comparable between freshly isolated hepatocytes and whole liver [4]. In the present study, changes in enzyme activity levels in the *in vitro* models of Cyp3a1, Cyp1a1/2, Cyp2a1, and Cyp2c12 compared to *in vivo*, corresponded with changes for gene expression values of the respective genes *Cyp3a3* (encoding for the Cyp3a1 protein [23]), *Cyp1a1/2*, *Cyp2a1*, and *Cyp2c40* (encoding for the Cyp2c12 protein [33]). All, except *Cyp1a1* (see above) were similar or more similar to liver in the modified model compared to the standard model.

The 6β, 7α, 16α, 2α and androstenedione hydroxylation products are the major testosterone metabolites in primary rat hepatocytes [20, 22, 28]. The testosterone metabolite profile in the present study was highly comparable to the profile reported by De Smet *et al*. [22]. They reported that addition of PB increased 7α- and 6β-hydroxytestosterone levels, and addition of DEX resulted in a small increase in levels of these testosterone metabolites. The formation of 6β-hydroxytestosterone has been related to enzyme activities of Cyp3a1, Cyp1a1 and Cyp1a2 [34, 35], whereas the 7α-hydroxytestosterone metabolite is mainly formed by Cyp2a1 (>95%) and is not, or only as a minor product, produced by other CYP450s [34, 35]. Sidhu *et al*. [26] reported that treatment of hepatocytes with both PB and DEX produced an additive effect on Cyp3a1 enzyme induction compared to either agent alone. These findings correspond with results presented here, where 7α- and 6β-hydroxytestosterone levels, reflecting enzyme activities of Cyp3a1, Cyp1a1/2 and Cyp2a1 were increased to levels almost similar to those in freshly isolated hepatocytes. However, a part of the 6β-hydroxytestosterone product formed in the modified model might result from the high expression of *Cyp1a1* compared to liver.

In line with the results of De Smet *et al*. [22], in the present study no inducing effect of the mixture of PB, DEX and β-NF on levels of 16α-hydroxytestosterone, 2α-hydroxytestosterone and androstenedione was observed. Levels of these metabolites were decreased upon addition of the inducer mixture. These results did not correspond with gene expression results, as Cyp2c mRNA levels, which encode for the Cyp2c11 enzyme [36], were equally declined compared to liver and mRNA levels of cyp2b1/2 were similar to liver in the modified model and highly decreased in the standard model. A possible explanation for this discordance might be that gene expression changes might precede changes in enzyme activity and thus are not yet reflected in results of the testosterone hydroxylation assay.

In summary, a modified *in vitro* hepatocyte system is presented in which the metabolic competence, as determined by gene expression analysis and enzyme activity analysis, is preserved to a better extent compared to *in vivo*. Genes encoding for enzymes within the Cyp3a, Cyp1a, Cyp2a, Cyp2b, and Cyp2c subfamilies, are indeed increased and for many the levels are almost equivalent to those in liver *in vivo*. The fact that the Cyp3a subfamily metabolizes more that 50% of all drugs and chemical compounds [23] indicates the importance of this optimal retention of

mRNA levels and enzyme activity in the modified model. Furthermore, the gene expression of phase II enzymes was generally closer to liver in the modified system. In conclusion, for toxico- and pharmacological studies, the modified sandwich-based hepatocyte system presented here offers an improvement over existing models with respect to sustaining metabolic competence *in vitro*. Whether compound-induced toxicity in the modified model indeed better represents the *in vivo* situation, is currently being investigated.

ACKNOWLEDGEMENTS

We wish to thank M. Schut, Dr. N. Treijtel, J. Bogaards, L. LeNoble and R. Doornbos for their help in hepatocyte isolation, culture techniques, the testosterone hydroxylation assay and real-time PCR analysis respectively.

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Chapter III

Toxicogenomics to evaluate coumarin-induced toxicity in primary rat hepatocytes and rats *in vivo*

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ABSTRACT

Sandwich-cultured primary rat hepatocytes are often used as an *in vitro* model in toxicology and pharmacology. Loss of liver specific functions, in particular the decline of cytochrome P450 (CYP450) enzyme activity, however, limits the value of this model for prediction of *in vivo* toxicity. In this study, we investigated whether a hepatic *in vitro* system with improved metabolic competence enhances the predictability for coumarin-induced *in vivo* toxicity by using a toxicogenomics approach. Therefore, primary rat hepatocytes were cultured in sandwich configuration in medium containing a mixture of low concentrations of CYP450 inducers, phenobarbital, dexamethasone, and β-naphthoflavone. A toxicogenomics approach was employed enabling comparison of similar mechanistic endpoints at the molecular level between *in vitro* and *in vivo*, namely compound-induced changes in multiple genes and signaling pathways. Toxicant-induced cytotoxic effects and gene expression profiles observed in hepatocytes cultured in modified medium and hepatocytes cultured in standard medium (without inducers) were compared to results from a rat *in vivo* study. Coumarin was used as a model compound because its toxicity depends on bioactivation by CYP450 enzymes. Metabolism of coumarin towards active metabolites, coumarin-induced cytotoxicity, and gene expression modulation were more pronounced in hepatocytes cultured in modified medium compared to hepatocytes cultured in standard medium. Additionally, more genes and biological pathways were similarly affected by coumarin in hepatocytes cultured in modified medium and *in vivo*. In conclusion, these experiments showed that for coumarin-induced toxicity sandwich-cultured hepatocytes maintained in modified medium better represent the situation *in vivo* compared to hepatocytes cultured in standard medium.

INTRODUCTION

To assess possible hepatotoxicity, conventional studies rely on the use of animal model systems to examine tissue toxin levels, changes in serum levels of hepatic enzymes, and histopathological changes [1, 2]. Simple, well established *in vitro* assays, such as primary hepatocytes, precision cut liver slices, and hepatic cell lines, are increasingly in demand for identifying potential hepatotoxicity in early stages of investigative toxicology and for decreasing attrition rates of drugs during lead optimization [3]. However, extrapolation of *in vitro* results to the *in vivo* situation remains a scientific challenge [4].

Toxicogenomics, the application of the genomics technologies in toxicology, would be particularly useful in the extrapolation from *in vitro* experiments to the *in vivo* situation. Extrapolations can be made at the molecular level, comparing similar mechanistic endpoints, namely compound-induced changes in multiple genes and signaling pathways [5, 6]. Several toxicogenomics-based studies have already been performed comparing rat hepatic *in vitro* models with the situation *in vivo* [7-9]. These studies concluded that to date no toxicogenomics-based *in vitro* system allowed for prediction of hepatotoxic responses *in vivo*. The main limitation of hepatic *in vitro* assays used in these toxicogenomics-based studies is the loss of liver-specific functions, in particular cytochrome P450 (CYP450) monooxygenase activities [7, 10]. Extrapolation from these *in vitro* models to the *in vivo* situation is therefore hampered when examining compounds for which toxicity depends on bioactivation by the CYP450 enzyme system.

In the aim to develop an *in vitro* toxicogenomics-based system, the relevance of a hepatocyte sandwich culture with improved metabolic competence towards prediction of *in vivo* toxicity was assessed. Sandwich-cultured hepatocytes were used because compared to other hepatocyte-based *in vitro* models, hepatocyte longevity is increased and a polarized cell and membrane architecture resembling *in vivo* is maintained for several weeks [11, 12]. Furthermore, rat hepatocytes maintained in sandwich configuration display a more optimal CYP450 inducibility [13, 14]. In a separate study performed by this laboratory, it was shown that in sandwich-cultured hepatocytes maintained in modified medium, enriched with low concentrations of the known CYP450 inducers phenobarbital (PB), dexamethasone (DEX), and β-naphthoflavone (β-NF), the metabolic competence, as reflected by retention of CYP450 enzyme activities and gene expression levels of several phase I and phase II enzymes, was enhanced [15]. In the present study, the effect of coumarin, a compound for which toxicity depends on metabolic activation by the CYP450 enzyme system [16, 17], on toxicity and gene expression profiles was studied in sandwich-cultured hepatocytes maintained in standard medium, the standard model (without inducers), and in sandwich-cultured hepatocytes maintained in modified medium, the modified model (containing enzyme inducers). Coumarin-induced cytotoxicity data and gene expression profiles generated in both *in vitro* models were compared to toxicity and gene expression profiles in rat liver in the *in vivo* study in which rats were exposed to coumarin, since *in vivo* verification of *in vitro* toxicogenomics data has been proposed to be a necessity to judge the value of the

in vitro model for *in vivo* toxicity prediction [18]. The expected differences of bioactivation of coumarin in the standard model versus the modified model were verified by measurement of coumarin and one of its metabolites in medium.

METHODS

Chemicals

Collagenase type B, Roche reagent kits, primer $p(DT)_{15}$, and Easyhyb were obtained from Roche (Mannheim, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), gentamycin, phosphate buffered saline (PBS), TRIzol™, yeast tRNA, human Cot-1 DNA were obtained from Invitrogen (Breda, The Netherlands). Insulin, glucagons, hydrocortisone, PB, DEX, β-NF, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and amino-allyl dUTP were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The RNeasy mini kit, the RNAse-free DNAse kit, and the QIAquick PCR purification kit were obtained from Qiagen (Westburg B.V., Leusden, The Netherlands). Cy3 and Cy5 monofunctional reactive dyes, and Poly(dA)·Poly(dT) were purchased from Amersham Biosciences (Roosendaal, The Netherlands). Coumarin, CAS-no 91-64-5; purity by HPLC minimum 99% according to the manufacturer, was obtained from Sigma-Aldrich. All other chemicals were of analytical grade.

Animals

Male Wistar rats (Crl: (WI) WU BR), 9-12 weeks of age, 180-250 g, were obtained from Charles River GmbH, Sulzfeld, Germany. During the acclimatization period and until sacrifice, animals were housed individually in macrolon cages with wire tops and sawdust bedding at 22 °C and 50-60% humidity. The light cycle was 12h light/12h dark. Feed and tap water were available ad libitum.

Animal treatment *in vivo*

Wistar rats, housed under conditions as described above, were injected intraperitoneally (i.p.) with 17.5 mg/kg b.wt. (low dose), 75 mg/kg b.wt. (mid dose), and 200 mg/kg b.wt. (high dose) coumarin dissolved in corn oil. Doses were defined in a range finding study. As a solvent control, only corn oil was injected (vehicle-treated control). In each dose group and vehicle-treated group, n=5 rats were included. Injection volume for each treatment was 10 mL/kg b.wt.. Body weight was recorded on day 0 and at 24h, just before sacrifice. Rats were anesthetized by inhalation of CO_2/O_2. Twenty-four hours after i.p. injection, animals were sacrificed by bleeding through the aorta abdominalis, the blood was collected in heparin tube, from which plasma was isolated for clinical chemistry. Thereafter, livers were immediately dissected, frozen in liquid nitrogen, and stored at -80 °C until further processing. A section of the liver was kept aside in formaline for pathological examination.

Clinical Chemistry

Serum alanine aminotransferase (ALAT) activity, serum aspartate aminotransferase (ASAT) activity, lactate dehydrogenase (LDH) leakage, serum alkaline phosphatase (ALP) activity, glucose, cholesterol, γ-glutamyl transferase (GGT) activity, and phospholipid levels were analyzed on a Hitachi 911 centrifugal analyzer using Roche reagent kits. Differences between plasma levels in treated and non-treated animals were defined as statistically significant at a *P* value below 0.01, determined by one-way ANOVA followed by Dunnett's test.

Culture of rat hepatocytes

Male Wistar rats similar to those used in the *in vivo* study and housed under identical conditions were used for hepatocyte isolation. Hepatocytes were isolated from livers from three individual rats according to a two-step collagenase perfusion technique as described by Seglen [19]. Hepatocyte preparations with viability greater than 85% as determined by Trypan Blue exclusion were used and cultured on collagen gel precoated 6-well plates at a density of 1.25×10^6 cells per well. Sandwich cultures were essentially prepared according to the method of Beken *et al*. [20]. Hepatocytes were allowed to attach for 4h in DMEM supplemented with 10% FCS, insulin (0.5 U/ml), glucagon (7 ng/ml), gentamycin (50 µg/ml). After attachment, dead cells were removed by washing and the upper collagen layer was applied. Cells were kept in standard medium consisting of DMEM containing 25 mM HEPES and 4.5 g/l D-glucose supplemented with insulin (0.5 U/ml), glucagon (7 ng/ml), hydrocortisone (7.5 µg/ml), and gentamycin (50 µg/ml). In the modified model, standard culture medium was modified by supplementation of an inducer mix that consisted of 1 mM PB, 10 µM DEX, and 5 µM β-NF [15]. PB was added as a concentrated stock solution in PBS. DEX and β-NF were added as concentrated stock solutions in dimethylsulphoxide (DMSO), resulting in a DMSO concentration of 0.2% (v/v). Standard and modified media were applied four hours after seeding the cells. Cultures were incubated at 37 °C in a humidified incubator gassed with 5% CO_2. Medium was changed on a daily basis during a period of 72h.

Hepatocyte treatment and cytotoxicity analysis

Hepatocytes cultured in either standard or modified medium for 72h were exposed to coumarin in a concentration range of 0 to 1000 µM dissolved in DMSO for 24h. The final concentration of DMSO was equalized in all culture media and did not exceed 0.5% (v/v). Cytotoxicity was determined employing the MTT reduction method [21]. LDH leakage was determined spectrophotometrically in pooled medium obtained from three wells per rat per dose group, on a Hitachi 911 centrifugal analyzer using Roche reagent kits. The final coumarin concentrations for the gene expression study selected were 0 µM (control), 70 µM (low dose), 200 µM (mid dose), and 600 µM (high dose). Doses corresponded to 0%, 10%, 20%, and 50% cytotox as determined by the MTT reduction assay, respectively (see results).

Gas chromatography to study metabolism of coumarin

Coumarin and the *o*-hydroxyphenylacetic acid (*o*-HPAA) metabolite were measured in standard and modified culture medium of hepatocytes exposed to coumarin for 24h using gas chromatography with flame ionization detection (GC-FID) as described [22, 23]. Samples (0.1 ml) were diluted with water to a final volume of 1 ml. To increase the extraction efficiency of *o*-HPAA, 0.5 g sodium chloride was added to each sample, following extraction with ethyl acetate. The extraction efficiencies of coumarin and *o*-HPAA were 30% and 65%, respectively. CM and *o*-HPAA were separated and quantitated using a Fisons HRGC 8650 gaschromatograph and a Varian VG-5ms column, 50 m x 0.25 mm. Quantification was accomplished by calculation of peak-area ratios relative to the *n*-tridecane (*n*-C13) internal standard, and use of the external standard curves. The same extraction procedure was applied to the external standards.

Total RNA isolation

RNA was extracted using TRIzol™ according to the manufacturer's protocol. TRIzol™ was added to frozen liver samples obtained from the *in vivo* study which subsequently were pulverized with mortar and pestle under liquid nitrogen before extraction. To obtain RNA from sandwich cultures, TRIzol™ was added on the upper collagen layer, and cells were collected. RNA was purified using the RNeasy mini kit including an additional DNAse digestion step. RNA concentration was calculated from the absorbance at 260 nm as measured spectrophotometrically. RNA quality was assessed by agarose gel electrophoresis.

Microarray design

A local reference design was used for microarray hybridization (Table 1). For the *in vivo* samples, tester RNA labeled with Cy5 from individual rat livers was combined with Cy3-labeled reference

Table 1. Experimental design.

in vivo	***in vitro***		
	Experiment 1 Rat 1	Experiment 2 Rat 2	Experiment 3 Rat 3
	Isolation of hepatocytes and preparation of collagen sandwich cultures for each rat		
	Incubation into standard or modified medium		
in vivo exposure to coumarin for 24h at 0, 17.5, 75, and 200 mg/kg b.wt.; n=5 per dose group	Exposure of cultures to coumarin for 24h at tester concentrations of 0 (reference control), 70, 200, and 600 µM		
Cy5 labeling of RNA from each individual coumarin treated rat	Cy5 labeling of RNA from coumarin treated cultures		
Cy3 labeling of pool RNA from control animals	Cy3 labeling of pool of RNA from respective control cultures		
Resulting datasets from *in vivo* and *in vitro* cultures combined			
Maximum missing values allowed: 2 out of 5 for the *in vivo* study 1 out of 3 for the *in vitro* studies			

RNA consisting of a pool of RNA extracted from livers obtained from five vehicle-treated, control rats. For the *in vitro* studies, Cy5 labeled tester RNA from one experiment was combined with Cy3 labeled reference RNA from the same experiment; hepatocytes in one experiment were obtained from one rat.

Microarray labeling

RNA samples were indirectly labeled according to the amino-allyl labeling procedure for microarrays from The Institute for Genomic Research (TIGR) (http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml). For each labeling reaction, 25 µg of total RNA was used as starting amount for reverse transcription of mRNA. Briefly, for reverse transcription, mRNA was selected by oligo dT priming with primer $p(DT)_{15}$. The reverse transcription reaction in which amino-allyl dUTP is incorporated, was conducted for three hours at 42 °C. Non-transcribed RNA was degraded by alkaline hydrolysis in a final concentration of 0.25 M NaOH for 30 minutes 37 °C. Thereafter, the mixture was neutralized with an equimolar amount of acetic acid. The cDNA was purified using the Qiagen QIAquick PCR purification kit. Columns were washed with 10 mM sodium-borate in 80% ethanol (pH 8.5). Column-bound cDNA was eluted two times in 30 µl MilliQ H_2O. Samples were labeled with either Cy3 (reference) or Cy5 (tester) monofunctional reactive dyes and afterwards cleaned from unincorporated Cy dyes using AutoseqG-50 sephadex chromatography columns.

Microarray hybridization

Samples were hybridized on rat oligonucleotide microarrays containing approximately 5800 different oligonucleotide fragments of 70 nucleotides in length (Qiagen Operon, Westburg B.V., Leusden, The Netherlands), spotted in duplicate at the Frank Holstege group (Utrecht genomics laboratory, Utrecht, The Netherlands) as described [24, 25]. Briefly, Cy3 and Cy5 labeled samples were combined according to the experimental design. To avoid non-specific hybridization, yeast tRNA, Poly(dA)·Poly(dT) and human Cot-1 DNA were added. Samples were dissolved in 110 µl Easyhyb hybridization buffer. After cDNA denaturation at 100 °C, human Cot-1 DNA was allowed to anneal for half an hour at 42 °C. The hybridization mixture was pipetted directly in the center of the hybridization chamber (Corning Life Sciences B.V., Schiphol-Rijk, The Netherlands). Slides were prehybridized according to the TIGR protocol and placed carefully on top. To keep the chamber moisturized during hybridization, a pre-cut paper drenched in MilliQ H_2O was put on top. Samples were hybridized overnight at 42 °C in a water bath. Slides were washed with sodium chloride sodium citrate (SSC) buffers decreasing in stringency. Microarrays were scanned using a Packard Scanarray confocal laser scanner (PerkinElmer, Boston, MA). Resulting TIFF images were loaded into Imagene 5.0 (Biodiscovery Inc. El Segundo, CA) and saved to further process and analyze the data.

Microarray quality criteria

Criteria for microarrays to be further analyzed consider the homogeneity of the spot signal intensities, the effect of bleaching of the fluorescence, the number of manually flagged (excluded) spots, the spatial distribution of the signals over the slide surface, the balance between Cy3 and Cy5 signal intensity, the number of saturated spots and the quality of the slide with respect to other slides of the experiment, as described by Heijne *et al*. [24].

Microarray data analysis

Flagged spots and controls were excluded from further analyses. Only spots on qualified microarrays with intensities higher than 1.5 times the intensity of their local background were included in data analysis. Ratios of the background-corrected intensities of tester over reference were calculated for each slide. To account for technical variations introduced during labeling or hybridization, data were normalized using the Lowess algorithm [26]. Normalized ratios were log-transformed with base two in SAS Enterprise guide V2 (SAS Institute Inc., Cary). The resulting data set was loaded into Excel (Microsoft Corporation, Redmond, WA). Replicate genes per array were averaged. For a gene to be included in the analysis for both *in vivo* and *in vitro* studies, a maximum of 30% missing values per gene were accepted. Two sample *T* tests (with random variance model) were performed using BRB-Array Tools Version 3.3.1 developed by Dr. R. Simon and A. Peng Lam [27]. To reduce false positives discovery, the nominal significance level of every univariate test was set at 0.001. To study whether similarities between biological processes could be observed between the *in vitro* models and *in vivo*, pathway analysis was performed using genes significantly expressed as determined by BRB ArrayTools, Genelists were uploaded on the website of the Database for Annotation, Visualization and Integrated Discovery (DAVID) [28]. Pathway analyses were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) [29]. A pathway was considered "triggered" when at least two genes within the pathway were significantly modulated by coumarin as assessed by means of the Fisher's exact test. Therefore, for each pathway and per system (*in vitro* and *in vivo*), the total number of genes that gave a signal on the microarray was divided into four groups: the number of genes not significantly modulated by coumarin and representing the pathway, not significantly modulated by coumarin and not representing the pathway, significantly modulated by coumarin and representing the pathway, and finally, significantly modulated by coumarin and not representing the pathway. Pathways were considered significant at a *P* value below 0.05. Furthermore, principal component analysis (PCA) was performed using Matlab software Version 6.5 (The MathWorks Inc., Natick).

RESULTS

In vivo toxicity

Hepatotoxicity *in vivo* was determined in rats treated with coumarin up to 200 mg/kg b.wt.. Clinical chemistry parameters showed that coumarin significantly increased the plasma levels of cholesterol at a dose level of 75 mg/kg b.wt., and AST, ALT, and GGT at a dose of 200 mg/kg b.wt.. The phospholipids level measured in plasma of rats exposed to 200 mg/kg b.wt. coumarin was significantly decreased as presented in Table 2 (data represent mean ± SD). Histopathological findings in rat liver show that at necropsy, the liver of one animal treated with 75 mg/kg b.wt. of coumarin was focally discolored, probably by focal congestion. Upon microscopy, only animals treated with 200 mg/kg showed slight to severe single cell necrosis and minimally, centrilobular and mononuclear cell infiltrate.

In vitro toxicity

Cytotoxicity of coumarin was determined in the sandwich-cultured hepatocyte model containing either standard medium or modified medium (Fig. 1). No cytotoxicity of coumarin (up to 1 mM) was detected in the standard system. However, in the modified system coumarin appeared to be cytotoxic in a dose-response manner. Based on these data, for the gene expression study, hepatocytes were exposed to coumarin at 0 µM (control), 70 µM (low dose), 200 µM (mid dose), and 600 µM (high dose) for 24h. In the modified system, the low, mid, and high dose resulted in 100-90%, 90-80%, and <50% viability, respectively. In the standard system, the viability was 100% at all concentrations of coumarin.

Coumarin metabolism

To verify that the improved metabolic competence of the modified model indeed affects the metabolism of coumarin and thus is responsible for differences in cytotoxicity of coumarin in

Table 2. Clinical chemistry parameters from n=5 coumarin-treated rats per treatment group in the *in vivo* study.

	Control	**Low dose**	**Mid dose**	**High dose**
Coumarin dose	**0 (mg/kg b.wt.) Vehicle control**	**17.5 (mg/kg b.wt.) Low dose**	**75 (mg/kg b.wt.) Mid dose**	**200 (mg/kg b.wt.) High dose**
ALP (U/l)	136 ± 13	151 ± 20	139 ± 31	163 ± 24
Cholesterol (mM)	1.71 ± 0.13	1.65 ± 0.21	2.26 ± 0.4**	1.38 ± 0.26
Glucose (mM)	10.41 ± 0.26	10.45 ± 0.35	10.72 ± 1.56	10.26 ± 0.89
ASAT (U/l)	68 ± 13	81 ± 21	59 ± 3	383 ± 244**
ALAT (U/l)	66 ± 21	61 ± 20	35 ± 3	264 ± 175**
GGT (U/l)	1.4 ± 0.4	1.6 ± 0.4	1.5 ± 0.3	3.3 ± 1.7**
LDH (U/l)	168 ± 92	342 ± 1.99	126 ± 26	421 ± 154
Phospholipids (mmol/l)	1.93 ± 0.12	1.73 ± 0.18	1.94 ± 0.27	1.44 ± 0.24**

**P<0.01 as determined by ANOVA followed by Dunnett's test.

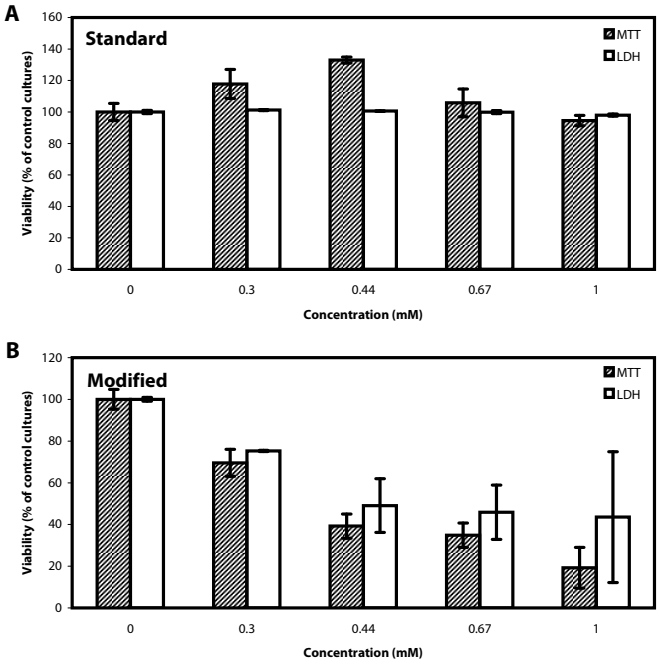

Figure 1. Cytotoxicity of coumarin in the standard model (A) and the modified model (B). MTT reduction is expressed as a percentage of the MTT determination of control hepatocyte cultures incubated with vehicle only. LDH retention is expressed as an inverted percentage of the LDH leakage in Triton-X treated hepatocyte cultures. Data are means ± SD (n=3).

standard culture medium and modified culture medium, both coumarin and the major coumarin metabolite reported to be detected in rat urine [23, 30, 31] were measured in both standard and modified culture medium 24h after exposure. A dose dependent increase in the formation of *o*-HPAA was exclusively measured in the modified culture medium, but no *o*-HPAA was present in the standard culture medium. Furthermore, for all dose groups, coumarin concentration was significantly lower in the modified medium compared to the standard medium (the reduction varied from 15% for the high dose group to 59% for the low dose group; data not presented).

Gene expression profiling

Analysis of significantly modulated genes In total, the criteria settings for microarray analysis used in this study resulted in datasets containing gene expression ratios of 1621 genes *in vivo*, 2536 in the standard system, and 2368 genes in the modified system. The large volume of data in the dataset was reduced by performing robust statistical analysis that separated genes which expression was actually changed by coumarin from genes that remained unchanged. Resulting datasets contained, regardless of dose-dependency, 321, 13, and 92 genes which significantly changed by coumarin *in vivo*, in the standard system, and the modified system, respectively. Overlap of the changed genes between both *in vitro* models and *in vivo* is presented by the

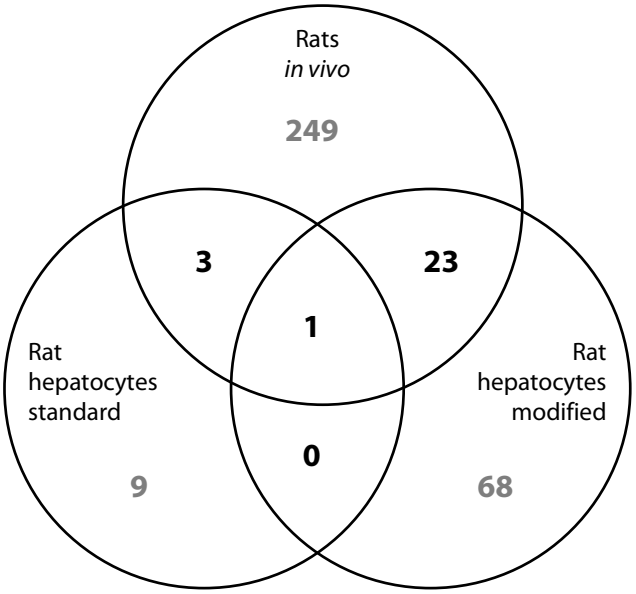

Figure 2. Circles in de Venn diagram represent the number of genes significantly changed *in vivo*, in the standard model (ST), and in the modified model (MOD). Overlaps contain the number of significantly modulated genes similar between systems.

Venn diagram in Fig. 2. Twenty-three genes were altered in both the modified model and *in vivo*, and only three genes in both the standard system and *in vivo*. One gene was altered in all systems, *in vitro* and *in vivo*. These 27 genes form the subset of genes significantly changed by coumarin which are listed in Table 3. In both *in vivo* and the *in vitro* models, most genes showed a dose-dependent down regulation (for 52 to 85% of the genes the Pearson correlation analysis coefficient is <-0.8; data not shown). When comparing the different systems with each other, the direction of modulation was always comparable between the standard and the modified *in vitro* models, and in most of the cases also between the *in vivo* model and one of the *in vitro* models.

Pathway analysis Pathways triggered by coumarin in at least two systems and significant in at least one are presented in Table 4. In the standard model, the modified model, and *in vivo*, one, eight, and six pathways were significantly triggered by coumarin, respectively. Four pathways, i.e. methionine metabolism, fatty acid metabolism, gamma-hexachlorocyclohexane degradation, and complement and coagulation cascades, were significant in both the modified system and *in vivo*. Only one pathway was significantly triggered in the standard system, but not *in vivo*.

Principal component analysis of whole data set The complete datasets of the *in vivo* and *in vitro* studies were used for PCA (Fig. 3). The two major components within the total variation between *in vivo* and both *in vitro* models, principal component (PC) #1 and PC #2, explain 40%

Table 3. Genes significantly changed by coumarin in at least two systems; *in vivo*, in the standard, or in the modified model.

		in vivo **(mg/kg b.wt.)**			**Standard *in vitro* model (μM)**			**Modified *in vitro* model (μM)**		
		17.5	**75**	**200**	**70**	**200**	**600**	**70**	**200**	**600**
AccNumber	**Symbol**	**Low**	**Mid**	**High**	**Low**	**Mid**	**High**	**Low**	**Mid**	**High**
NM_030850	Bhmt	-1.00 *	-2.41*	-1.63*	0.17	-0.58	-1.82	-1.55*	-1.53*	-1.08
M35266	Cdo1	0.14	0.69*	-0.35	-0.25	-0.81	-1.36	-0.66*	-2.05	-2.38*
NM_017074	Cth	-0.09	-0.34	-2.21*	0.05	-0.49	-1.18	-0.62*	-1.07	-1.18*
M13646	CYP3A2	0.52	0.27	-1.03*	NM	NM	NM	-0.58*	-0.42	-0.32
X69834	Serpina3m	-0.01	-0.23	-0.93*	-0.02	-0.14	-0.40	-0.75*	-0.72	-0.55
NM_130433	Acaa2	0.08	0.68*	0.37	0.01	-0.25	-0.75	-0.79*	-1.60	-1.86
NM_017321	Ratireb	0.25	0.88*	0.23	0.11	0.14	-0.07	-0.47	-1.07*	-1.04
NM_080892	Selenbp2	-0.39	-0.39	-1.33*	-0.09	-0.02	-0.70	-0.87	-2.18*	-2.71
NM_012541	Cyp1a2	-0.15	-0.28	-1.28*	-0.14	-0.36	-0.23	-0.92	-2.08*	-3.01*
NM_017306	Dci	0.62	1.91*	1.49*	0.05	-0.01	-0.17	-0.18	-0.59*	-0.79
NM_012826	Azgp1	0.12	0.16	-0.87*	-0.20	-0.50*	-0.75*	-0.48	-1.06*	-1.60
D17310	D17310	0.08	0.50*	-0.18	0.15	0.17	-0.33	-0.75	-2.13*	-2.70
NM_031736	Slc27a2	0.41	1.42*	0.72	-0.02	-0.34	-0.41	-0.43	-0.99*	-1.15
NM_031760	Abcb11	0.00	0.11	-0.93*	-0.15	-0.27	-0.66	-0.70	-1.53*	-1.90*
NM_030826	Gpx1	0.11	-0.44	-1.29*	-0.09	-0.21	-0.80	-0.72	-1.25*	-1.43*
NM_138884	Akr1d1	0.48	1.48*	0.92	-0.06	-0.35	-0.93	-0.47	-1.21*	-1.18
NM_012699	Dnajb9	0.20	0.16	1.02*	-0.10	-0.11	0.72	0.27	1.35*	2.04
AB009463	Lrp3	-0.08	-0.27	-0.45*	-0.05	-0.09	-0.45	-0.50	-1.00*	NM
NM_022513	NM_022513	-0.13	-0.47*	-0.24	0.04	-0.19	-0.67	-0.74	-1.33*	-1.04
D50559	Sc4mol	0.01	0.25	-1.20*	-0.02	0.02	-0.68	-0.32	-0.84	-1.30*
X60822	Mat1a	-0.51	0.76*	0.25	0.07	-0.53	-0.80	-0.68	-2.20	-2.52*
NM_012559	Fgg	0.35	1.08	1.19*	0.10	-0.35	-0.92	-0.78	-0.89	-0.58*
NM_016987	Acly	0.01	-1.11	-1.93*	0.05	-0.28	-0.61	-0.10	-0.69	-1.00*
NM_017134	Arg1	-0.18	-0.13	0.76*	-0.40	-0.16	-0.23	-0.67	-1.33	-1.63*
NM_017332	Fasn	-0.62	-2.06*	-2.56*	0.05	-0.56	-1.05*	-0.02	0.09	-0.16
K01934	Thrsp	-1.02	-3.08*	-3.47*	-0.08	-1.07	-2.05*	-1.19	-1.07	-0.64
X56228	Tst	0.00	-0.49	-1.11*	-0.08	-0.17	-0.51*	-0.10	-0.73	-1.46

*Significant values ($P<0.001$); Values are log transformed ratios corrected for the control; NM, not measured.

of the variance in the data set. The results show that genomics responses induced by the low and mid dose in the standard system could not be distinguished from controls, whereas a dose-response effect of coumarin *in vivo* and in the modified system was evident along the PC#2 axis, explaining14% of the variance in the whole data set. Along the PC#1 axis, 26% of the variance in the data was explained by genes reacting differently *in vivo* and *in vitro*.

Table 4. Pathways triggered by coumarin *in vivo*, in the standard model, or in the modified model.

	In vivo		Standard model		Modified model	
Pathway	**significant/ signal**	***P* value**	**significant/ signal**	***P* value**	**significant/ signal**	***P* value**
Fatty acid metabolism	12/34	0.024*	0/35	1.000	4/36	0.049*
Tryptophan metabolism	11/28	0.013*	0/26	1.000	3/27	0.085
Complement and coagulation cascades	10/28	0.036*	0/29	1.000	4/27	0.019*
Citrate cycle (TCA cycle)	6/11	0.011*	0/11	1.000	2/11	0.065
Gamma-Hexachlorocyclohexane degradation	8/15	0.004*	0/14	1.000	3/14	0.015*
Urea cycle and metabolism of amino groups	2/8	0.492	0/9	1.000	2/9	0.045*
Nitrogen metabolism	3/8	0.199	0/7	1.000	2/7	0.028*
Selenoamino acid metabolism	2/3	0.102	0/7	1.000	2/7	0.028*
Cysteine metabolism	3/6	0.096	0/6	1.000	2/5	0.014*
Methionine metabolism	3/4	0.026*	0/6	1.000	3/6	0.001*
Glutathione metabolism	4/13	0.246	2/16	0.003*	1/15	0.449

*$P<0.05$ calculated by the Fisher's exact test; the significant/signal column represents the number of genes in the pathway significantly altered by coumarin versus the number of genes in that pathway present in the complete dataset.

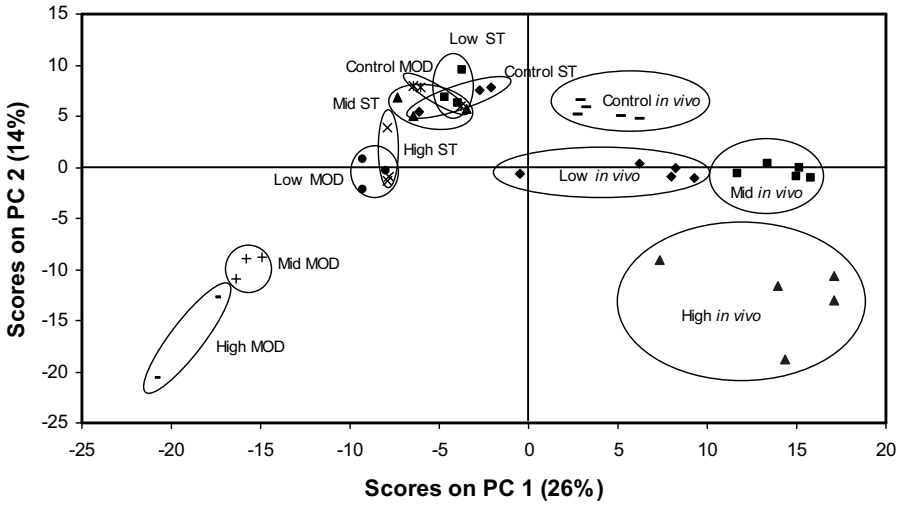

Figure 3. Principal component analysis of the complete dataset of expression profiles resulting from *in vitro* and *in vivo* hepatotoxicant-treatment of hepatocytes and rats. Two principal components were generated and plotted for each system and dose group. Dose groups are distinguished by circles and identified within (*in vivo*) or outside (ST, the standard model; MOD, the modified model) each circle.

DISCUSSION

To investigate the relevance of hepatocyte sandwich cultures towards mimicking aspects of *in vivo* toxicity, cytotoxicity measures and gene expression profiles were compared with data from an *in vivo* rat study. Coumarin, a compound for which toxicity depends on metabolism by the CYP450 enzyme system, was used as a model compound. Specifically, CYP450 enzymes of the 1A and 2E subfamily convert coumarin to the toxic metabolite coumarin 3,4-epoxide (CE) [16, 17]. CE rearranges spontaneously to the more stable *o*-hydroxyphenylacetaldehyde (*o*-HPA). Both CE and *o*-HPA are assumed to contribute to the hepatotoxicity of coumarin in rats as they conjugate with critical cellular macromolecules [22, 30, 32-35].

Prior to engaging into gene expression studies, it was hypothesized that an increased metabolic competence of sandwich cultures would improve metabolic conversion of coumarin to toxic metabolites, thereby increasing the resemblance with aspects of *in vivo* coumarin-induced hepatotoxicity. Therefore, sandwich-cultured hepatocytes were either maintained in a standard medium or in a modified medium that was enriched with a mixture of low concentrations of known CYP450 inducers in order to enhance metabolic capacity [15]. Findings in this study showed that both traditional measures of toxicity and coumarin-induced gene expression in the modified model were more pronounced and closer to *in vivo* compared to the standard model.

In vivo, coumarin toxicity was indicated by increased plasma ALT, AST, and GGT levels, primarily at the highest dose level of 200 mg/kg b.wt.. Moreover, hepatotoxicity did manifest itself as severe single cell necrosis and minimal centrilobular necrosis, as observed by histopathological observations in livers of rats treated with 200 mg/kg coumarin. These findings agree with other studies which report that administration of coumarin in doses ranging from 125 to 500 mg/kg results in severe centrilobular necrosis after 24h [31, 34, 35].

In vitro, coumarin appeared to be cytotoxic in hepatocytes cultured in modified medium, whereas no cytotoxicity was measured in the standard model. In our laboratory, it has been shown that gene expression of *CYP1A2* and *CYP2E1* in the modified model is closer to *in vivo* compared to the standard model in which gene expression of these genes is highly down-regulated. Furthermore, upon addition of the inducer mixture, enzyme activities of the 1A subfamily were increased to levels comparable to *in vivo* [15]. As coumarin is converted by CYP1A and CYP2E to *o*-HPA [16, 17], this metabolite possibly causes the cytotoxicity in the modified model. It was shown earlier that the compounds in the inducer mixture, PB, DEX, and β-NF increase the formation of *o*-HPA and other coumarin metabolites, enhancing coumarin toxicity [36, 37]. In the present study, these findings were confirmed by measurement of the coumarin metabolite *o*-HPAA, which is formed after oxidation of *o*-HPA. After 24h of coumarin exposure, a dose dependant formation of *o*-HPAA was exclusively observed in hepatocytes cultured in the modified model, whereas no *o*-HPAA was formed in the standard model. Furthermore, coumarin clearance was significantly higher in the modified culture medium compared to the standard culture medium in all dose groups.

Analysis of gene expression revealed that both in vivo and *in vitro*, genes are affected by coumarin at dose levels where no toxicity occurs as determined by traditional toxicology measures. This suggests that gene expression changes may very well be more sensitive indicators of potential adverse effects, as was indicated before by Heinloth *et al*. [38]. Considerably more genes were significantly altered and similarly affected by coumarin in both the modified system and *in vivo* compared to the standard system. In this study, not all affected genes are discussed in detail. It is of more relevance to investigate analogies in the biological pathways these genes trigger. The four pathways that were statistically significantly triggered both *in vivo* and in the modified model and the significant differentially expressed genes within these pathways will be discussed.

Betaine-homocysteine methyltransferase (*Bhmt*) and CTL target antigen (*Cth*), a putative cystathione gamma lyase enzyme, both down-regulated *in vivo* and in the modified system, appear in the methionine metabolism pathway. It has been shown that down-regulation of *Bhmt* and *Cth* impairs conversion of homocysteine to methionine and cysteine, respectively, resulting in increased homocysteine levels in the cell [39, 40]. Homocysteine accumulation is associated with impaired liver function, necrosis, liver cirrhosis, and fibrogenesis [39, 41].

Expression of *CYP1A2*, a gene which represents both the gamma-hexachlorocyclohexane degradation pathway and the fatty acid metabolism pathway, is significantly down-regulated *in vivo* and in the modified model. This CYP450 belongs to the CYP1A subfamily that converts coumarin to toxic metabolites. Therefore, down-regulation of gene expression of this enzyme could be interpreted as a classical negative feedback loop, turning out in the present case to prevent additional liver injury.

Fibrinogen gamma polypeptide (*fgg*) is significantly up-regulated *in vivo* and significantly down-regulated in the modified model. This gene is part of the complement and coagulation cascade, to which coumarin is associated as coumarin derivatives, such as warfarin, are well known for their anticoagulant properties [42]. One possible explanation of the significant, but different response of particular genes to coumarin *in vivo* and *in vitro* can be illustrated by fgg. *Fgg* is mainly expressed in hepatocytes. Up-regulation of *fgg*, as occurred *in vivo*, would be expected in terms of toxicity as fibrinogen levels are increased following liver injury [43]. Fibrinogen synthesis has shown to be stimulated in hepatocytes by factors excreted by extrahepatic tissues or nonparenchymal cells [44]. *In vitro*, these factors are absent. Therefore, stimulation of fibrinogen synthesis in hepatocyte cultures *in vitro* may only occur when these factors are added to the culture medium.

Additional to the statistical analysis of microarray data which resulted in a list of significantly modulated genes, PCA was performed. PCA allows inclusion of all genes into analysis, preserving their interrelationships. Results showed that even though differences between *in vitro* and *in vivo* remain evident, the dose-response effect with respect to gene expression of coumarin in the modified system is more similar to *in vivo* compared to the standard system.

Dose levels *in vivo* were compared with the coumarin concentrations applied *in vitro*. Using pharmacokinetic data from literature, a distribution coefficient of coumarin of 3.33 L/kg was estimated [22, 31, 45]. If assumed that the *in vivo* bioavailability of coumarin is 100% after i.p. injection, this results in *in vivo* target organ concentrations of 36 µM, 154 µM, and 411 µM for the low, mid, and high *in vivo* doses, respectively, which are in the same range as the *in vitro* concentrations of 70 µM, 200 µM, and 600 µM. Thus, although not identical, the range of estimated plasma dose levels *in vivo*, and therefore, potential target organ concentrations were comparable to the dose level range employed in the culture media *in vitro*.

It is important to note that in addition to coumarin, the inducer mixture may also influence gene expression and that these effects interact with each other. In the design of the present study, however, effect of the inducer mixture on gene expression was filtered out because matching controls were used, i.e. both test and control samples are from hepatocytes cultured in the modified system. Nonetheless, it remains possible that the inducer mixture affected the expression of some genes in the modified system in such way that no additional effect of coumarin could have been detected.

Furthermore, effects on gene expression in the highest dose group of the modified model may not be solely attributed to coumarin. The highest coumarin dose group in this model resulted in a 50% loss of cell viability as measured by the MTT reduction assay. In a sandwich configuration, dead cells remain between the two layers of collagen. As a consequence, necrosis caused by coumarin exposure in one cell can affect gene expression in viable neighbor cells [46]. Excluding the high dose group of the modified model from analysis, still 80% of the genes in Table 2 are retained. Moreover, the methionine metabolism and gamma-hexachlorocyclohexane degradation pathways are still significantly triggered by coumarin.

In summary, our experiments have shown that the metabolism of coumarin towards active metabolites, coumarin-induced toxicity, gene expression profiles, and consequently, biological pathways in the modified system containing sandwich-cultured hepatocytes with enhanced metabolic capacity better represent the situation *in vivo* compared to conventional sandwich-cultured hepatocytes. This highlights the need for a metabolically competent, toxicogenomics-based, hepatocyte *in vitro* system.

ACKNOWLEDGEMENTS

We would like to thank M. Schut, Dr. N. Treijtel, and W. Maas for their help in hepatocyte isolation and culture techniques, M. van den Wijngaard for sample isolation of the *in vivo* study, Dr. W. Heijne for his help with the experimental design, A. de Kat Angelino-Bart, M. Havekes, and Dr. F. Schuren at the microarray facility, M. Dansen for microarray data quality analysis, A. Freidig for help with pharmacokinetic modeling, and Dr. S. Bijlsma for assistance in principal component analysis. Financial support provided by Servier Nederland B.V. is greatly appreciated.

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Chapter IV

Interspecies differences in coumarin hepatotoxicity: a toxicogenomics-based parallelogram approach

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ABSTRACT

A compound for which marked species differences have been reported in laboratory animals and humans is coumarin. In rats, coumarin is metabolized by cytochrome P450s to highly toxic metabolites. In humans, coumarin is mainly metabolized to non-toxic metabolites. Therefore, reports on adverse effects caused by coumarin in humans are rare. However, gene expression profiling has shown to be a sensitive technique able to identify adverse effects of compounds at dose levels thought to be non-toxic as determined by traditional measures of toxicity. In the present study, a toxicogenomics-based parallelogram approach was used to compare either toxic or non-toxic effects of coumarin on gene expression in human hepatocytes relevant for the situation *in vivo*. To this purpose, gene expression profiling was performed on human hepatocytes treated with coumarin and results were compared to a previously performed coumarin *in vivo* and *in vitro* rat toxicogenomics study. No cytotoxicity was observed in human hepatocytes, whereas rats showed clear toxic effects *in vitro* as well as *in vivo*. In all three systems, coumarin affected coagulation; this indicates relevant responses in cases of human exposure. However, no pathways and processes related to hepatotoxicity in rats were observed in human hepatocytes. Still, repression of energy-consuming biochemical pathways and impairment of mitochondrial function were observed in human hepatocytes treated with 600 µM of coumarin, possibly indicating toxicity. In conclusion, the present study shows the potential of toxicogenomics to sensitively identify compound-induced (adverse) effects and underscores the need to use human hepatocytes to predict chemical-induced responses in humans.

INTRODUCTION

Liver-based *in vitro* systems, such as primary hepatocytes, precision cut liver slices, and hepatic cell lines are valuable tools for studying hepatotoxicity. Many studies use liver material isolated from laboratory animals, especially rats. However, due to compound-related metabolism-mediated species differences in response to toxicants, these *in vitro* models are not always suitable to predict effects in human liver *in vivo*. Therefore, human hepatocytes in culture are considered as the closest model to predict hepatotoxicity actually occurring in livers of humans *in vivo* [1-3].

The toxicology of coumarin merits special attention with regard to its marked species differences in both metabolism and toxicity [4-6]. The use of coumarin was banned in the USA in 1954 based on reports of hepatotoxicity in rats and was recommended for withdrawal from use in the UK in 1965 [4, 5, 7]. Consecutive research revealed that coumarin metabolism in rats and other species is quite different compared to metabolism in man [4]. The severe hepatotoxicity caused by coumarin in rats depends on conversion of coumarin to the toxic metabolites coumarin 3,4-epoxide (CE) and *o*-hydroxyphenylacetaldehyde (*o*-HPA) [8-13]. Metabolism of coumarin is cytochrome P450-dependent and is suggested to occur in the rat via CYP450s of the 1A and 2E subfamilies [14, 15].

In humans, coumarin 7-hydroxylation (7-HC), catalyzed by hepatic CYP2A6, is the major route of coumarin biotransformation in most subjects [4, 5, 15-17]. 7-Hydroxycoumarin and its glucuronide and sulfate conjugates are non-toxic and water soluble. By that, they are readily excreted in the urine following an oral dose in most subjects [7, 14, 16]. Reports of adverse effects in humans resulting from coumarin administration are therefore rare [5, 7, 13]. Still, genetic polymorphisms in CYP2A6 can result in deficiency of the 7-HC pathway [5] while also other minor routes comprising biotransformation via human CYP1A and 2E subfamilies can still result in formation of the toxic metabolites CE and *o*-HPA [6].

The introduction of toxicogenomics, the use of genomics techniques in toxicology, enabled evaluation of thousands of endpoints simultaneously. An increasing number of *in vitro* studies have successfully applied this approach to identify toxicant-induced signatures [18-23]. One of the expectations of toxicogenomics is its increased sensitivity to detect potential adverse effects of compounds compared to conventional toxicology assays. Heinloth *et al*. [24] confirmed this expectation as they observed that doses of the hepatotoxicant acetaminophen which were considered to be non-toxic on the basis of traditional measures of toxicity caused changes in gene expression indicative of early adverse cellular effects and cell stress responses that indeed underlie development of toxicity at higher dose levels. These observations may have important implications for safety assessment of drugs and environmental agents [24].

The objective of the present study was to use a toxicogenomics-based parallelogram approach to evaluate the relevance of coumarin-induced transcriptomics responses in primary human hepatocytes for the situation in humans *in vivo* in terms of toxicity or non-toxic

beneficial/pharmacological effects. To this purpose, coumarin-induced gene expression profiles in primary human hepatocytes cultured in sandwich configuration were compared to results obtained from a previously performed study in which sandwich-cultured rat hepatocytes and rats *in vivo* were exposed to coumarin [25] (see **Chapter III**).

METHODS

Chemicals

Ca^{2+}- and Mg^{2+}-free Hank's Buffered Salt Solution (HBSS), Dulbecco's Minimal Essential Medium (DMEM), Foetal Calf Serum (FCS), penicillin-streptomycin, and TRIzol™ were obtained from Invitrogen, Breda, The Netherlands. Bovine Serum Albumin (BSA), ascorbic acid, collagenase Type IV, insulin, dexamethasone (DEX), Trypan Blue, Percoll, coumarin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich, Zwijndrecht, The Netherlands. BD™ ITS+ Premix and rat tail collagen I were obtained from BD Biosciences, Alphen aan den Rijn, The Netherlands. The RNeasy minelute kit and RNeasy mini spin columns were obtained from Qiagen, Westburg B.V. (Leusden, The Netherlands). Cyanine 5-cytosine triphosphate (CTP) and cyanine 3-CTP were purchased from Perkin Elmer (Boston, MA). Agilent's low RNA input fluorescent linear amplification kit and the hybridisation solution were obtained from Agilent Technologies (Palo Alto, CA). Coumarin, CAS-no 91-64-5; purity by HPLC minimum 99% according to the manufacturer, was obtained from Sigma-Aldrich. All other chemicals were of analytical grade.

Culture of human hepatocytes

Human hepatocytes were obtained from resection specimens of patients undergoing partial hepatectomy for colorectal metastases in an otherwise normal liver at the University Hospital Maastricht, the Netherlands. Surgery was performed as described before [26] and in none of the cases hepatic inflow occlusion was applied during liver transaction. All patients provided written informed consent and the study was approved by the Medical Ethics Committee of the University Hospital Maastricht. Isolation of human hepatocytes from resection specimens was performed according to the method described by LeCluyse *et al*. [27]. This method has been adopted by an interlaboratory consortium sponsored by the European Centre for the Validation of Alternative Methods (ECVAM) for the isolation and cultivation of primary human hepatocytes for testing the potential of new drugs to induce liver enzyme expression [27, 28]. Essentially, directly after removal of the liver tissue, an encapsulated wedge, weighing approximately 50-100 g, was cut off. Vessels on the cut surface were immediately flushed with ice cold wash buffer consisting of Ca^{2+}- and Mg^{2+}-free HBSS supplemented with 0.5 mM EDTA, 0.5% (w/v) BSA, and 50 µg/ml ascorbic acid. Liver tissue was transported to the laboratory in ice cold buffer. Transportation time did not exceed 15 min. After a two-step collagenase perfusion, first with wash buffer, then

with digestion medium consisting DMEM supplemented with 0.05% (w/v) Collagenase Type IV and 0.5% BSA, hepatocytes were dispersed from the digested liver and collected in ice cold attachment medium (5% FCS and penicillin-streptomycin (100 U/ml and 100 µg/ml, respectively), 0.1 U/ml insulin, and 1 µM DEX in DMEM). After passing of the cell suspension through a 100 µm nylon mesh, the suspension was washed by low speed centrifugation three times at 75 x g for 5 min. Pellets were resuspended in ice cold suspension medium after each centrifugation step. Viability was assessed by Trypan blue exclusion. Hepatocyte preparations with viability greater than 75% were included for further studies. Cell suspension with viability below 85% was purified using a Percoll gradient, as previously described [27]. Cells were cultured on collagen gel precoated 12-well plates at a density of 6.5 x 10^5 cells per well. Human hepatocyte sandwich cultures were essentially prepared according to the method of Beken *et al*. [29]. After attachment for 4h in attachment medium, dead cells were removed by washing and the upper collagen layer was applied. Thereafter, cells were kept in DMEM containing 0.1 µM dexamethasone, 6.25 µg/ml insulin, 6.25 µg/ml transferin, and 6.25 ng/ml selenium (ITS+) [30]. Cultures were incubated at 37 ºC in a humidified incubator gassed with 5% CO_2. Medium was changed on a daily basis during a period of 72h.

Hepatocyte treatment and cytotoxicity analysis

At 72h, five independent human hepatocyte cultures were exposed to two concentrations of coumarin (200 and 600 µM) for 24h. Donor information is provided in Table 1. The coumarin concentrations used were identical to those used in a previous study in which rat hepatocytes were exposed [25]. Coumarin was dissolved in dimethylsulfoxide (DMSO). The final DMSO concentration in culture medium was 0.2% (v/v). Cytotoxicity was determined employing the MTT reduction method [31] on hepatocytes originating from human individuals # 2, 4, and 5.

Total RNA extraction

After the 24h coumarin treatment, TRIzol™ was added onto the upper collagen layer and cells were collected. RNA was purified using the RNeasy minelute kit including an additional DNA digestion step. The RNA concentration was determined spectrophotometrically. RNA quality was determined using the Bioanalyzer (Agilent Technologies, Palo Alto, CA). All samples con-

Table 1. Primary human hepatocyte donor information.

Donor	Gender	Age (years)	Cell viability (%)
1	M	76	85
2	M	74	91
3	M	73	90
4	F	42	86
5	F	75	94

The cell viability was assessed by the trypan blue exclusion assay; M, male; F, female.

tained intact total RNA with a 28S/18S rRNA ratio>1.5. RNA extractions of hepatocyte cultures exposed to coumarin of five human individuals were used for microarray analysis.

Labeling

RNA samples from control hepatocyte cultures and hepatocytes cultures exposed to the two concentrations of coumarin were labeled with cyanine 5-CTP. Cyanine 5-CTP labeled samples from one individual were hybridized against cyanine 3-CTP labeled RNA samples from control hepatocyte cultures from the same individual. As a result, control samples labeled with cyanine 5-CTP hybridized against cyanine 3-CTP samples are a self-self hybridization. Labeling was performed using Agilent's low RNA input fluorescent linear amplification kit following manufacturer's instruction. Briefly, double-stranded cDNA was synthesized using molony murine leukemia virus-reverse transcriptase (MMLV-RT) with T7 promoter primer, starting with 1 µg of total RNA. Cyanine-labelled cRNA targets were transcribed using T7 RNA Polymerase. The amplified cRNA was purified using RNeasy mini spin columns. Synthesized cRNA products were quantified spectrophotometrically.

Hybridization

For microarray hybridization, cyanine 5-samples and cyanine 3-samples were combined. cRNAs were fragmented at 60 ºC for 30 min with fragmentation solution followed by hybridization on Agilent 22K format 60-mer oligo microarrays (G4110B for human from Agilent Technologies, Palo Alto, CA) for 17h at 60 ºC with Agilent hybridization solution. Arrays were washed according to manufacturer's instruction. Microarrays were scanned using a Packard Scanarray Express confocal laser scanner (PerkinElmer, Boston, MA). Resulting TIFF images were loaded into Imagene 5.0 (Biodiscovery Inc. El Segundo, CA) to further process and collect the gene expression data.

Microarray data analysis

Data were transferred into GeneSight 4.1 (Biodiscovery Inc. El Segundo, CA). Flagged spots, consisting of poor quality spots and negative and positive control spots, were excluded. For each spot, median local background intensity was subtracted from the median spot intensity and spots from low expression genes (with a net intensity of <40 in both channels), were excluded from further analysis. These background-corrected median intensities were log transformed by base 2. Data were normalized using the Lowess algorithm [32]. Resulting gene expression ratios were loaded into Excel (Microsoft Corporation, Redmond, WA). For further analysis, 60% of present values were used.

For MAPPFinder analysis [33], a subset of significantly modulated genes was created as follows: genes within the complete microarray dataset were determined significantly differentially expressed compared to control hepatocyte cultures if fold changes were either greater than 1.5 or smaller than -1.5 and if this differential expression was significant according to a two-sided

Student's *T* test (*P* values < 0.05) [34]. Over-represented biochemical pathways and biological processes within the subset of differentially expressed genes compared to the complete dataset of genes were determined using MAPPFinder [33]. Pathways and processes were retrieved from GenMAPP (version 2.1, Gladstone Institutes 2000-2006 [35]) and included Gene Ontology (GO) terms [36], Kyoto Encyclopedia for Genes and Genomes (KEGG) pathways [37], BioCarta pathways [38], and BiGCaT [39] pathways. These pathways and processes were determined significantly over-represented when *Z* scores were greater than 2 and *P* values were below 0.1. The minimally required number of changed genes was set at three. Specificity of GO terms was increased by removing parent terms from the list in cases where both a parent and a child term were present and the presence of the parent term was due entirely to genes meeting the criterion for the child term [33].

For T-profiler, the complete data set of genes was assembled into the analysis without pre-selecting only significantly modulated gene expressions. 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 within the complete data set of genes (http://www.t-profiler.org) [40]. Other than MAPPFinder analysis, no pre-selection of subsets of (differentially expressed) genes is needed. In the present study, the predefined gene groups are based on membership of a specific GO category, part of a pathway defined by the KEGG, or other gene-sets (GenMAPP, BioCarta, or manually curated).

RESULTS

At both 200 μM and 600 μM, coumarin did not give rise to any signs of cytotoxicity in human hepatocytes as measured by the MTT reduction assay.

Individual gene expression

The number of genes significantly modulated by either 200 μM or 600 μM of coumarin in human hepatocytes is presented in the Venn diagram in Fig. 1. Treatment with 200 μM coumarin resulted in significant modulation of 198 individual genes; 619 individual genes are significantly modulated by 600 μM coumarin. The 135 genes in the overlap of the Venn diagram are significantly modulated by both concentrations. For both doses, all these genes were modulated in the same direction. For most genes a dose-dependent response in modulation was observed.

Pathway analysis

MAPPFinder analysis was used for identifying over-represented biochemical pathways and biological processes in the subset of significantly modulated genes in human hepatocytes. Results are presented in Table 2. Only one biochemical pathway, namely cholesterol biosynthesis, was down-regulated upon treatment of human hepatocytes with 200 μM coumarin. Treatment of

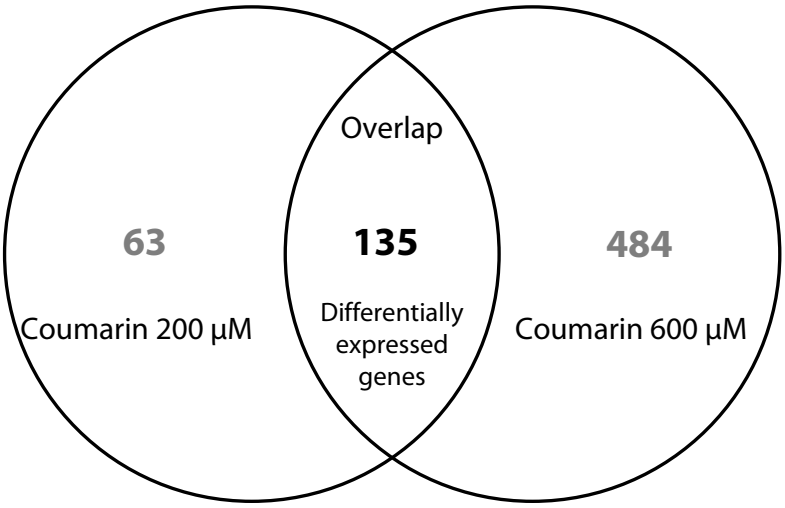

Figure 1. Venn diagram showing the number of genes significantly modulated by coumarin. The overlap contains the genes significantly modulated by both concentrations. Genes within the overlap are all modulated in similar directions by both concentrations.

human hepatocytes with 600 µM coumarin resulted in down-regulation of eight biochemical pathways and biological processes, including alcohol metabolism, lipid metabolism, oxidoreductase activity, and urea cycle and metabolism of amino groups.

Using T-profiler, coumarin-induced biochemical pathways and biological processes were identified in the complete data set of genes. T-profiler results are presented in Table 3. Three biochemical pathways and biological processes were predominant after exposure of human hepatocytes to 200 µM coumarin, including down-regulation of complement and coagulation cascades. Forty pathways and processes were affected in human hepatocytes by a concentration of 600 µM coumarin. Results showed up-regulation of transcription and protein folding related pathways. Furthermore, complement and coagulation cascades, urea cycle related pathways, lipid metabolism pathways, oxidoreductase activity, and metabolism of xenobiotics by CYP450 were among the pathways which were down-regulated upon exposure of human hepatocytes to 600 µM of coumarin.

Results of the coumarin rat study. Results of the previously performed coumarin rat study relevant for completion of the parallelogram approach in the present study will be shortly described. For more detailed method description and results, refer to **Chapter III** and the original publication [25]. Clinical chemistry and histopathology determinations showed hepatotoxicity of coumarin in rats *in vivo*. In rat hepatocytes *in vitro*, 200 µM of coumarin resulted in viability loss of 10 to 20%, whereas 600 µM of coumarin resulted in more than 50% cell death.

Pathway analysis in the coumarin rat study was performed by uploading lists of differentially expressed genes in the Database for Annotation, Visualization and Integrated Discovery (DAVID) [41] whereafter KEGG pathways were retrieved, as described [25]. In short, the pathway

Table 2. Biochemical pathways and biological processes over-represented by the subset of significantly expressed genes as determined using MAPPFinder.

	Source	Number changed	Number measured	Number in GO	*Z* score	*P* value	Regulation
200 µM Coumarin							
Cholesterol biosynthesis	GenMAPP	4	12	15	7.6	0.005	Down
600 µM Coumarin							
Alcohol metabolism (P)	GO	22	113	245	6.9	0.046	Down
Isoprenoid metabolism (P)	GO	3	3	16	7.4	0.042	Down
Pyridoxal phosphate binding (F)	GO	5	8	10	7.3	0.043	Down
Transferase activity, transferring nitrogenous groups (F)	GO	7	16	32	7.0	0.046	Down
Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor (F)	GO	11	39	96	6.5	0.057	Down
Urea cycle and metabolism of amino groups	KEGG	6	15	20	5.4	0.006	Down
Nuclear receptors in lipid metabolism and toxicity	BioCarta	7	25	33	4.5	0.044	Down
Fatty acid omega oxidation	BiGCaT	4	11	15	4.1	0.096	Down

GO terms are assigned to a biological process (P), a molecular function (F), or a cellular component (C).

results presented show KEGG pathways triggered in at least two systems (*in vitro* and *in vivo*) and significant in at least one (P value<0.05, Fisher's exact test). Pathways significant in rats *in vivo* as well as in rat hepatocytes *in vitro* included fatty acid metabolism, complement and coagulation cascades, gamma-hexachlorocyclohexane degradation, and methionine metabolism. Urea cycle and metabolism of amino groups, nitrogen metabolism, seleno-amino acid metabolism, and cysteine metabolism were additionally significantly triggered in the rat hepatocyte *in vitro* model.

DISCUSSION

As gene expression changes have been shown to be more sensitive indicators of potential adverse effects [24], in the present study, the gene expression profiling was used to identify coumarin-induced gene expression profiles in human hepatocytes and interpret these profiles with respect to potential toxicity. In a toxicogenomics-based parallelogram approach coumarin-

Table 3. Biochemical pathways and biological processes scored using T-Profiler.

	Source	*T* value	*E* value	Mean	Orfs
200 µM Coumarin					
Cadmium ion binding (F)	GO	5.25	0.00	0.94	6
Aldo-keto reductase activity (F)	GO	4.15	0.04	0.92	4
Complement and coagulation cascades	KEGG	-4.62	0.00	-0.16	66
600 µM Coumarin					
Transcription (P)	GO	5.42	0.00	0.12	1033
Response to unfolded protein (P)	GO	4.87	0.00	0.35	38
Regulation of transcription, DNA-dependent (P)	GO	4.80	0.00	0.10	1435
Protein folding (P)	GO	4.24	0.03	0.17	188
Metabolism (P)	GO	-4.93	0.00	-0.03	346
Urea cycle (P)	GO	-5.03	0.00	-0.69	6
Fatty acid metabolism (P)	GO	-5.23	0.00	-0.20	54
Lipid metabolism (P)	GO	-5.37	0.00	-0.08	197
Zinc ion binding (F)	GO	6.12	0.00	0.11	1714
Nucleic acid binding (F)	GO	5.50	0.00	0.14	669
Metal ion binding (F)	GO	4.49	0.01	0.10	1674
DNA binding (F)	GO	4.42	0.01	0.11	912
Lyase activity (F)	GO	-4.39	0.01	-0.11	90
Oxidoreductase activity (F)	GO	-4.84	0.00	-0.02	420
Lipid transporter activity (F)	GO	-4.96	0.00	-0.25	33
Nucleus (C)	GO	5.74	0.00	0.09	3041
Peroxisome (C)	GO	-4.51	0.01	-0.16	56
Extracellular region (C)	GO	-4.84	0.00	-0.03	390
Mitochondrion (C)	GO	-5.67	0.00	-0.03	543
GO_0005739 (mitochondr) (C)	GO	-4.15	0.02	-0.06	154
Pores ion channels	KEGG	3.65	0.05	0.73	4
Metabolism of xenobiotics by cytochrome P450	KEGG	-3.97	0.01	-0.13	56
Propanoate metabolism	KEGG	-4.04	0.01	-0.18	36
Bile acid biosynthesis	KEGG	-4.24	0.00	-0.19	37
Glycine, serine and threonine metabolism	KEGG	-4.32	0.00	-0.18	42
Arginine and proline metabolism	KEGG	-4.49	0.00	-0.16	55
Urea cycle and metabolism of amino groups	KEGG	-4.67	0.01	-0.30	22
Complement and coagulation cascades	KEGG	-4.98	0.00	-0.16	65
Valine, leucine and isoleucine degradation	KEGG	-5.07	0.00	-0.21	48
Fatty acid metabolism	KEGG	-6.97	0.00	-0.31	46
Glycine, serine, and threonine metabolism	GenMAPP	-3.88	0.05	-0.26	220
Extrinsic prothrombin activation pathway	Manually curated	-3.98	0.03	-0.38	11
Fibrinolysis pathway	Manually curated	-4.33	0.01	-0.54	7
Valine, leucine, and isoleucine degradation	GenMAPP	-4.34	0.01	-0.28	22

Adult liver vs fetal liver GNF2	Manually curated	-5.45	0.00	-0.17	71
Tryptophan metabolism	GenMAPP	-5.55	0.00	-0.24	44
Human mito DB-6-2002	Manually curated	-5.56	0.00	-0.04	392
Bile acid biosynthesis	GenMAPP	-5.95	0.00	-0.42	20
Mitochondr	Manually curated	-6.62	0.00	-0.06	405
Fatty acid metabolism	GenMAPP	-7.11	0.00	-0.33	43

Positive and negative *T* values indicate predominant up- and down-regulation of genes within the term, respectively; significant GO terms have an *E* value (Bonferroni corrected *P* value [40]) below 0.05; mean represents the mean log 2 expression value of all genes within the term measured in the complete data set (Orfs); GO terms are assigned to a biological process (P), a molecular function (F), or a cellular component (C).

induced gene expression profiles in human hepatocytes were compared to toxicogenomics results in rat hepatocytes and rats *in vivo* exposed to coumarin in a previously performed study [25] in order to predict effects on gene expression in humans. Concentrations of coumarin used in human hepatocytes in the present study were similar to those used in the rat hepatocytes previously [25].

As expected and also reported by others [42], a conventional assay in toxicology, the MTT reduction assay which measures mitochondrial function, did not indicate signs of toxicity in human hepatocytes treated with 600 µM coumarin. In rat hepatocytes, however, this dose resulted in severe cytotoxicity, killing more than 50% of the cultured cells [25]. Considering results on gene expression, we were primarily interested in the biochemical pathways and biological processes affected and not the individual genes modulated after coumarin exposure. We, among others, [43], consider that the interrelation between genes reflected in these underlying pathways and processes gives more biological relevant information about the mode of action of compounds. Therefore, two approaches were used for identifying these biochemical pathways and biological processes. First, MAPPFinder [33] was applied to determine over-representation of pathways and processes within the subset of significantly modulated genes which were identified by prior data analysis. Second, T-profiler analysis [40] was performed to extract predominant biochemical pathways and biological processes from the complete data set of genes without the need for prior data analysis to pre-select of a subset of genes. Within the parallelogram approach, biological responses of coumarin in human hepatocytes as deduced from both pathway analysis methods were evaluated for their potential toxicity or non-toxic beneficial/ pharmacological effects.

The previously performed rat toxicogenomics study focused on the relevance of coumarin-induced gene expression profiles in rat hepatocytes cultured in sandwich configuration for the situation in rats *in vivo* [25]. To that purpose, sandwich-cultured rat hepatocytes *ex vivo* as well as rats *in vivo* were exposed to coumarin. Pathway analysis retrieved KEGG pathways for which the expression of contained genes changed in rat hepatocytes upon coumarin treatment.

These pathways could be compared to KEGG pathways obtained in the present study in human hepatocytes exposed to coumarin using both MAPPFinder and T-profiler analysis.

Complement and coagulation cascades have been shown to be affected by coumarin in rat hepatocytes *in vitro* and rats *in vivo* [25]. In the present study, gene expression changes which could be indicative for this effect were observed in human hepatocytes, as is shown in the T-profiler results upon treatment with 200 µM and 600 µM coumarin. Additional to this result, T-profiler analysis shows down-regulation of the extrinsic prothrombin activation pathway and fibrinolysis pathway in human hepatocytes. The down-regulation of these pathways is related to the therapeutic potential of coumarin and its derivatives 4-hydroxycoumarin and warfarin, well-known for their anticoagulation properties [7] and not to toxicity. Coumarin anticoagulants are increasingly used long term in patients to prevent thromboembolism [44, 45]. This is achieved by down-regulation of the vitamin K coagulation pathways [44-46]. With respect to this known therapeutic action of coumarin, toxicogenomics data from the present human hepatocyte study, together with our previous data obtained in rat hepatocytes *in vitro* and rats *in vivo* [25] can be used along the parallelogram approach to corroborate effects in humans *in vivo*. As coumarin is used in human subjects as a drug for its anticoagulation properties [7, 44], these results suggest relevance for *in vitro* reflecting the human *in vivo* situation.

Fatty acid metabolism, one of the major liver functions, has been shown to be repressed upon coumarin exposure of both rat hepatocytes *in vitro* and rats *in vivo* [25]. In the present study, T-profiler analysis indicated enrichment of differential expression of genes within this KEGG pathway upon treatment of human hepatocytes with 600 µM coumarin. Like the effect on complement and coagulation cascades, the completion of the parallelogram along the *in vitro* interspecies and *in vitro-in vivo* intraspecies axes suggests relevance of this effect for humans *in vivo*.

The KEGG pathway urea cycle and metabolism of amino groups was triggered in rat hepatocytes in the coumarin rat study [25] and was observed in both the MAPPFinder and T-profiler results in human hepatocytes treated with 600 µM coumarin in the present study. Like fatty acid metabolism, urea cycle and metabolism of amino groups comprises one of the major functions of the liver. As this pathway was not shown to be triggered in rat livers *in vivo* 25, the parallelogram was completed only along the *in vitro* interspecies axes. Coumarin-induced modulation of urea cycle and metabolism of amino groups may only occur in hepatocytes as an *in vitro* artifact, lacking relevance for *in vivo*.

Additional to fatty acid metabolism and urea cycle and metabolism of amino groups affected by coumarin, human hepatocytes in the present study showed coumarin-induced modulation of other major liver functions and energy-consuming biochemical pathways including lipid metabolism, oxidoreductase activity, metabolism of xenobiotics by CYP450, metabolism of several amino acids, and bile acid biosynthesis. All these are down-regulated upon coumarin exposure. One must be careful in the analysis of the observed responses in human hepatocytes and distinguish between toxicity and benign homeostatic adjustments [24], however, down-

regulation of these energy-consuming biochemical pathways might indicate (first signs of) toxicity in human hepatocytes.

Furthermore, down-regulation of bile acid biosynthesis may protect hepatocytes from bile acid toxicity. Coumarin affects mitochondrial function as observed in the T-profiler results upon treatment of human hepatocytes with 600 µM of coumarin in the present study. These results were not obvious in the coumarin rat study. Impairment of mitochondrial function is related to toxicity and is a known toxic endpoint of the necrotizing hepatotoxicant acetaminophen [24, 47]. Although no effects on mitochondrial function are observed using the traditional MTT reduction assay in human hepatocytes treated with coumarin, down-regulation of genes associated with mitochondria and mitochondrial function might, alike the repression of liver specific functions above, indicate first signs of toxicity albeit upon treatment with the highest concentration, 600 µM. However, effects of coumarin on the pathways designated as toxic effects of coumarin in the previously performed coumarin rat study [25], like impairment of methionine metabolism which is associated with liver failure, necrosis, liver cirrhosis and fibrogenesis [48, 49], were not observed in human hepatocytes in the present study.

The marked species differences in coumarin metabolism resulting in differences in hepatotoxicity between man and rats [4-6], are reflected in differences in toxic responses at the level of traditional measures of toxicity and in gene expression changes between human and rat hepatocytes in the present study. These findings underscore the need for using primary human hepatocytes for *in vitro* testing of compounds in order to be able to predict human exposure. Of course, the limited and irregular availability of primary human hepatocytes poses a problem, but the major improvements in methods and techniques for isolation of primary human hepatocytes enables isolation of cells with higher viability, as well as using smaller liver segments, and allows longer periods of time between obtaining a liver sample and isolation [27]. Others also report the inter-individual variability between donors as a disadvantage of the use of human hepatocytes which causes a wide variation in responses to compounds [50]. Despite this, human hepatocytes are arguably more reflective of the variability in susceptibility to different agents in a human population [51, 52].

In summary, identical to results observed in the coumarin rat study, the well-known property of coumarin in humans *in vivo*, namely, down-regulation of complement and coagulation cascades was observed in human hepatocytes. The parallelogram approach could not be completed for the hepatotoxic effect of coumarin in rats on methionine metabolism, given interspecies differences in metabolism. Still, repression of energy-consuming biochemical pathways and impairment of mitochondrial function, which have been related to hepatotoxicity caused by the necrotizing hepatotoxic acetaminophen, were observed in human hepatocytes treated with coumarin in the present study and might indicate toxicity. However, these effects are only observed in human hepatocytes treated with the highest concentration of coumarin and are therefore unlikely to be relevant for the situation in humans *in vivo*. Results on pathway and process analysis described here were predominantly observed by T-profiler analysis and not

by MAPPFinder analysis, indicating low-level modulation of gene expression. In more detail: bioinformatics approaches in toxicogenomics that do not rely upon statistical pre-selection of gene sets before proceeding towards biological bioinformatics (T-profiler) likely perform better than approaches for which an initial pre-selection needs to be performed (MAPPFinder).

In conclusion, the present study underscores the sensitivity of the toxicogenomics approach in identification of coumarin-induced biological pathways and processes either related to defined toxicities or non-toxic. Furthermore, although the toxicogenomics-based parallelogram approach aids in prediction of human compound-induced liver injury, future hepatocyte-based *in vitro* studies investigating modes of action of compounds should consider human hepatocyte-based models as the preferable model to account for inter-species differences in e.g. metabolism and toxicity of compounds.

ACKNOWLEDGEMENTS

We acknowledge the useful discussions with Dr. H. Wortelboer and W. Maas on the human hepatocyte isolation technique. We are grateful to Dr. N. Treijtel and K. Mathijs for their assistance in the hepatocyte isolation procedure.

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Chapter V

Toxicogenomics to identify pathways and processes reflecting thioacetamide hepatotoxicity in rats *in vivo*

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ABSTRACT

Thioacetamide is a potent hepatotoxicant which provokes hepatic necrosis. Previous toxicogenomics studies have identified subsets of thioacetamide-induced genes as markers of potential toxicity. Interpretation of subsets of genes affected upon thioacetamide exposure within a biological context, e.g. by using pathway analysis tools might increase the information that can be retrieved from toxicogenomics studies. The objective of the present study therefore was to identify biological pathways and processes as markers of potential hepatotoxicity upon thioacetamide administration. Male Wistar rats were treated intraperitoneally with thioacetamide in a low, moderate, and a high dose. Liver toxicity of thioacetamide was confirmed at 6h and 24h after administration by histopathological analysis and clinical chemistry measurements. DNA microarrays were employed to identify dose- and time-dependent gene expression profiles. Subsets of genes affected were translated to their underlying pathways and processes using MAPPFinder pathway analysis. Liver toxicity was most pronounced in the moderate and high dose groups at 24h, as reflected by traditional toxicity measures, the gene expression data, and the biological pathways and processes affected by thioacetamide. Only at 24h phenotypic anchoring showed correlation of gene expression results with clinical chemistry parameters. Both the moderate and high dose at 24h showed pathways and processes involved in thioacetamide hepatotoxicity including response to wounding, blood coagulation, inflammation, lipid metabolism, and cholesterol metabolism. The present thioacetamide toxicogenomics study shows the added value of pathway and process analysis and phenotypic anchoring, rather than single gene analysis, for interpreting the gene expression data in a relevant toxicological context.

INTRODUCTION

The introduction of the toxicogenomics approach several years ago enabled elucidation of multiple effects by simultaneous measurement of thousands of genes and pathways induced by toxicants in cells and organisms [1-3]. In recent years, toxicogenomics studies have identified toxic modes-of-action of compounds and have enabled clustering or discrimination of toxicant classes based on the mechanisms of toxicity at the single gene level [2-7]. Recently, a study was performed by Minami *et al*., which aimed at identifying marker genes for hepatotoxic chemicals that cause zone-3 necrosis in livers of rat [8]. One of the compounds used to identify marker genes for necrosis was thioacetamide ($CH3\text{-}C(S)NH_2$). Thioacetamide is a potent hepatotoxicant which is used as a compound in leather, textile, and paper industries as an accelerator in the vulcanization of buna rubber and as a stabilizer for motor fuels. Thioacetamide provokes necrosis as it is metabolized in the liver to reactive intermediate(s) which covalently bind to hepatic macromolecules [9-11]. In a follow-up study, Minami *et al*. determined dose- and time-specific effects on gene expression profiles of thioacetamide in particular, thereby selecting sensitive markers for thioacetamide toxicity [12].

Minami *et al*. identified single genes affected by thioacetamide, suggested to be related to toxicity, however, information was lacking on the relation of these genes to each other, their shared involvement in and the consequent influence of thioacetamide on biochemical pathways and biological processes within the cell [8, 12]. Investigating whether interrelation exists between toxicity markers and subsets of toxicant-affected genes by applying biochemical pathway and biological process mapping approaches has been proposed in order to increase the biological information that can be retrieved from a toxicogenomics study [13]. One tool that assists in identifying possibly affected biological processes and pathways is MAPPFinder [14], a program for analyzing microarray data on functional groups known as gene ontology (GO) terms [15] and biological pathways (MAPPs) [14].

Insights into biochemical pathways of toxicity and disease processes can be further increased by phenotypic anchoring of toxicogenomics results with suitable conventional assays [16, 17]. For instance, toxicant-induced gene expression changes can be correlated to results obtained from clinical chemistry measurements. Subsets of genes which strongly correlate with clinical chemistry parameters indicating toxicity may indicate specific toxicological mechanisms of the compound. Furthermore, translation of these subsets of genes to the pathways and processes to which they belong can distinguish true pathways of toxicity from other, non-specific or unrelated to toxicity, pathways [17].

The objective of the present study was to identify biological pathways and processes as markers of potential hepatotoxicity in a toxicogenomics model of thioacetamide *in vivo*. For this purpose, thioacetamide was administrated intraperitoneally (i.p.) to rats in a low, moderate, and high dose. To confirm toxicity, liver histopathology and clinical chemistry data were obtained at 6h and 24h after i.p. administration. Gene expression was translated to biochemical pathways

and biological processes using MAPPFinder. To evaluate potential hepatotoxicity of the thioacetamide-induced biochemical pathways and biological processes, phenotypic anchoring was performed by correlation of gene expression results with clinical chemistry data.

METHODS

Chemicals

Roche reagent kits and primer $p(DT)_{15}$ were purchased from Roche (Mannheim, Germany). TRIzol™ was obtained from Invitrogen (Breda, The Netherlands). Amino-allyl dUTP was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The RNeasy mini kit, the RNAse-free DNAse kit, and the QIAquick PCR purification kit were obtained from Qiagen (Westburg B.V., Leusden, The Netherlands). Cy3 and Cy5 monofunctional reactive dyes and AutoseqG-50 sephadex chromatography columns were purchased from Amersham Biosciences (Roosendaal, The Netherlands). Thioacetamide, CAS-no 62-55-5; purity minimum 99% according to the manufacturer, was obtained from Sigma-Aldrich. All other chemicals were of analytical grade.

Animals

Male Wistar rats (Crl: (WI) WU BR) aged 10 weeks (180-250 g), were obtained from Charles River GmbH, Sulzfeld, Germany. Animals were kept under controlled conditions according to international guidelines and national legislation regarding proper care and ethical use of animals. During the acclimatization period and until sacrifice, animals were housed individually in macrolon cages with wire tops and sawdust bedding at 22 °C and 50-60% humidity. The light cycle was 12h light/12h dark. Feed and tap water were available ad libitum.

Animal treatment

Forty animals were assigned to 8 groups (5 rats per group). Rats were injected intraperitoneally (i.p.) at dose levels of 7.4 mg/kg b.wt. (low dose), 66.7 mg/kg b.wt. (mid dose), and 200 mg/kg b.wt. (high dose) thioacetamide dissolved in saline. As a solvent control, only vehicle was injected (0 mg/kg b.wt.). Effective doses have previously been defined in a range finding study and correspond with doses used in other studies [8, 18]. Injection volume for each treatment was 10 ml/kg b.wt.. At the indicated time (6h and 24h post thioacetamide administration) rats were anesthetized by inhalation of CO_2/O_2. Animals were sacrificed by bleeding through the aorta abdominalis from which blood was collected in heparin tubes. Plasma was isolated for clinical chemistry. Thereafter, livers were immediately dissected, frozen in liquid nitrogen, and stored at -80 °C until further processing. A section of the liver was kept aside in formalin for pathological examination.

Clinical Chemistry

Serum alanine aminotransferase (ALT) activity, aspartate aminotransferase (AST) activity, lactate dehydrogenase (LDH) leakage, alkaline phosphatase (ALP) activity, glucose, cholesterol, γ-glutamyl transferase (GGT) activity, and phospholipid levels were analyzed on a Hitachi 911 centrifugal analyzer using Roche reagent kits. Differences between plasma levels in treated and non-treated animals were defined as statistically significant at a *P* value below 0.01, determined by one-way ANOVA followed by Dunnett's test.

Total RNA isolation

RNA was extracted using TRIzol™ according to the manufacturer's protocol. In the *in vivo* study, TRIzol™ was added to frozen liver samples which were subsequently pulverized with mortar and pestle under liquid nitrogen before extraction. RNA was purified using the RNeasy mini kit including an additional DNAse digestion step. RNA concentrations were determined spectrophotometrically. RNA quality was assessed by agarose gel electrophoresis.

Microarray design

A local reference design was used for microarray hybridization. Tester RNA labeled with Cy5 from individual rat livers was combined with reference RNA consisting of a pool of RNA extracted from livers obtained from five non-exposed, control rats.

Microarray labeling

RNA samples were indirectly labeled according to the amino-allyl labeling procedure for microarrays from The Institute for Genomic Research (TIGR)[19]. For each labeling reaction, 25 µg of total RNA was used as starting amount for reverse transcription of mRNA. Briefly, for reverse transcription, mRNA was selected by oligo dT priming with primer $p(DT)_{15}$. The reverse transcription reaction, by which amino-allyl dUTP is incorporated, was conducted for three hours at 42 °C. Non-transcribed RNA was degraded by alkaline hydrolysis in 2 µL 2.5M NaOH for 30 minutes at 37 °C. Thereafter, the mixture was neutralized with an equimolar amount of acetic acid. The cDNA was purified using the Qiagen QIAquick PCR purification kit. Columns were washed with 10 mM sodium-borate in 80% ethanol (pH 8.5). Column-bound cDNA was eluted two times in 30 µL MilliQ H_2O. Samples were labeled with either Cy3 (reference) or Cy5 (tester) monofunctional reactive dyes and afterwards cleaned from unincorporated Cy dyes using AutoseqG-50 sephadex chromatography columns.

Microarray hybridization

Samples were hybridized on rat oligonucleotide microarrays containing approximately 5800 different oligonucleotide fragments (Qiagen Operon, Westburg B.V., Leusden, The Netherlands), spotted in duplicate at the Utrecht Genomics Laboratory, Utrecht, The Netherlands as described [20, 21]. The hybridization protocol was performed as described previously [22]. Microarrays were

scanned using a Packard Scanarray confocal laser scanner (PerkinElmer Life And Analytical Sciences, Inc., Boston, USA). Resulting TIFF images were loaded into Imagene 5.0 (Biodiscovery Inc., Los Angeles, USA) and saved to further process and analyze the data.

Microarray quality criteria

Criteria for microarrays to be included into further analysis consider the homogeneity of the spot signal intensities, the effect of bleaching of the fluorescence, the number of manually flagged (excluded) spots, the spatial distribution of the signals over the slide surface, the balance between Cy3 and Cy5 signal intensity, the number of saturated spots and the quality of the slide with respect to other slides of the experiment, as described by Heijne *et al*. [20].

Microarray data analysis

Flagged spots and controls were excluded from further analyses. Only spots on qualified microarrays with intensities higher than 1.5 times the intensity of their local background were included in data analysis. Ratios of the background-corrected intensities of tester over reference were calculated for each slide. To account for technical variations introduced during labeling or hybridization, data were normalized using the Lowess algorithm [23]. Normalized ratios were log-transformed with base two in SAS Enterprise guide V2 (SAS Institute Inc., Cary, USA). The resulting data set was loaded into Excel (Microsoft Corporation, Redmond, WA, USA). Replicate genes per array were averaged. Two missing values per gene were accepted. The complete data set of genes was assembled into principal component analysis (PCA), performed using GeneSight software Version 4.1 (Biodiscovery Inc. El Segundo, CA).

Gene expression within the complete data set was correlated to the clinical chemistry parameters by calculating the Spearman rank order coefficient using GEPAS [24, 25]. Correlation was significant when the correlation coefficient was greater than 7.5 (positive correlation) or smaller than -7.5 (negative correlation) and *P* values were below 0.001. For each parameter, a subset of correlated genes was generated. Furthermore, subsets of significantly expressed genes were selected in each time point and dose group, all compared to the group of control rats treated with vehicle only. Genes were determined significant if fold changes were above or below a threshold of 1.5 and when two-sided Student's *T* test *P* values were below 0.05. This approach appears to be a favorable option for identifying differentially expressed genes [26].

Over-represented pathways and processes were determined in clinical chemistry parameter correlated genes and significantly expressed genes per time point and dose group using MAPPFinder (Version 2.0, Gladstone Institutes 2000-2006) [27]. MAPPs and GO terms were significantly over-represented when *Z* scores were greater than 2 and when the and when *P* values were below 0.25. Furthermore, the number of genes changed within a certain MAPP or GO term had to be either greater than 5 or less then 100 to exclude GO terms that are either too specific or too general. Within the over-represented GO terms, specificity was increased by removing parent terms from the list in cases where both a parent and a child term were present and the presence of the parent term was due entirely to genes meeting the criterion for the child term [14].

Table 1. Histopathological findings and clinical chemistry parameters.

	6h post TA administration				24h post TA administration			
TA dose group (mg/kg BW)	**0 (control)**	**7.4 (low)**	**66.7 (mid)**	**200 (high)**	**0 (control)**	**7.4 (low)**	**66.7 (mid)**	**200 (high)**
Histopathological findings								
Focally discolored liver (%)	0	0	60	0	0	0	80	100
Centrilobular hepatocellular necrosis (%)	0	0	80	0	0	0	100	100
Periportal inflammatory cell infiltrate (%)	0	0	100	0	0	0	0	100
Clinical chemistry parameters								
ALP (U/l)	147 (±13)	134 (±7)	140 (±20)	159 (±15)	172 (±13)	146 (±30)	167 (±24)	181 (±31)
Cholesterol (mmol/l)	1.82 (±0.32)	1.66 (±0.12)	1.61 (±0.24)	1.8 (±0.25)	2.02 (±0.13)	1.65** (±0.19)	1.56** (±0.16)	1.7* (±0.22)
Glucose (mmol/l)	10.65 (±1.51)	9.91 (±0.18)	10.34 (±0.87)	12.37* (±0.85)	11.08 (±1.25)	10.72 (±0.45)	8.11** (±0.39)	7.36** (±0.47)
AST (U/l)	63 (±6)	69 (±8)	138** (±45)	74 (±15)	74 (±8)	65 (±5)	1829 (±631)	9500 (±12810)
ALT (U/l)	62 (±5)	62 (±12)	72 (±11)	63 (±4)	61 (±6)	45 (±11)	776** (±442)	834** (±432)
GGT (U/l)	2.5 (±1.5)	1.2 (±0.5)	1.4 (±0.3)	1.2 (±0.4)	1 (±0.3)	1.5 (±0.4)	1.9 (±0.6)	2.6** (±0.9)
LDH (U/l)	116 (±24)	180 (±64)	477 (±280)	368 (±280)	421 (±112)	333 (±116)	5425 (±2019)	34588 (±51018)
Phospholipids (mmol/l)	1.9 (±0.27)	1.74 (±0.16)	1.61 (±0.15)	1.87 (±0.25)	2.11 (±0.21)	1.69** (±0.17)	1.34** (±0.11)	1.44** (±0.11)

Histopathological findings are given as percentages of the animals in which a certain parameter was observed per dose group (n=5); for clinical chemistry parameters: *P<0.05, **P<0.01; statistical significance was calculated compared to control levels; clinical chemistry parameters are expressed either in Units per liter or mmol per liter, data are presented as means (±SD).

RESULTS

Histopathology and clinical chemistry

Histopathology and clinical chemistry parameters were determined in rats 6h and 24h after i.p. administration of thioacetamide in a low, moderate, and high dose. Three histopathological parameters were determined: focally discoloring of the liver, centrilobular hepatocellular necrosis, and periportal inflammatory cell infiltrate. Table 1 presents the percentages of animals per dose group in which certain histopathological parameters were observed. At 6h after administration of thioacetamide, the moderate dose group already showed focally discoloring, centrilobular necrosis and inflammatory cell infiltrates in the majority of the animals. No effects

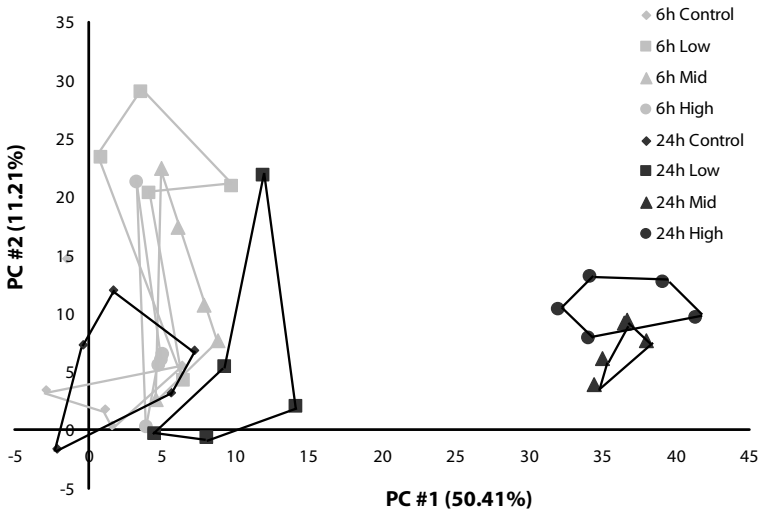

Figure 1. Principal component analysis of the complete dataset of genes containing expression values of rats at 6h or 24h after treatment with a low, moderate, or high dose of thioacetamide. Two principal components were generated and plotted for each time point and dose group.

of thioacetamide on histopathology parameters were observed after 6h in the other dose groups. 24h after treatment of rats with a moderate and high dose of thioacetamide, focally discolored liver and hepatocellular necrosis were demonstrated in almost all animals. After 24h, periportal inflammatory cell infiltrate was only observed in the high dose group.

Clinical chemistry data showed that at 6h, glucose levels and AST activities of the moderate and high dose group, respectively, were significantly elevated. At 24h, cholesterol and phospholipid levels were significantly decreased in all dose groups; glucose levels were significantly decreased in the moderate and high dose groups. Significantly increased levels of ALT activities (in the moderate and high dose) and GGT (in the high dose) were observed at 24h. Mean AST activity and LDH levels were extremely, though not significantly, elevated in the moderate and high dose 24h after thioacetamide administration. Clinical chemistry data are presented in Table 1.

Principal component analysis of all genes

To visualize treatment time- and dose-dependent differences in complete gene expression profiles, the unsupervised method principal component analysis (PCA) was performed (Fig. 1). PCA showed limited differences between gene expression profiles of all dose groups at 6h after thioacetamide administration, and in the control and low dose group at the 24h time point. However, the 24h moderate and high dose groups were clearly discriminated from the other test groups, indicating that in these treatment groups major changes in gene expression occured (Fig. 1).

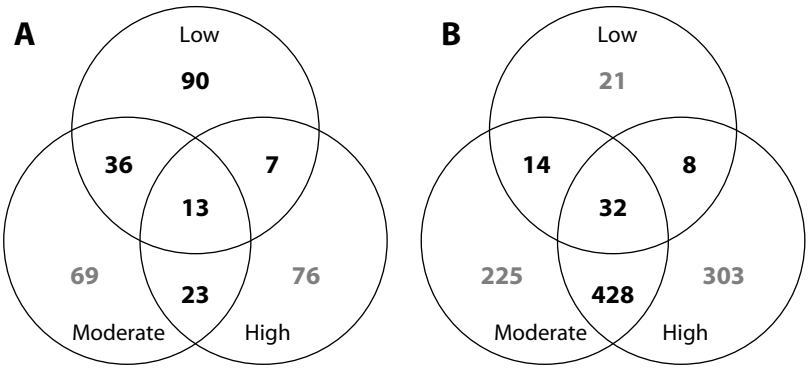

Figure 2. Circles in the Venn diagram represent the number of genes significantly changed in the low, moderate, or high dose group at 6h (A) and in the low, mid, or high dose group at 24h (B). Intersections represent the number of genes significantly modulated in more than one dose group.

Gene expression analysis towards individual genes

Criteria settings for filtering genes resulted in final datasets with 1638 genes for the 6h and 2161 genes for the 24h exposure period. Determination of significantly modulated genes resulted in 146, 141 and 119 genes modulated by, respectively, the low, moderate, and high dose at the 6h time point. At 24h, 75, 699, and 771 gene expressions were significantly changed by thioacetamide in the low, moderate, and high dose, respectively. Overlap of the changed gene expressions between dose groups 6h and 24h post thioacetamide administration is presented by the Venn diagram in Fig. 2A and Fig. 2B.

At 6h and 24h, respectively 79 and 482 genes were significantly modulated in at least two dose groups as presented in the intersections of the Venn diagrams. At each time point, all of these genes except one were modulated in similar directions. At 6h, dose-responsiveness was only observed in genes within the intersection between the moderate and high dose group. Gene expression levels in the high dose group were more extreme. At 24h, dose-responsiveness was observed within the low and the moderate or high dose group; more extreme values were observed at higher dose levels. Almost no difference in gene expression values was observed in genes significantly modulated in both the mid and high dose group at 24h, possibly indicating that a maximum change in gene expression profile already occurred at a moderate dose.

Minami *et al*. selected sixteen potential toxicity marker genes for hepatocellular necrosis which were confirmed to be sensitive markers for thioacetamide hepatotoxicity in livers of Sprague-Dawley rats exposed to 50, 150, and 400 mg/kg b.wt. thioacetamide administrated i.p. for 24h [8, 12]. For twelve out of these sixteen genes, gene expression was measured in the current study at 24h, and eleven of these genes were significantly modulated. These genes and their expression values are listed in Table 2. One, ten, and six genes were significantly expressed upon

Table 2. Gene expression of potential toxicity marker genes as selected by Minami *et al*. [8] at 24h.

Genbank Accession #	Gene Symbol	7.4 mg/kg BW Low	66.7 mg/kg BW Mid	200 mg/kg BW High
D25233	Rb1	0.02	-0.35	-0.41
NM_012883	Ste	0.03	-1.06*	-2.62*
AF095449	Hadhsc	0.07	-1.21*	-1.65*
NM_013043	Tgfb1i4	0.35	-1.81*	-2.43*
NM_031614	Txnrd1	0.20	0.79*	1.17*
NM_012771	Lyz	0.37	2.84*	2.15*
NM_012923	Ccng1	0.00	1.10*	1.97*
NM_012580	Hmox1	-0.06	1.40*	NM
AY017337	Nme3	0.09	-0.66*	NM
Z11690	Avpr1a	-0.11	-1.92*	NM
NM_031732	Sult1a2	0.22	-1.19*	NM
NM_017000	Nqo1	0.97*	0.12	0.48

*Significant values (see methods); gene values are log-transformed ratios corrected for the control; NM, not measured.

administration of the low, moderate, and high dose, respectively. The direction of modulation of all these genes was similar to results presented by Minami *et al*. [12].

Identification of over-represented MAPPs and GO terms

Table 3 shows the GO terms over-represented within the thioacetamide-induced gene expression profiles. At 6h, only the highest dose affected amino acid metabolism and related biological processes. At 24h, the moderate dose resulted in over-representation of carboxylic acid metabolism, response to wounding, and cholesterol biosynthesis. Molecular functions affected by this dose group included vitamin binding and chymotrypsin activity. Most GO terms were affected by the high dose group 24h after thioacetamide administration. Predominantly, response to wounding, blood coagulation and related biological processes were affected.

Significant MAPPs were observed only 24h after thioacetamide administration. Administration of the moderate dose resulted in over-representation of the tryptophan metabolism pathway. Twenty out of thirty genes measured on the array were changed, resulting in a *Z* score of 3.89 and a *P* value of 0.006. The ribosomal proteins pathway was over-represented in the high dose group. This dose resulted in significant modulation of 30 out of 51 genes measured on the array, resulting in a *Z* score of 2.90 and a *P* value of 0.178.

Phenotypic anchoring of clinical chemistry results

To distinguish true toxic gene expression responses, gene expression within the complete data set was correlated to the clinical chemistry results. Only four, two, two, and three genes were correlated to the clinical chemistry parameters ALP, AST, LDH, and phospholipids, respectively. No correlated genes were found in the parameters cholesterol, glucose, ALT, and GGT in livers of rats 6h after thioacetamide administration. The low number of genes correlated to clinical

Table 3. Over-represented GO terms after administration of thioacetamide.

GO ID	GO Name	Number Changed	Number Measured	Number in GO	*Z* score	*P* value
6h high dose (200 mg/kg b.wt.)						
Biological Process						
19752	Carboxylic acid metabolism	23	95	252	6.02	0.01
9308	Amine metabolism	14	54	175	4.93	0.13
6519	Amino acid and derivative metabolism	15	50	164	5.82	0.09
6520	Amino acid metabolism	13	41	136	5.65	0.09
9064	Glutamine family amino acid metabolism	5	7	20	6.17	0.01
24h moderate dose (66.7 mg/kg b.wt.)						
Biological Process						
19752	Carboxylic acid metabolism	50	102	252	3.64	0.09
9611	Response to wounding	21	35	116	3.49	0.21
6695	Cholesterol biosynthesis	9	11	15	3.49	0.21
Molecular Function						
19842	Vitamin binding	13	17	47	3.87	0.05
4263	Chymotrypsin activity	11	14	119	3.68	0.09
24h high dose (200 mg/kg b.wt.)						
Biological Process						
9605	Response to external stimulus	23	39	140	3.73	0.06
9611	Response to wounding	24	35	116	4.09	0.01
6954	Inflammatory response	13	16	61	3.81	0.04
7596	Blood coagulation	11	14	31	3.35	0.23
51701	Response to other organism	26	42	131	3.57	0.11
9613	Response to pest, pathogen or parasite	25	41	124	3.40	0.19
Molecular Function						
3735	Structural constituent of ribosome	31	51	373	3.78	0.05
Cellular Component						
5783	Endoplasmic reticulum	57	113	238	3.36	0.21

GO analysis was performed using GenMAPP and MAPPFinder [14]; over-represented GO terms have *Z* scores>2, *P* values<0.25, and 5 to 100 changed genes; GO terms are arranged as parent terms and their related child terms; GO terms are arranged in a hierarchy and listed as parent terms with their strongly related child terms downstream.

chemistry parameters at the 6h time point did not allow for MAPPFinder analysis. At 24h after thioacetamide administration, however, five out of eight clinical chemistry parameters were highly correlated to the gene expression results. Results showing the number of correlated genes and the biochemical pathways and biological processes of which these genes are part are presented in Table 4. Over-represented GO terms include carboxylic acid metabolism and its child term amino acid metabolism, several GO terms related to lipid metabolism, and monooxygenase activity. Over-represented MAPPs include complement and coagulation cascades, tryptophane metabolism, mitochondrial fatty acid betaoxidation, and cholesterol biosynthesis.

Table 4. GO terms and MAPPs in subsets of genes correlated to clinical chemistry parameters.

Clinical Chemistry parameters	Number of correlated genes	GO terms		Local MAPPs
ALP	0	None		None
Cholesterol	7	None		None
Glucose	301	Carboxylic acid metabolism	P	Complement and Coagulation Cascades KEGG
		Amino acid metabolism	P	
		Sulfur amino acid metabolism	P	
AST	220	Carboxylic acid metabolism	P	Tryptophane Metabolism KEGG
		Vitamin binding	F	
		Generation of precursor metabolites and energy	P	
ALT	146	Lipid metabolism	P	Tryptophane Metabolism KEGG
		Fatty acid metabolism	P	Mitochondrial fatty acid betaoxidation
		Generation of precursor metabolites and energy	P	
		Electron transport	P	
		Oxidoreductase activity	F	
		Monooxygenase activity	F	
		Unspecific monooxygenase activity	F	
		Acyl-CoA dehydrogenase activity	F	
		Microsome	C	
GGT	20	None		None
LDH	240	Carboxylic acid metabolism	P	Tryptophane Metabolism KEGG
		Amino acid metabolism	P	
		Cholesterol metabolism	P	
Phospholipids	179	Lipid biosynthesis	P	Cholesterol Biosynthesis
		Steroid metabolism	P	
		Steroid biosynthesis	P	
		Cholesterol biosynthesis	P	

GO analysis was performed using GenMAPP and MAPPFinder [14]; over-represented GO terms have *Z* scores>2, *P* values<0.25, and 5 to 100 changed genes; P, Biological Process; F, Molecular Function; C, Cellular Component.

DISCUSSION

In the present study, the toxicogenomics approach was applied to identify biological processes and pathways affected by the necrotic compound thioacetamide in male Wistar rats. To this purpose, male Wistar rats, 10 weeks of age, were treated with thioacetamide at a low (17.5 mg/kg b. wt.), moderate (66.7 mg/kg b. wt.), and high (200 mg/kg b. wt.) dose level. To evaluate biochemical pathways and biological processes for their hepatotoxicity, phenotypic anchor-

ing of toxicogenomics data with traditional toxicity markers was performed using correlation analysis.

Overall histopathology and clinical chemistry findings showed that effects by thioacetamide were highest at 24h rather than at 6h after i.p. administration. These findings confirm other studies following the time course of changes induced by thioacetamide, which reported that the maximal toxicity peaks at 24h [8, 12, 28, 29]. At 6h after treatment, histopathological abnormalities and effects on clinical chemistry parameters were clearly observed at the mid dose group, and almost not at the high dose group. So, traditional toxicity measures did not demonstrate dose-dependent responses at this time point. The severity of toxicity as shown by the traditional toxicology markers at 24h upon administration of moderate and high doses was confirmed by gene expression profiling using PCA (Fig. 1). Furthermore, compared to 6h after thioacetamide administration, at 24h, the number of thioacetamide-modulated genes in the moderate and high dose group was higher. At this time point, a dose-responsive increase of the number of genes was observed. Furthermore, clinical chemistry parameters appear conserved across Wistar and Sprague-Dawley rat strains, as our LDH, AST activity, and ALT activity levels were similar to levels observed in Sprague-Dawley rats, 5 weeks of age, treated with comparable doses of thioacetamide for 24h [12].

Additional verification of thioacetamide hepatotoxicity in the present study was performed by comparison of gene expression results to a thioacetamide study performed by Minami *et al*. [12]. A large number of the genes confirmed to be sensitive markers of thioacetamide hepatotoxicity in livers of Sprague-Dawley rats as shown by Minami *et al*. [8, 12] were also significantly expressed in the present study. The direction of modulation of the genes presented in Table 2 was similar to results presented by Minami *et al*. [8], despite that a different rat strain was used. Therefore, not only clinical chemistry parameters, but also potential hepatotoxic marker genes can be extrapolated between Wistar and Sprague-Dawley rat strains, as has been suggested by others as well [5].

In the present study, transcriptomic responses to the treatment with thioacetamide were interpreted in the context of the underlying biochemical pathways and biological processes to increase the biological information that can be retrieved from a toxicogenomics experiment [13, 30]. MAPPFinder is a pathway analysis tool that integrates different initiatives which developed databases containing biochemical pathways and biological processes, defined as GO terms and MAPPs [14]. MAPPFinder can be applied to analyze over-representation of these GO terms and MAPPs within a predefined subset of genes.

The most predominant processes affected by thioacetamide are observed at 24h after thioacetamide administration of the high dose. Results show over-representation of response to wounding, which is also over-represented in the moderate dose at 24h, and the strongly interrelated pathways inflammatory response and blood coagulation. Over-representation of these GO terms is suggested to be a consequence of necrosis [18, 31, 32]. GO term analysis is thus concordant with histopathological findings at the 24h exposure period, as centrilobular

hepatocellular necrosis was observed in all animals in both the moderate and the high dose at this time point, and inflammatory cell infiltrate was evident in all animals at 24h treatment with the highest dose. The thioacetamide-induced effects on wounding, inflammation, blood coagulation, and consequently necrosis will be described in the following section with special focus on individual genes involved in these processes which are significantly expressed in the present study and have been well-documented in literature.

At the priming phase of liver regeneration which follows the onset of necrosis in response to thioacetamide-induced liver injury [18, 31, 32], differential expression of a variety of genes involved in the cytokine network, e.g. increases in liver mRNA and serum levels of tumor necrosis factor (TNF) and interleukin-6 (IL-6), has been reported [33, 34]. Furthermore, the transcription factors nuclear factor-kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) have shown to be activated [33, 35, 36]. Differential expression of these genes in rat livers upon treatment with thioacetamide is confirmed in the present study. Our findings show up-regulation of TNF in both the moderate and high dose at 24h. Additionally, IL-6, STAT3, and NF-κB were up-regulated in the high dose group at 24h. Cell cycle progression in liver regeneration is featured by an increase in e.g. cyclin D1 [33, 37], shown to be up-regulated at 24h by both a moderate and high dose of thioacetamide in the present study. An important linkage between cytokines and growth factors may be the activation of matrix metalloproteinases (MMPs) by cytokines [33]. MMPs degrade components of the extracellular matrix, allowing hepatocyte proliferation [38]. This process might be activated in the present study as MMP14 is up-regulated by the high dose of thioacetamide at 24h. Up-regulation of expression of numerous genes encoding for both small and large subunits of ribosomal proteins observed in our study stimulates protein synthesis and allows faster cell growth [39].

Exclusively 24h after thioacetamide administration, clinical chemistry parameters correlated with gene expression results. For glucose, AST, ALT, LDH, and phospholipids, correlated genes could be translated to over-represented GO terms and pathways. These results underscore the importance of studying the interrelationship between genes by GO term and pathway analysis as the relatively low number of genes correlated with ALT (146) resulted in the highest number of over-represented GO terms (9) and pathways (2). Vice versa, the highest number of correlated genes was observed for glucose (301), resulting in only three over-represented GO terms and one over-represented MAPP.

Carboxylic acid metabolism, affected by thioacetamide in the moderate dose at 24h, is related to amino acid metabolism which was correlated to both the clinical chemistry parameters glucose and LDH. The major biological processes lipid metabolism and biosynthesis with their child terms fatty acid metabolism and cholesterol metabolism and biosynthesis, which is affected by the moderate dose of thioacetamide at 24h, are correlated to ALT, LDH, and phospholipids. Cholesterol is synthesized predominantly in the liver. Decreased cholesterol production must be avoided because cholesterol supply is required for many cellular functions. Nonetheless, excess cholesterol can form solid crystals that kill cells. A constant, balanced, cholesterol level

within the cell is therefore highly important to maintain normal cell functions [40]. Impairment of these pathways upon thioacetamide administration and their high correlation with traditional measures of toxicity implicate their role in thioacetamide hepatotoxicity.

Those pathways and processes which were strongly correlated to clinical chemistry parameters 24h after thioacetamide administration can be defined under oxidoreductase activity. However, these pathways and processes were not over-represented in the subset of genes significantly modulated by thioacetamide. Oxidoreductase activity with its child terms monooxygenase acitivity and unspecific monooxygenase activity were correlated to ALT 24h after thioacetamide administration. Metabolism via CYP2E1 appears to be the primary mechanism underlying thioacetamide-induced liver injury [9]. In the present study, the monooxygenase enzyme CYP2E1 is down-regulated by both the mid dose and high dose at the 24h time point. Down-regulation of gene expression of this enzyme could be interpreted as a classical negative feedback loop, turning out in the present case to prevent additional liver injury.

In conclusion, the present study showed that biological information on thioacetamide hepatotoxicity can be increased and specified by translation of transcriptomic data to underlying biochemical pathways and biological processes. Further specification of the hepatotoxic responses is obtained by phenotypic anchoring of the gene expression results by histopathological findings and clinical chemistry parameters, which could only be performed at 24h after thioacetamide administration. The present study confirmed the onset of necrosis, response to wounding, inflammatory response and blood coagulation as consequences of thioacetamide-induced liver injury. Furthermore, cholesterol biosynthesis and pathways related to lipid metabolism were predominant toxic responses to thioacetamide exposure. Clearly, analysis of the modulated gene sets in respect to biochemical pathways and biological processes verified for their toxic potential by phenotypic anchoring has an added value for interpreting the gene expression data in a relevant toxicological context.

ACKNOWLEDGEMENTS

We would like to thank M. van den Wijngaard for sample isolation of the *in vivo* study, Dr. W. Heijne for his help with the experimental design, A. de Kat Angelino-Bart, M. Havekes, and Dr. F. Schuren at the microarray facility, and M. Dansen for microarray data quality analysis.

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Chapter VI

Rat to human interspecies comparison of thioacetamide hepatotoxicity *in vitro* using toxicogenomics

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ABSTRACT

The toxicogenomics approach is promising for interspecies comparisons of the responsiveness to toxic compounds, as it allows simultaneous comparison of the expression of numerous genes. Translation of gene modulation to the genetic pathways or biological processes in which these genes participate represents the next level for interspecies comparison. These pathway and process analyses enable direct comparisons between species as they use common vocabularies, e.g. gene expression. Furthermore, different sets of modulated genes between species may be part of similar pathways and processes. In the present study, it is investigated whether interspecies comparison by toxicogenomics tools at the level of pathways and processes may be the preferable approach above comparisons of sets of modulated genes. Therefore, primary human and rat hepatocytes cultured in sandwich configuration were treated with the well-known hepatotoxicant thioacetamide. Gene expression profiles generated by DNA microarrays were compared at the gene level and at the pathway and process level. For the latter, two approaches were used, MAPPFinder and T-profiler. Whereas no cytotoxicity was observed in human and rat hepatocytes, thioacetamide treatment did result in modulation of gene expression. However, only eight significantly modulated genes were found for both human and rat cells. Comparison at the pathway and process level showed highly comparable results between species: thioacetamide mainly induced cholesterol biosynthesis and cholesterol-related pathways and processes in both human and rat hepatocytes. In conclusion, present results underscore the value of biochemical pathway and biological process analysis in rat-human interspecies extrapolation *in vitro*.

INTRODUCTION

To characterize interspecies differences and/or similarities in the responsiveness to toxic compounds at the whole genome level, the toxicogenomics approach is considered promising. This approach enables comparison of multiple endpoints, as thousands of genes induced by hepatotoxicants can be measured simultaneously [1, 2]. It has already been proposed that the biological information that can be retrieved from toxicogenomics studies can be increased by translation of the individual gene expression changes to their underlying biochemical pathways and biological processes [3]. In a recent study, it was suggested that a higher level of agreement would be obtained between intra-site, inter-site and inter-platform toxicogenomics data when using biochemical pathway and biological process analyses as alternative methods of gene list concordance [4].

For interspecies comparisons made at the level of modulated genes, only lists of orthologue genes, genes with the same function in both species, can be used. This is problematic for the linkage of human and rat genes, as compared to humans, the rat genome is poorly annotated. Only less than 50% of the annotations of the human and rat genome can be matched (resourcerer database [5]). Therefore, to investigate agreement between species, it may be better to compare at the biochemical pathway level using a common vocabulary: tools in which subsets of genes are clustered within biochemical pathways and biological processes [3]. Furthermore, toxic compounds might target different genes between species which nevertheless are components of similar biochemical pathways or biological processes [6, 7].

As rat primary hepatocyte-based *in vitro* cultures are frequently used to model humans, rat-to-human comparison of hepatic responses as analyzed by toxicogenomics may aid in improving interspecies comparisons. Primary human and rat hepatocyte cultures differ particularly in their morphological and functional alterations over time in culture [8-10]. This specifically concerns the stability of enzyme activity and gene expression levels of cytochrome P450 isoenzymes (CYP450s). In sandwich-cultured human hepatocytes – hepatocytes cultured between two layers of collagen mimicking the extracellular matrix – only a moderate decline in enzyme activities is observed until day 9 in culture, whereas most individual CYP450s rapidly decline in sandwich-cultured rat hepatocytes using a conventional culture medium [11].

The objective of the present study was to investigate whether analysis of biochemical pathways and biological processes as deduced from gene expression profiles in human and rat hepatocytes following treatment with a hepatotoxic compound can improve interspecies comparison using the toxicogenomics response. To this purpose, sandwich-cultured human hepatocytes as well as sandwich-cultured rat hepatocytes maintained in either a conventional "standard" culture medium or a metabolically more competent "modified" culture medium [12, 13] were treated with two concentrations of the hepatotoxicant thioacetamide.

Significantly modulated genes as well as biochemical pathways and biological processes induced by thioacetamide were identified and compared between species. Over-representation

of pathways and processes was determined in the subset of significantly expressed genes using MAPPFinder [14] and in the complete dataset of genes without pre-selection of a subset using T-profiler [15].

METHODS

Chemicals

Ca^{2+}- and Mg^{2+}-free Hank's Buffered Salt Solution (HBSS), Dulbecco's Minimal Essential Medium (DMEM), Foetal Calf Serum (FCS), penicillin-streptomycin, Phosphate Buffered Saline (PBS), and TRIzol™ were obtained from Invitrogen, Breda, The Netherlands. Bovine Serum Albumin (BSA), ascorbic acid, collagenase Type IV, insulin, dexamethasone (DEX), Percoll, phenobarbital (PB), β-naphthoflavone (β-NF), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich, Zwijndrecht, The Netherlands. BD™ ITS+ Premix and rat tail collagen I were obtained from BD Biosciences, Alphen aan den Rijn, The Netherlands. The RNeasy minelute kit and RNeasy mini spin columns were obtained from Qiagen, Westburg B.V. (Leusden, The Netherlands). Cyanine 5-cytosine triphosphate (CTP) and cyanine 3-CTP were purchased from Perkin Elmer (Boston, MA). Agilent's low RNA input fluorescent linear amplification kit and the hybridisation solution were obtained from Agilent Technologies (Palo Alto, CA). Thioacetamide, CAS-no 62-55-5; purity minimum 99% according to the manufacturer, was obtained from Sigma-Aldrich. All other chemicals were of analytical grade.

Culture of human hepatocytes

Human hepatocytes were obtained from resection specimens of patients undergoing partial hepatectomy for colorectal metastases in an otherwise normal liver at the University Hospital Maastricht, the Netherlands. Surgery was performed as described before [16] and in none of the cases hepatic inflow occlusion was applied during liver transaction. All patients provided written informed consent and the study was approved by the Medical Ethics Committee of the University Hospital Maastricht. For patient information, refer to Table 1. Isolation of human hepatocytes from resection specimens was performed according to the method described by LeCluyse *et al*. [17]. This method has been adopted by an interlaboratory consortium sponsored by the European Centre for the Validation of Alternative Methods (ECVAM) for the isolation and cultivation of primary human hepatocytes for testing the potential of new drugs to induce liver enzyme expression [17, 18]. Essentially, directly after removal of the liver tissue, an encapsulated wedge, weighing approximately 50-100 g, was cut off. Vessels on the cut surface were immediately flushed with ice cold wash buffer consisting of Ca^{2+}- and Mg^{2+}-free HBSS supplemented with 0.5 mM EDTA, 0.5% (w/v) BSA, and 50 µg/ml ascorbic acid. Liver tissue was transported to the laboratory in ice cold buffer. Transportation time did not exceed 15 min. After a two-step collagenase perfusion, first with wash buffer, then with digestion medium consisting DMEM

Table 1. Primary human hepatocyte donor information.

Donor	Gender	Age (years)	Cell viability (%)*
1	M	76	85
2	M	74	91
3	M	73	90
4	F	42	86
5	F	75	94

*The cell viability was assessed by the Trypan Blue exclusion assay after Percoll purification; M, male; F, female.

supplemented with 0.05% (w/v) collagenase Type IV and 0.5% BSA, hepatocytes were dispersed from the digested liver and collected in ice cold attachment medium (5% FCS and penicillin-streptomycin (100 U/ml and 100 µg/ml, respectively), 0.1 U/ml insulin, and 1 µM DEX in DMEM). After passing the cell suspension through a 100 µm nylon mesh, the cells were washed by low speed centrifugation three times at 75 x g for 5 min. Pellets were resuspended in ice cold suspension medium after each centrifugation step. Hepatocyte preparations with viability greater than 75%, as assessed by Trypan Blue exclusion, were included for further studies (see Table 1). Cell suspension with viability below 85% were purified using a Percoll gradient, as previously described [17]. Human hepatocyte sandwich cultures were essentially prepared according to the method of Beken *et al*.[19]. Cells were cultured on collagen gel precoated 12-well plates at a density of 6.5 x 10^5 cells per well. After attachment for 4h in attachment medium, dead cells were removed by washing and the upper collagen layer was applied. Thereafter, cells were kept in DMEM containing 0.1 µM DEX, 6.25 µg/ml insulin, 6.25 µg/ml transferin, and 6.25 ng/ml selenium (BD™ ITS+ Premix) [8]. Cultures were incubated at 37 °C in a humidified incubator gassed with 5% CO_2 in air. Medium was changed on a daily basis during a period of 72h.

Culture of rat hepatocytes

Male Wistar rats (Crl: (WI) WU BR), 9-12 weeks of age, 180-250 g, were obtained from Charles River GmbH, Sulzfeld, Germany. During the acclimatization period and until sacrifice, animals were housed individually in macrolon cages with wire tops and sawdust bedding at 22 °C and 50-60% humidity. The light cycle was 12h light/12h dark. Feed and tap water were available ad libitum. Hepatocytes were isolated according to a two-step collagenase perfusion technique as described by Seglen [20, 21] with minor modifications [21]. Hepatocyte preparations with viability greater than 85% as determined by Trypan Blue exclusion were used and cultured on collagen gel precoated 6-well plates at a density of 1.3 x 10^6 cells per well. Sandwich cultures of rat hepatocytes were prepared similar to sandwich cultures of human hepatocytes as described above with culture conditions as described previously [13]. To increase the metabolic competence of conventional rat sandwich cultures, media was supplemented with 1 mM PB, 10 µM DEX, and 5 µM β-NF [12]. PB was added as a concentrated stock solution in PBS. DEX and β-NF were added as concentrated stock solutions in dimethylsulphoxide (DMSO). The final concentration of DMSO was equalized in all culture media and did not exceed 0.2% (v/v). Cultures were incubated at 37

°C in a humidified incubator gassed with 5% CO_2. Medium was changed on a daily basis during a period of 72h.

Hepatocyte treatment and cytotoxicity analysis

After 72h of culture, five independent human hepatocyte cultures and three independent rat hepatocyte cultures were exposed to two concentrations of thioacetamide (1.33 and 4 mM) for 24h. Thioacetamide was dissolved in culture medium. Control cultures were maintained in medium only. These *in vitro* concentrations were based on *in vivo* plasma concentrations from a previous study in which rats were exposed to thioacetamide for 24h [22]. Using pharmacokinetic data from literature, a distribution coefficient of thioacetamide of 0.62 L/kg was estimated [23]. Assuming that bioavailability of thioacetamide is 100% after i.p. injection, a maximum *in vivo* plasma concentration of 1.43 mM can be calculated for the *in vivo* moderate dose of 66.7 mg/kg b.wt., and 4.29 mM for the *in vivo* high dose of 200 mg/kg b.wt.. Cytotoxicity was determined by applying the MTT reduction method 24 on hepatocytes from human subjects 2, 4, and 5 and on all rat hepatocyte cultures, either conventionally cultured (standard culture) or cultured in medium to increase the metabolic competence (modified culture).

Total RNA extraction

Following removal of the culture medium, TRIzol™ was added onto the upper collagen layer and cells were collected. RNA was purified using the RNeasy minelute kit including an additional DNA digestion step. RNA concentrations were determined spectrophotometrically by UV-absorbance at 260 nm. RNA quality was determined using the Bioanalyzer (Agilent Technologies, Palo Alto, CA). All samples contained intact total RNA with a 28S/18S rRNA ratio>1.5, and were used for microarray analysis.

Labeling

RNA samples from negative control hepatocyte cultures and cultures exposed to thioacetamide were labeled with cyanine 5-CTP. Cyanine 5-CTP labeled samples from one individual (rat or human) were hybridized against the cyanine 3-CTP labeled RNA sample from negative control hepatocyte cultures of the same individual. As a result, control samples labeled with cyanine 5-CTP hybridized against cyanine 3-CTP samples can be considered as a self-self hybridization. Labeling was performed using Agilent's low RNA input fluorescent linear amplification kit following manufacturer's instruction. Briefly, double-stranded cDNA was synthesized using molony murine leukemia virus-reverse transcriptase (MMLV-RT) with T7 promoter primer, starting with 1 µg of total RNA. Cyanine-labelled cRNA targets were transcribed using T7 RNA Polymerase. The amplified cRNA was purified using RNeasy mini spin columns and synthesized cRNA products were quantified spectrophotometrically.

Hybridization

For microarray hybridization, cyanine 5-labeled samples and cyanine 3-labeled samples were combined. cRNAs were fragmented at 60 ºC for 30 min with fragmentation solution followed by hybridization on Agilent 22K format 60-mer oligo microarrays (G4130A for rat and G4110B for human from Agilent Technologies, Palo Alto, CA) for 17h at 60 ºC with Agilent hybridization solution. Arrays were washed according to manufacturer's instruction. Microarrays were scanned using a Packard Scanarray Express confocal laser scanner (PerkinElmer, Boston, MA). Resulting TIFF images were loaded into Imagene 5.0 (Biodiscovery Inc. El Segundo, CA) to further process and collect the gene expression data.

Data analysis

Data were transferred to GeneSight 4.1 (Biodiscovery Inc. El Segundo, CA). Flagged spots, consisting of poor quality spots and negative and positive control spots, were excluded. For each spot, median local background intensity was subtracted from the median spot intensity and spots from low expression genes (with a net intensity of <40 in both channels), were excluded from further analysis. These background-corrected median intensities were log transformed by base 2. Data were normalized using the Lowess algorithm [25]. Resulting gene expression ratios were loaded into Excel (Microsoft Corporation, Redmond, WA). For further analysis, only genes were included for which at least 60% of the arrays gave an expression ratio. For rat and human cells, a subset of significantly modulated genes was selected by performing a Student's *T* test ($P<0.05$) on genes with mean fold changes above or below a threshold of 1.5 compared to control cultures [26]. Similar human and rat genes, orthologues, were identified based on gene symbol or based on orthologues as provided by The Computational Biology and Functional Genomics Laboratory at the Dana-Farber Cancer Institute and Harvard School of Public Health [5]. Over-represented biochemical pathways and biological processes were identified in the subset of significantly modulated genes versus the complete dataset of genes using MAPPFinder (version 2.0, Gladstone Institutes 2000-2006) [14, 27]. Biochemical pathways and biological processes were determined significantly over-represented when *Z* scores were greater than 2 and *P* values were below 0.2 [3, 14]. Furthermore, T-profiler [15] was used to identify over-representation of biochemical pathways and biological processes in the complete dataset of genes without pre-selecting only significantly modulated 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 within the complete data set of genes [15]. To determine significance, a corrected *P* value, *E* value, is generated. Pathways and processes were significant when *E* values were below 0.05. In the present study, the biochemical pathways and biological processes are based on membership of a specific Gene Ontology (GO) category [6] or part of a pathway defined by GenMAPP or by the Kyoto Encyclopedia of Genes and Genomes (KEGG) [28].

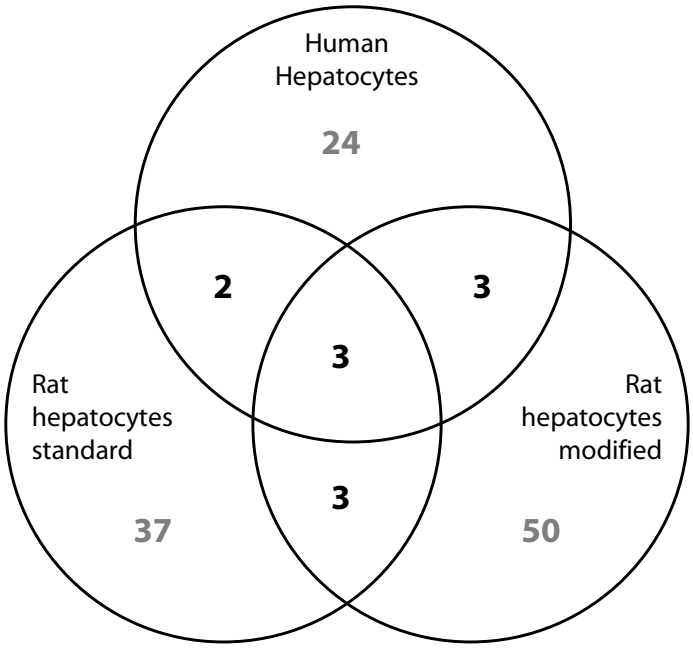


Figure 1. Venn diagram. Each circle presents the number genes significantly changed after treatment regardless of the concentration. In the overlap, the number of significantly changed genes between human hepatocytes and rat hepatocytes cultured in standard or modified medium is presented.

RESULTS

No cytotoxicity of thioacetamide could be determined by the MTT reduction assay in human hepatocytes and in rat hepatocytes cultured in standard or modified culture medium upon treatment with 1.33 and 4 mM thioacetamide.

Gene expression modulation

The number of significantly modulated genes was determined in human and rat hepatocytes treated with 1.33 mM and 4 mM thioacetamide for 24h. Gene expression modulation was more pronounced in rat hepatocytes compared to human hepatocytes. The low and high concentrations resulted in modulation of 47 and 56 genes in human hepatocytes, 113 and 134 genes in rat hepatocytes cultured in standard medium, and 143 and 144 genes modulated in rat hepatocytes cultured in modified medium. In order to compare significant gene expression changes between rat and human hepatocytes, only the orthologues were used. Rat orthologues were found for only 30% of all human genes. The number of modulated orthologues is presented in a Venn diagram (Fig. 1). The eight genes modulated in both rat and human hepatocytes are specified in Table 2. Six genes were modulated in a similar direction; however, the expression

Table 2. Genes significantly modulated by thioacetamide in both human and rat hepatocytes.

			Human hepatocytes		Rat hepatocytes			
					Standard		Modified	
Gene name	**Symbol Human/ Rat**	**Genbank ID Human/Rat**	**1.33 mM**	**4 mM**	**1.33 mM**	**4 mM**	**1.33 mM**	**4 mM**
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	HMGCS1/ Hmgcs1	NM_002130/ NM_017268	1.06*	0.85*	1.80*	-0.34	2.03	2.23*
Isopentenyl-diphosphate delta isomerase	IDI1/ Idi1	NM_004508/ NM_053539	0.68*	0.68*	1.22*	0.56	1.40*	1.27*
Squalene epoxidase	SQLE/ Sqle	NM_003129/ NM_017136	0.880*	0.81*	0.77*	0.63*	1.06*	1.06*
Farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltranstransferase, geranyltranstransferase)	FDPS/ Fdps	NM_002004/ NM_031840/	0.716*	0.44*	0.68*	0.36	0.80	0.78
Early growth response 1	EGR1/ Egr1	NM_001964/ NM_012551	0.63*	0.25	0.75	0.67	2.23*	2.27
Hydroxysteroid (17-beta) dehydrogenase 7	HSD17B7/ Hsd17b7	NM_016371/ NM_017235	0.81*	0.61*	0.24*	0.37*	0.52	0.60*
Angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	ACE2/ Ace2	NM_021804/ NM_001012006	0.73*	0.05	-0.60	-0.27	0.06	0.24
Selenocysteine lyase	SCLY/ Scly	AF175767/ NM_001007755	0.08	0.61*	-0.22	-0.25	-0.87*	-0.67

*Significant values (fold change>1.5 and *P* value<0.05 as calculated by a Student's *T* test); Values are base 2 log transformed expression ratios corrected for control ratios.

of two genes, *ACE2*/*Ace2* and *SCLY*/*Scly*, were up-regulated in human hepatocytes and down-regulated in rat hepatocytes.

Pathway analysis

MAPPFinder was used to identify biochemical pathways and biological processes over-represented in the subset of significantly modulated genes versus the complete dataset of genes. Results are presented in Table 3. This analysis resulted in over-representation of GO terms only in rat hepatocytes treated with 1.33 mM thioacetamide in standard culture medium and rat hepatocytes treated with 4 mM thioacetamide in modified culture medium. Over-represented GO terms were: steroid biosynthesis, sterol metabolism, sterol biosynthesis, cholesterol metabolism, cholesterol biosynthesis, isoprenoid metabolism, and isoprenoid biosynthesis. The GenMAPP pathway cholesterol biosynthesis was over-represented in both human and rat

Table 3. Pathways and processes over-represented in the subset of significantly modulated genes retrieved using MAPPFinder.

Pathway or process	Human hepatocytes		Rat hepatocytes			
			Standard		Modified	
	1.33 mM	4 mM	1.33 mM	4 mM	1.33 mM	4 mM
GenMAPP						
Steroid biosynthesis	5.14	7.15*	-0.31	-0.24	-0.29	2.92
	(0.29)	(0.15)	(1.00)	(1.00)	(1.00)	(0.80)
Cholesterol biosynthesis	19.56*	13.35*	17.93*	6.30*	13.61*	17.38*
	(0.00)	(0.00)	(0.00)	(0.05)	(0.00)	(0.00)
Circadian exercise	1.27	2.10	1.31	-0.44	3.45	4.84*
	(1.00)	(1.00)	(1.00)	(1.00)	(0.66)	(0.00)
GO						
Steroid biosynthesis	7.85	8.31	14.75*	1.37	7.61	10.91*
	(1.00)	(1.00)	(0.02)	(1.00)	(1.00)	(0.09)
Sterol metabolism	6.57	2.05	16.24*	-0.10	8.42	12.65*
	(1.00)	(1.00)	(0.02)	(1.00)	(1.00)	(0.05)
Sterol biosynthesis	10.78	3.62	20.47*	2.22	7.95	12.65*
	(0.99)	(1.00)	(0.00)	(1.00)	(1.00)	(0.05)
Cholesterol metabolism	4.44	-0.40	14.48*	1.71	6.50	10.78*
	(1.00)	(1.00)	(0.02)	(1.00)	(1.00)	(0.09)
Cholesterol biosynthesis	7.92	-0.234	18.75*	2.42	5.58	8.24
	(1.00)	(1.00)	(0.00)	(1.00)	(1.00)	(1.00)
Isoprenoid metabolism	8.38	-0.11	12.36*	-0.27	7.58	7.10
	(1.00)	(1.00)	(0.05)	(1.00)	(1.00)	(1.00)
Isoprenoid biosynthesis	10.31	-0.09	13.24*	-0.26	8.13	7.62
	(1.00)	(1.00)	(0.05)	(1.00)	(1.00)	(1.00)

*Over-represented pathways and processes (*Z* score>2; *P* values<0.2).

systems at all incubations. Treatment of human hepatocytes with 4 mM thioacetamide resulted in additional up-regulation of the steroid biosynthesis pathway retrieved from GenMAPP. Furthermore, the GenMAPP pathway circadian exercise was over-represented upon administration of 4 mM thioacetamide to rat hepatocytes cultured in modified medium.

T-profiler analysis was used to identify the transcriptional regulation of biochemical pathways and biological processes in the complete dataset of genes. T-profiler results are presented in Table 4. The GenMAPP pathway sterol biosynthesis, the KEGG pathway biosynthesis of steroid and the GO term cholesterol biosynthesis were over-represented in all systems and incubations. In all systems and incubations, the GO term cholesterol biosynthesis was up-regulated. Treatment of human hepatocytes with 1.33 mM thioacetamide resulted in additional down-regulation of metabolism of xenobiotics by cytochrome P450 (KEGG). Furthermore, treatment of rat hepatocytes with 1.33 mM thioacetamide in modified culture medium resulted in up-regulation of the GO term isoprenoid biosynthesis. Results in rat hepatocytes cultured in modified medium show over-representation of a manually curated geneset by T-profiler, mitochondrion, involving genes expressed in mitochondria.

Table 4. Pathways and processes identified by T-profiler.

Pathway or process	Human hepatocytes		Rat hepatocytes			
			Standard		Modified	
	1.33 mM	4 mM	1.33 mM	4 mM	1.33 mM	4 mM
GenMAPP						
Sterol biosynthesis	5.04* (0.00)	4.69* (0.00)	5.76* (0.00)	4.13* (0.02)	6.24* (0.00)	5.74* (0.00)
KEGG						
Biosynthesis of steroids	5.36* (0.00)	4.61* (0.00)	6.02* (0.00)	4.54* (0.00)	5.73* (0.00)	5.61* (0.00)
Metabolism of xenobiotics by cytochrome P450	-3.82* (0.02)	-1.60 (1.00)	-1.00 (1.00)	0.62 (1.00)	-0.53 (1.00)	0.77 (1.00)
GO						
Cholesterol biosynthesis	4.89* (0.00)	4.85* (0.00)	5.77* (0.00)	4.41* (0.01)	6.33* (0.00)	5.77* (0.00)
Isoprenoid biosynthesis	3.90 (0.11)	2.56 (1.00)	3.63 (0.22)	2.45 (1.00)	4.56* (0.00)	3.50 (0.33)
Manually curated						
Mitochondrion	-1.35 (1.00)	-2.01 (1.00)	-2.19 (1.00)	-2.34 (1.00)	-4.24* (0.01)	-3.96* (0.03)

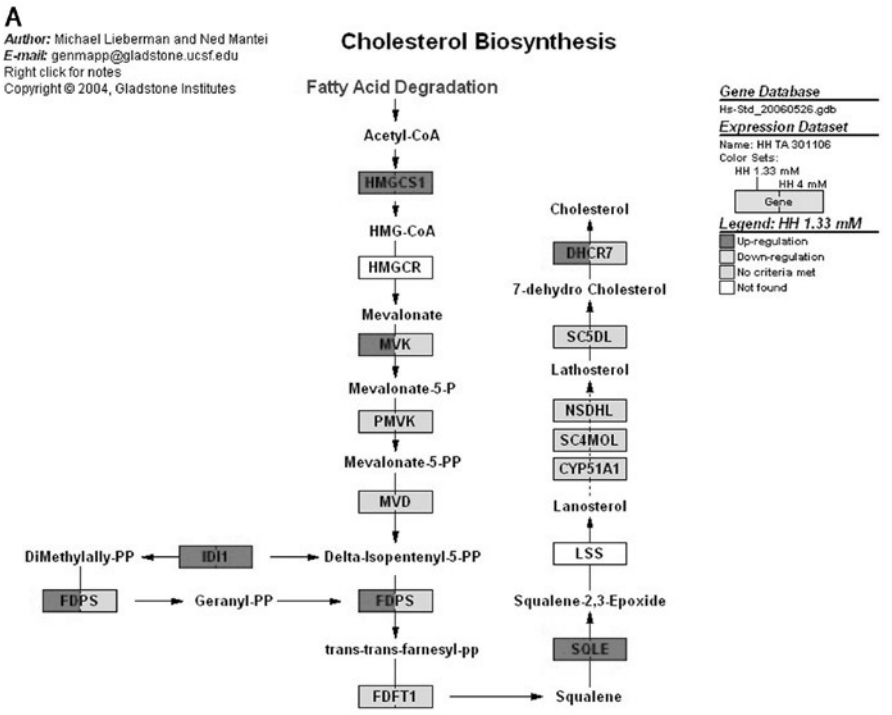
*Significant pathways and processes (*Z* score>2; *E* values<0.05); manually curated gene sets are provided by T-profiler.

The cholesterol biosynthesis pathway, as retrieved from GenMAPP, is illustrated in Fig. 2 for human hepatocytes (Fig. 2A) and for rat hepatocytes either cultured in standard or modified medium (Fig. 2B). The significance of cholesterol biosynthesis in both human and rat hepatocytes is emphasized although not all genes modulated by thioacetamide in humans and rats are similar. For MAPPFinder analysis, over-representation of the cholesterol biosynthesis pathway was determined by the significantly modulated genes (dark grey) versus all measured genes (light grey); for T-profiler analysis, over-representation of the cholesterol biosynthesis pathway was determined by specific values of all colored genes (either dark grey or light grey) within the geneset.

DISCUSSION

In the present study, we provide arguments that interspecies comparisons of toxicogenomics responses should not be restricted to comparison of sets of individually modulated genes, but integrated at the biochemical pathway and biological process levels. Toxicogenomics studies in general benefit from pathway analysis tools which enable interpretation of gene expression data within a biological context by translation of the information on significantly modulated genes into the pathways and processes in which these genes take part [3]. It has already been suggested that for intrasite, intersite and interplatform comparisons of toxicogenomics data, a

A


Author: Michael Lieberman and Ned Mantei
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Cholesterol Biosynthesis
Fatty Acid Degradation
Acetyl-CoA
HMGCS1
HMG-CoA
HMGCR
Mevalonate
MVK
Mevalonate-5-P
PMVK
Mevalonate-5-PP
MVD
DiMethylally-PP
IDI1
Delta-Isopentenyl-5-PP
FDPS
Geranyl-PP
FDPS
trans-trans-farnesyl-pp
FDFT1
Squalene
SQLE
Squalene-2,3-Epoxide
LSS
Lanosterol
CYP51A1
SC4MOL
NSDHL
Lathosterol
SC5DL
7-dehydro Cholesterol
DHCR7
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Expression Dataset
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HH 4 mM
Gene
Legend: HH 1.33 mM
Up-regulation
Down-regulation
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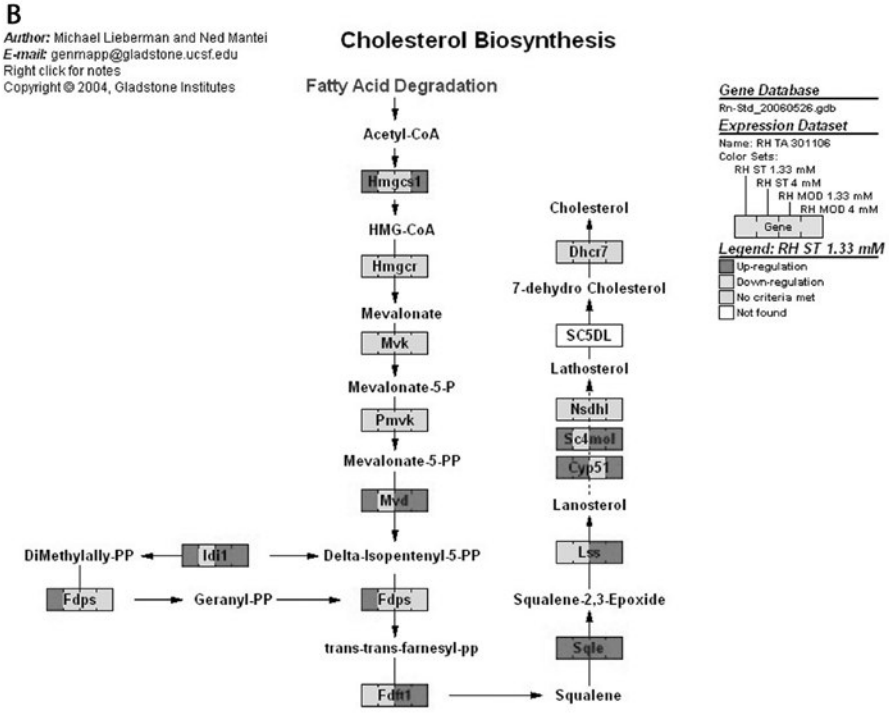
Not found

B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Cholesterol Biosynthesis
Fatty Acid Degradation
Acetyl-CoA
Hmgcs1
HMG-CoA
Hmgcr
Mevalonate
Mvk
Mevalonate-5-P
Pmvk
Mevalonate-5-PP
Mvd
DiMethylally-PP
Idi1
Delta-Isopentenyl-5-PP
Fdps
Geranyl-PP
Fdps
trans-trans-farnesyl-pp
Fdft1
Squalene
Sqle
Squalene-2,3-Epoxide
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Cyp51
Sc4mol
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Lathosterol
Sc5dl
7-dehydro Cholesterol
Dhcr7
Cholesterol
Gene Database
Rn-Std_20060526.gdb
Expression Dataset
Name: RH TA 301106
Color Sets:
RH ST 1.33 mM
RH ST 4 mM
RH MOD 1.33 mM
RH MOD 4 mM
Gene
Legend: RH ST 1.33 mM
Up-regulation
Down-regulation
No criteria met
Not found

Figure 2. The GenMAPP cholesterol biosynthesis pathway containing human genes (A) and their rat homologues (B). The MAPP is retrieved from www.genmapp.org. Dark grey colored genes were significantly modulated and included in pathway analysis using MAPPFinder. Light grey colored genes were present on the microarray, but not significantly modulated according to our criteria. (A) Squares representing genes are divided in half; the first half representing human hepatocytes exposed to 1.33 mM thioacetamide; the second half representing human hepatocytes exposed to 4 mM thioacetamide. (B) Squares representing genes are divided in four sections; the first section representing hepatocytes cultured in standard medium exposed to 1.33 mM thioacetamide; the second section representing hepatocytes cultured in standard medium exposed to 4 mM thioacetamide; the third section representing hepatocytes cultured in modified medium exposed to 1.33 mM thioacetamide; the fourth section representing hepatocytes cultured in modified medium exposed to 4 mM thioacetamide.

higher level of agreement would be detected using these biochemical pathway and biological process analyses as alternative methods of gene list concordance [4]. Specifically for interspecies comparison, unlike comparison of gene lists which for a large part differ in nomenclature between species, biochemical pathway and process analysis would be ideal because of the fact that the vocabulary of the majority of GO terms, but also biochemical pathways defined by GenMAPP and KEGG, are common to several organisms [6, 7]. Furthermore, different sets of genes affected by toxicants between species might in fact be components of common pathways or processes.

With the aim to investigate interspecies differences and/or similarities in hepatotoxic responses, in the present study, primary human and rat hepatocytes were exposed to the well-described hepatotoxicant thioacetamide, whose toxic metabolites, formed through conversion of thioacetamide by CYP2E1, cause hepatocellular necrosis [23, 29, 30]. Besides gene lists of significantly modulated genes, biochemical pathways and biological processes as defined by the GO consortium, KEGG, and GenMAPP were obtained by using two pathway analysis tools, MAPPFinder and T-profiler. MAPPFinder was applied to determine over-represented biochemical pathways and biological processes within a pre-defined subset of significantly modulated genes [14].

T-profiler was performed to detect transcriptional regulation of biochemical pathways and biological processes within the complete dataset of genes [15].

Despite no effects of thioacetamide on cell viability in human and rat hepatocytes as measured by the MTT reduction assay, the transcript levels of many genes were significantly modulated. This finding indicates the sensitivity of the toxicogenomics approach to detect transcriptional responses of compounds, as was shown before [12, 31]. The effect of thioacetamide treatment on modulation of gene expression was highest in rat hepatocytes cultured in modified medium. This agrees with the increased competence of these cells to metabolize compounds as compared to rat hepatocytes cultured in standard culture medium [13]. Compared to rat hepatocytes, fewer genes are modulated by thioacetamide in human hepatocytes. This may be due to the larger genetic variability between human hepatocyte cultures retrieved from individuals varying in age and sex (Table 1), whereas the rat hepatocyte cultures were prepared from genetically identical Wistar rats. For example, CYP450s are highly genetically polymorphic

in humans [9, 32]. Increasing the number of human hepatocyte cultures, and thereby the power of the toxicogenomics study, will increase the number of modulated genes. Investigation of inter-individual gene expression variation between human hepatocyte cultures, however, was beyond the scope of the present study. A higher concentration of thioacetamide did only result in a small increase of the number of significantly modulated genes. This lack in dose responsiveness did not necessarily interfere with the goals of the present study: interspecies comparison at the genetic pathway and biological process level.

In the present study, comparison between species of genes significantly modulated upon thioacetamide treatment showed only eight genes which were significantly modulated in both human and rat hepatocytes. Five out of these eight genes, *HMGCS1*/*Hmgcs1*, *IDI1*/*Idi1*, *SQLE*/*Sqle*, *FDPS*/*Fdps*, and *HSD17B7*/*Hsd17b7*, have a role in steroid and sterol metabolism-related genetic pathways and biological processes. Specifically, these genes are all part of the cholesterol biosynthesis pathway [33] and have all been up-regulated by thioacetamide in both human and rat hepatocytes. Furthermore, an additional gene, *EGR1*/*Egr1*, is up-regulated in human hepatocytes and rat hepatocytes cultured in modified culture medium upon treatment with 1.33 mM of thioacetamide. The expression of *EGR1*/*Egr1* is induced by cytokines, growth factors, ischemic injury, and tissue damage [34]. EGR1/Egr1 has been reported to promote liver injury [35]. Therefore, as thioacetamide induces the expression of EGR1/Egr1 and as EGR1/Egr1 induces liver injury, thioacetamide may induce a direct hepatotoxic effect by inducing *EGR1*/*Egr1* in human and rat hepatocytes.

Two genes are regulated in opposite directions in human and rat hepatocytes upon treatment with thioacetamide, *ACE2*/*Ace2* and *SCLY*/*Scly*. ACE2/Ace2 belongs to the angiotensin converting enzyme family and catalyzes the cleavage of angiotensin [2] to angiotensin 1-7 [36]. *ACE2*/*Ace2* is up-regulated in human hepatocytes and down-regulated in rat hepatocytes cultured in standard medium, but slightly (and not significantly) up-regulated in rat hepatocytes cultured in modified medium. In normal liver tissue, expression of *ACE2*/*Ace2* is low [37]. However, in human and rat, major up-regulation of hepatic *ACE2*/*Ace2* expression levels has been shown after chronic liver injury [36]. In the present study, this effect is only observed in human hepatocytes. SCLY/Scly is a lyase involved in selenoamino acid metabolism. After treatment with thioacetamide, *SCLY*/*Scly* is up-regulated in human hepatocytes and down-regulated in rat hepatocytes cultured in modified medium (also slightly but not significantly down-regulated in rat hepatocytes cultured in standard medium). Activity of the SCLY/Scly enzyme is known to be high in liver compared to other tissues [38]. No findings in literature were found on the specific up- or down-regulation of *SCLY*/*Scly* in relation to the response to thioacetamide or to liver toxicity.

In spite of the low number of similar significantly modulated genes upon thioacetamide treatment between species, genetic pathways and biological processes, all containing large numbers of genes, showed highly similar results between human and rat hepatocytes. MAPPFinder was used to identify over-represented genetic pathways and biological processes within

the set of modulated genes [3]. All over-represented pathways and processes in both human and rat hepatocytes showed strong relation to cholesterol metabolism and biosynthesis. The GO term steroid biosynthesis, over-represented in rat hepatocytes, is as a parent term related to its child terms sterol metabolism, sterol biosynthesis, cholesterol metabolism, and cholesterol biosynthesis. Steroid biosynthesis is over-represented as a GenMAPP pathway in human hepatocytes exposed to 4 mM of thioacetamide. Isoprenoid metabolism and biosynthesis is related to cholesterol biosynthesis as reactions within the cholesterol biosynthesis pathway transform mevalonate to isopentenyl diphosphate, which is the five carbon precursor of all isoprenoids [35]. Cholesterol biosynthesis represents one of the major functions of the liver. A constant and well-balanced, cholesterol level within the cell is highly important for maintaining normal cell functions. Thioacetamide results in up-regulation of the cholesterol biosynthesis pathway/process. Excess cholesterol can form solid crystals that kill cells [39]. One pathway, the circadian exercise pathway which is over-represented in rat hepatocytes, seems to lack biological relevance to liver responses as it normally represents genes that are time and exercise-dependently regulated in skeletal muscle. Still, this pathway may be over-represented due to its relation to cholesterol biosynthesis, as it contains the *IDI1*/*Idi1* gene, which is part of the cholesterol biosynthesis pathway.

Furthermore, analysis with T-profiler (which is performed with all genes, thus without preselection of a subset of genes) also resulted in identification of GO terms, KEGG pathways, and GenMAPP pathways related to cholesterol biosynthesis. An additional pathway significant in human hepatocytes treated with 1.33 mM thioacetamide is metabolism of xenobiotics by cytochrome P450. Down-regulation of this pathway, as was observed in the present study, may be interpreted as a classical negative feedback loop [12], which may in the present case prevent liver injury. Furthermore, in rat hepatocytes cultured in modified medium, a set containing genes expressed in mitochondria was down-regulated. Physiological effects on mitochondria were not yet seen in the results of the MTT cytotoxicity assay (which is a measure of mitochondrial damage); therefore, these effects on gene expression might precede the disruption of mitochondrial function, a well-known toxic effect.

In conclusion, the results in the present study indicate that interspecies comparison of transcriptomic data by a pathway analysis approach is highly preferable to comparison at the level of selected lists of modulated genes. Significantly modulated gene list comparisons resulted in agreement of only eight genes upon thioacetamide treatment of human and rat hepatocytes. Pathway analysis using either MAPPFinder (over-representation within the subset of significantly modulated genes) or T-profiler (over-representation in the complete dataset of genes) showed highly similar results between species: thioacetamide mainly induced genes involved in cholesterol biosynthesis and cholesterol-related pathways and processes in both human and rat hepatocytes. Present results underscore the value of biochemical pathway and biological process analysis in rat-human interspecies extrapolation *in vitro*.

ACKNOWLEDGEMENTS

We acknowledge the useful discussions with W. Maas on the human hepatocyte isolation technique. We are grateful to Dr. N. Treijtel and K. Mathijs for their assistance in the hepatocyte isolation procedure.

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Chapter VII

Interspecies and *in vitro-in vivo* comparison of acetaminophen-induced gene expression profiles

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ABSTRACT

The frequent use of rodent hepatic *in vitro* systems in pharmacological and toxicological investigations challenges both the extrapolation of *in vitro* results to the situation *in vivo* and interspecies extrapolation from rodents to humans. The toxicogenomics approach may aid in evaluating the relevance of these model systems for human risk assessment by direct comparison of toxicant-induced gene expression profiles and infers mechanisms between several systems. In the present study, in a parallelogram approach acetaminophen (APAP) was used as a model compound to compare gene expression profiles between *in vitro* experiments using rat and human hepatocytes and between rat *in vitro* and *in vivo*. In previous chapters, comparison at the level of modulated biochemical pathways and biological processes rather than at that of genes appears preferable as it increases the overlap between various systems. Pathway analysis by T-profiler revealed similar biochemical pathways and biological processes repressed in rat and human hepatocytes *in vitro* and rat liver *in vitro-in vivo*. Pathways included repression of energy-consuming biochemical pathways, mitochondrial dysfunction, and down-regulation of oxidoreductase activity. Completion of the parallelogram approach suggests that these common pathways and processes are relevant for APAP-induced human liver injury. In conclusion, the present study is the first to reveal that robust APAP-induced toxicogenomics results which were previously found in rats and mice are relevant to the situation in humans as well.

INTRODUCTION

Toxicological and pharmacological assessments of hepatotoxic risks of compounds and drugs in humans rely on extrapolation from animal experiments. Increasingly, studies in well-established hepatic *in vitro* assays, such as primary hepatocytes, liver slices, and hepatic cell lines, precede *in vivo* animal experiments in an attempt to identify potential hepatotoxicity in early stages of toxicity testing and to decrease attrition rates of drugs during lead optimization [1]. The general use of rodent hepatic *in vitro* systems challenges not only the extrapolation of *in vitro* results to the situation *in vivo* [2], but also interspecies extrapolation from rodents to humans [3-5].

One of the most extensively studied hepatotoxicants is acetaminophen or paracetamol (N-acetyl-para-aminophenol; APAP). Despite the numerous experiments performed to identify the toxic mode-of-action of APAP, the exact mechanism by which this compound induces liver injury is not completely clear [6, 7]. In the recent past, toxicogenomics and -proteomics approaches have been applied to livers from APAP treated mice and rats in order to aid in unraveling its mechanisms of liver cell injury [8-12]. Besides contributing to the identification of the mode-of-action of compounds, the toxicogenomics approach would be particularly suitable for comparison of compound-induced effects, e.g. induced by different drugs or compounds, between *in vitro* systems (e.g. cancer cell lines and primary cells), between species, or between the *in vitro* and *in vivo* situation, as the approach allows comparison of thousands of similar endpoints, being it gene expression levels, between situations. Toxicogenomics studies have previously been performed for identification of (in)consistencies in profiles between APAP and other compounds inducing similar toxicological endpoints of hepatotoxicity [13], between subtoxic and overtly toxic doses [10], and between primary human hepatocytes and HepG2 cells [14]. Recently, a multicenter study of APAP hepatotoxicity was performed to uncover robust genomic signatures of APAP-induced toxicity in mice [12]. None of these toxicogenomics studies seized the challenge to apply the toxicogenomics approach for comparing acute toxic effects of APAP both *in vitro* and *in vivo* and in rodent and human, using a parallelogram approach in order to predict mechanisms of human liver injury.

The aims of the present study therefore were (1) to compare APAP-induced gene expression profiles from rat liver cells *in vitro* with *in vivo*, (2) to perform interspecies extrapolation based upon rat-human *in vitro* models, and (3) to predict mechanistic changes as they could occur *in vivo* in man using a parallelogram approach. To this purpose, APAP-induced gene expression profiles in sandwich-cultured primary human and rat hepatocytes were compared to a rat *in vivo* study publicly available on the internet (GEO database, Heinloth *et al*., 2006, see methods section).

METHODS

Chemicals

Ca^{2+}- and Mg^{2+}-free Hanks's Buffered Salt Solution (HBSS), Dubecco's Minimal Essential Medium (DMEM), Foetal Calf Serum (FCS), penicillin-steptomycin, Phosphate Buffered Saline (PBS), and TRIzol™ were obtained from Invitrogen, Breda, The Netherlands. Bovine Serum Albumin (BSA), ascorbic acid, collagenase Type IV, insulin, dexamethasone (DEX), Percoll, phenobarbital (PB), β-naphthoflavone (β-NF), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased form Sigma-Aldrich, Zwijndrecht, The Netherlands. BD™ ITS+ Premix and rat tail collagen I were obtained from BD Biosciences, Alphen aan den Rijn, The Netherlands. The RNeasy MinElute kit and RNeasy mini spin columns were obtained from Qiagen, Westburg B.V. (Leusden, The Netherlands). Cyanine 5-cytosine triphosphate (CTP) and cyanine 3-CTP were purchased from Perkin Elmer (Boston, MA, USA). Agilent's low RNA input fluorescent linear amplification kit and the hybridization solution were obtained form Agilent Technologies (Palo Alto, CA, USA). Acetaminophen, CAS-no 103-90-2; purity minimum 99% according to the manufacturer, was obtained from Sigma-Aldrich.All other chemicals were of analytical grade.

Culture of human hepatocytes

Human hepatocytes were obtained from resection specimens of patients undergoing partial hepatectomy for colorectal metastases in an otherwise normal liver at the University Hospital Maastricht, the Netherlands. Surgery was performed as described before [15] and in none of the cases hepatic inflow occlusion was applied during liver transaction. All patients provided written informed consent and the study was approved by the Medical Ethics Committee of the University Hospital Maastricht. Isolation of human hepatocytes from resection specimens was performed according to the method described by LeCluyse *et al*. [16]. This method has been adopted by an inter-laboratory consortium sponsored by the European Centre for the Validation of Alternative Methods (ECVAM) for the isolation and cultivation of primary human hepatocytes for testing the potential of new drugs to induce liver enzyme expression [16, 17]. Essentially, directly after removal of the liver tissue, an encapsulated wedge, weighing approximately 50-100 g, was cut off. Blood vessels on the cut surface were immediately flushed with ice cold wash buffer consisting of Ca^{2+}- and Mg^{2+}-free HBSS supplemented with 0.5 mM EDTA, 0.5% (w/v) BSA, and 50 µg/ml ascorbic acid. Liver tissue was transported to the laboratory in ice-cold buffer. Transportation time did not exceed 15 min. After a two-step collagenase perfusion, first with wash buffer, then with digestion medium consisting DMEM supplemented with 0.05% (w/v) Collagenase Type IV and 0.5% BSA, hepatocytes were dispersed from the digested liver and collected in ice-cold attachment medium (5% FCS and penicillin-streptomycin (100 U/ml and 100 µg/ml, respectively), 0.1 U/ml insulin, and 1 µM DEX in DMEM). After passing of the cell suspension through a 100 µm nylon mesh, the suspension was washed by low speed centrifugation three times at 75 x g for 5 min. Pellets were resuspended in ice cold suspension medium after

each centrifugation step. Viability was assessed by Trypan blue exclusion. Hepatocyte preparations with viability greater than 75% were included for further studies. Cell suspension with viability below 85% was purified using a Percoll gradient, as previously described [16]. Cells were cultured on collagen gel precoated 12-well plates at a density of 6.5 x 10^5 cells per well. Human hepatocyte sandwich cultures were essentially prepared according to the method of Beken *et al*.[18]. After attachment for 4h in attachment medium, dead cells were removed by washing and the upper collagen layer was applied. Thereafter, cells were kept in DMEM containing 0.1 µM DEX, 6.25 µg/ml insulin, 6.25 µg/ml transferin, and 6.25 ng/ml selenium (BD™ ITS+ Premix) [19]. Cultures were incubated at 37 °C in a humidified incubator gassed with 5% CO_2 in air. Medium was changed on a daily basis during a period of 72h.

Culture of rat hepatocytes

Male Wistar rats (Crl: (WI) WU BR), 9-12 weeks of age, 180-250 g, were obtained from Charles River GmbH, Sulzfeld, Germany. During the acclimatization period and until sacrifice, animals were housed individually in macrolon cages with wire tops and sawdust bedding at 22 °C and 50-60% humidity. The light cycle was 12h light/12h dark. Feed and tap water were available ad libitum. Hepatocytes were isolated according to a two-step collagenase perfusion technique as described by Seglen [20] with minor modifications [21]. Hepatocyte preparations with viability greater than 85% as determined by Trypan Blue exclusion were used and cultured on collagen gel precoated 6-well plates at a density of 1.3 x 10^6 cells per well. Sandwich cultures of rat hepatocytes were prepared similar to sandwich cultures of human hepatocytes as described above with culture conditions as described previously [22]. An inducer mix used to increase the metabolic competence of conventional rat sandwich cultures consisted of 1 mM PB, 10 µM DEX, and 5 µM β-NF [23]. PB was added as a concentrated stock solution in PBS. DEX and β-NF were added as concentrated stock solutions in dimethylsulphoxide (DMSO). The final concentration of DMSO was equalized in all culture media and did not exceed 0.2% (v/v). Cultures were incubated at 37 °C in a humidified incubator gassed with 5% CO_2 in air. Medium was changed on a daily basis during a period of 72h.

Hepatocyte treatment and cytotoxicity analysis

After 72h of culture, five independent human hepatocyte cultures and three independent rat hepatocyte cultures were exposed to two concentrations of APAP (5 and 10 mM) for 24h. APAP was dissolved in culture medium. Control cultures were maintained in medium only. Cytotoxicity was determined by the MTT reduction method [24] on hepatocytes from subjects 4, 5, and 6 (Table 1) and on all rat hepatocyte cultures either conventionally cultured (standard culture) or cultured in medium to increase the metabolic competence (modified culture).

Table 1. Primary human hepatocyte donor information.

Donor	Gender	Age (years)	Cell viability (%)
2	M	74	91
3	M	73	90
4	F	42	86
5	F	75	94
6	M	68	96

The cell viability was assessed by the trypan blue exclusion assay; M, male; F, female.

Total RNA extraction

Following removal of culture medium, TRIzol™ was added onto the upper collagen layer and cells were collected. RNA was purified using the RNeasy MinElute kit including an additional DNA digestion step. RNA concentrations were determined spectrophotometrically by UV-absorbance at 260 nm. RNA quality was determined using the Bioanalyzer (Agilent Technologies, Palo Alto, CA). All samples contained intact total RNA with a 28S/18S rRNA ratio>1.5 and a RIN number>8. RNA extractions of hepatocyte cultures exposed to APAP of five human individuals and three rats were used for microarray analysis.

Labeling

RNA samples from control hepatocyte cultures and cultures exposed to APAP were labeled with cyanine 5-CTP. Cyanine 5-CTP labeled samples from one individual (rat or human) were hybridized against cyanine 3-CTP labeled RNA samples from control hepatocyte cultures of the same individual. As a result, control samples labeled with cyanine 5-CTP hybridized against cyanine 3-CTP samples can be considered as a self-self hybridization. Labeling was performed using Agilent's low RNA input fluorescent linear amplification kit following manufacturer's instruction. Briefly, double-stranded cDNA was synthesized using molony murine leukemia virus-reverse transcriptase (MMLV-RT) with T7 promoter primer, starting with 1 µg of total RNA. Cyanine-labelled cRNA targets were transcribed using T7 RNA Polymerase. The amplified cRNA was purified using RNeasy mini spin columns. Synthesized cRNA products were quantified spectrophotometrically.

Hybridization

For microarray hybridization, cyanine 5-labeled samples and cyanine 3-labeled samples were combined. cRNAs were fragmented at 60 ^{o}C for 30 min with fragmentation solution followed by hybridization on Agilent 22K format 60-mer oligo microarrays (G4130A for rat and G4110B for human from Agilent Technologies, Palo Alto, CA) for 17h at 60 ^{o}C with Agilent hybridization solution. Arrays were washed according to manufacturer's instruction. Microarrays were scanned using a Packard Scanarray Express confocal laser scanner (PerkinElmer, Boston, MA). Resulting TIFF images were loaded into Imagene 5.0 (Biodiscovery Inc. El Segundo, CA) to further process and collect the gene expression data.

Data analysis

Data were transferred to GeneSight 4.1 (Biodiscovery Inc. El Segundo, CA). Flagged spots, consisting of poor quality spots and negative and positive control spots, were excluded. For each spot, median local background intensity was subtracted from the median spot intensity and spots from low expression genes (with a net intensity of <40 in both channels), were excluded from further analysis. These background-corrected median intensities were log transformed by base 2. Data were normalized using the Lowess algorithm [25]. Resulting gene expression ratios were loaded into Excel (Microsoft Corporation, Redmond, WA). For further analysis, 60% of values per gene had to be available. For rat and human cells, a subset of significantly modulated genes was selected by performing a Student's *T* test ($P<0.05$) on genes with mean fold changes above or below a threshold of 1.5 compared to control cultures [6]. Similar human and rat genes, orthologues, were identified based on gene symbol or based on orthologues as provided by Resourcerer database of The Computational Biology and Functional Genomics Laboratory at the Dana-Farber Cancer Institute and Harvard School of Public Health [26]. *In vivo* data from male F344/N rats, 8-12 weeks old, were retrieved from the Gene Expression Omnibus (GEO) database on the NCBI website [27], series GSE5860, submitted by Heinloth, A.N. and Paules, R.S., September 19, 2006. The data we obtained from their study represent log 10 expression ratios of mRNA from rats single-dosed by oral gavage with 1.5 g/kg b.wt. APAP in methyl cellulose hybridized against mRNA from rats oral gavaged with vehicle only. Three rats were used per APAP-treated and vehicle group. Rats were sacrificed 24h following dosing. No information on necropsy procedures could be obtained from the study description on the NCBI website. Hybridization was performed in a dye-swap design on Agilent-011868 Rat Oligo Microarrays G4130A. Refer to the NCBI website for more information on the APAP rat *in vivo* study regarding animals, study design, and data. We averaged the Log 10 expression data of dye-swap array pairs and calculated fold-changes. Genes with fold changes greater than 1.5 and below 0.667 were considered modulated. Modulated genes were considered significant when average Log 10 expression values minus two times their standard deviation were greater than zero.

MAPPFinder (version 2.0, Gladstone Institutes 2000-2006, 28) was used to identify biochemical pathways and biological processes over-represented in the subset of significantly modulated genes versus the complete dataset of genes in all systems [29]. For a pathway or process to be over-represented, the minimum of significantly modulated genes within the pathway of process had to be 5. Furthermore, over-represented pathways and processes had *Z* scores greater than 2 and *P* values below 0.2 [29]. Pathways and processes provided by MAPPFinder included Gene Ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, GenMAPP pathways, and pathways created by BiGCat [30].

T-profiler [31] was used to identify transcriptional regulation of biochemical pathways and biological processes in the complete data set of genes without pre-selecting only significantly modulated 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 within the complete data

set of genes [31]. To determine significance, a Bonferroni corrected *P* value, *E* value, is generated. Pathways and processes were significant when *E* values were below 0.05. Pathways and processes provided by T-profiler included GO terms, KEGG pathways, and gene sets including GenMAPP pathways, gene sets from BioCarta, T-profiler curated (manually curated) gene sets and gene sets retrieved from literature.

RESULTS

Cytotoxicity was determined using the MTT reduction assay. After exposure to 5 mM APAP, no cytotoxicity was observed in human and rat hepatocytes, the latter maintained in either standard or modified culture medium. Exposure to 10 mM APAP resulted in on average 10% loss in viability in human hepatocytes and in on average 20% loss in viability in rat hepatocytes cultured in modified culture medium. At 10 mM, no cytotoxicity was observed in rat hepatocytes cultured in standard culture medium, without enzyme inducers.

Treatment of human hepatocytes with 5 and 10 mM APAP resulted in significant modulation of 1624 genes (991 and 1401 modulated genes after treatment with 5 or 10 mM, respectively, all 767 overlapping genes were regulated in the same directions). APAP treatment resulted in significant modulation of 368 genes in rat hepatocytes cultured in standard medium (208 and 207 genes modulated after treatment with 5 or 10 mM, respectively, 47 genes in the overlap were, with 2 exceptions, regulated in similar directions), whereas 1289 genes were significantly changed in rat hepatocytes cultured in modified medium (327 and 1132 modulated genes after treatment with 5 or 10 mM, respectively, all 172 genes in the overlap were regulated in the same directions). *In vivo* treatment of rats with 1.5 g/kg b.wt. APAP resulted in significant modulation of 1349 genes, as derived from the Heinloth dataset.

Overlap of significantly modulated genes between rats *in vivo* and rat hepatocytes *in vitro* is shown in Fig. 1. Overlap is highest between rat hepatocytes cultured in standard and modified medium. When comparing with *in vivo*, more commonly modulated genes were found in rat hepatocytes cultured in modified medium as compared to those cultured in standard medium. However, only 18 out of 43 genes common between the modified system and *in vivo* are regulated in the same directions. Furthermore, only 2 out of the 5 significantly modulated genes in the standard system and *in vivo* are regulated in a similar direction. The two genes significantly modulated by all three systems are down-regulated *in vitro* and up-regulated *in vivo*. This indicates that rat hepatocytes cultured in modified medium better represent effects induced by APAP in rats *in vivo* in contrast to the cells cultured in standard medium, at least whenever the expression of single genes is concerned, irrespective of the direction and magnitude of compound-induced expression changes.

In order to compare the gene expression results in rat and human hepatocytes, only rat-human orthologues were used, based on gene symbol and the Resourcerer database. The

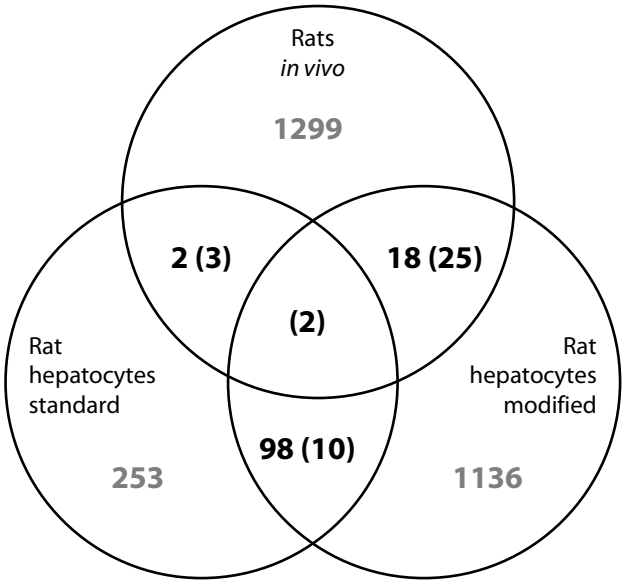

Figure 1. Venn diagram representing the number of significantly modulated genes in rat hepatocytes cultured in standard medium, rat hepatocytes cultured in modified medium, and rats *in vivo* treated with APAP. The overlap represents genes significantly changed between systems, regardless of the concentration or dose used. Numbers between brackets represent the number of genes regulated in opposite directions between systems.

Venn diagram in Fig. 2 presents the number of significantly modulated genes between human hepatocytes and rat hepatocytes cultured in modified medium, and between rat hepatocytes cultured in modified medium and rats *in vivo*. These results show that overlap is highest between human hepatocytes and rat hepatocytes in modified culture medium, and that most genes in the overlap are regulated in the same direction in both species.

To compare the functional effects of APAP on biochemical pathways and biological processes in human hepatocytes, rat hepatocytes and rats *in vivo*, initially, MAPPFinder was used. Table 2 shows that the subset of significantly modulated genes represents only a small number of functional gene sets for each system. Genes significantly modulated in rat hepatocytes cultured in standard medium showed no interrelation in such way that over-representation of any single pathway could be identified, at least no pathways contained with GenMAPP/KEGG/GO. No common gene sets were observed between rat hepatic systems. For only two systems, human hepatocytes and rat hepatocytes cultured in modified medium, significantly modulated genes complied with GO terms. APAP treatment of human hepatocytes with 5 and 10 mM resulted in over-representation of 20 and 13 GO terms, respectively. Rat hepatocytes cultured in modified medium treated with 10 mM APAP showed over-representation of two GO terms. No common GO terms were induced by APAP in human and rat hepatocytes.

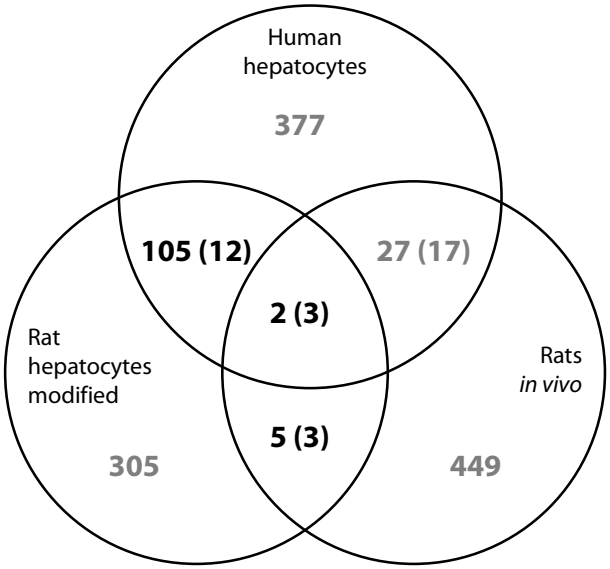

Figure 2. Venn diagram representing the number of significantly modulated genes in human hepatocytes, rat hepatocytes cultured in modified medium, and rats in vivo treated with APAP. Only rat and human genes for which orthologues could be compared are presented. The overlap represents genes significantly changed between systems, regardless of the concentration or dose used. Numbers between brackets represent the number of genes regulated in opposite directions between systems.

Application of T-profiler, a pathway analysis method for which no pre-selection of subsets of genes is needed [31], to the data sets in the present study resulted in identification of large numbers of biochemical/cellular pathways and biological processes in which genes were differentially expressed, enabling thorough functional comparison between systems. Tables 3 to 5 show pathways and processes enriched in at least two systems: human hepatocytes, rat

Table 2. Gene sets overrepresented upon treatment with at least one concentration or dose of APAP as determined by MAPPFinder.

	Source	**Human hepatocytes**	**Rat hepatocytes in standard medium**	**Rat hepatocytes in modified medium**	**Rats *in vivo***
Cholesterol biosynthesis	GenMAPP	+	-	-	-
Mitochondrial fatty acid betaoxidation	GenMAPP	-	-	+	-
Matrix metalloproteinases	GenMAPP	-	-	-	+
Circadian Exercise	GenMAPP	+	-	-	-
Urea cycle and metabolism of amino groups	KEGG	+	-	-	-
Tryptophane metabolism	KEGG	-	-	+	-
Fatty acid omega oxidation	BiGCat	+	-	-	-

Over-represented pathways (+) have *Z* scores above 2 and *P* values below 0.2.

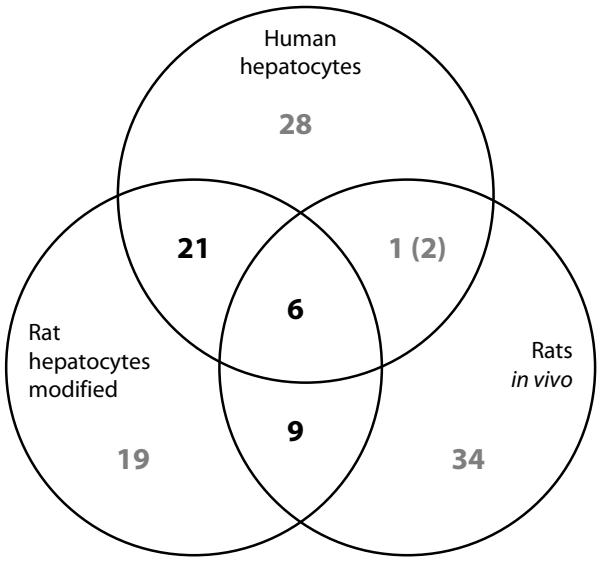

Figure 3. Venn diagram representing the number of predominant biochemical pathways and biological processes in the complete data set of genes as determined by T-profiler in human hepatocytes, rat hepatocytes cultured in modified medium, and rats *in vivo* treated with APAP. The overlap represents biochemical pathways and biological processes that are shared between systems, regardless of the concentration or dose used. Numbers between brackets represent the number of pathways and processes regulated in opposite directions between systems.

hepatocytes (either standard or modified), or rats *in vivo*. Eighteen predominant biochemical pathways and biological processes were identified in rat hepatocytes cultured in standard medium, of which eight were found in at least one other system. Six of these eight pathways were regulated in opposite directions compared to regulation in the other systems. Due to the lack of correspondence of APAP-induced biochemical pathways and biological processes in rat hepatocytes cultured in standard medium with the other systems as identified by T-profiler, this rat hepatocyte-based system was excluded from the Venn diagram in Fig. 3. This Venn diagram in Fig. 3 shows the number of biochemical pathways and biological processes as modified by APAP and identified by T-profiler per system and the overlap of pathways and processes between human hepatocytes and rat hepatocytes cultured in modified medium, and between rat hepatocytes in cultured in modified medium and rats *in vivo*. Comparisons were made regardless concentrations/doses of APAP. Overlap between human hepatocytes and rat hepatocytes cultured in modified medium, is highest. Unlike the results on significantly modulated genes, overlap between systems at the level of T-profiler identified pathways and processes is higher between rat hepatocytes and rats *in vivo* as compared to human hepatocytes and rats *in vivo*. The six pathways and processes in the centre of the Venn diagram included metabolism in general, lipid and fatty acid metabolism, electron transport, processes occurring in peroxisomes and metabolism of amino acids (valine, leucine, and isoleucine degradation).

Human hepatocytes and rat hepatocytes cultured in modified medium share APAP-modulated biochemical pathways and processes related to major liver functions, fatty acid metabolism, bile acid biosynthesis, glycolysis/gluconeogenesis, amino acid metabolism (L-phenylalanine catabolism, lysine degradation, glycine, serine, threonine, tyrosine, and tryptophan metabolism), and pathways and processes occurring in mitochondria, electron transporter activity, and related to oxidoreductase activity and metabolism of xenobiotics by CYP450. Pathways and processes predominant in both rat hepatocytes cultured in modified medium and rats *in vivo* were also related to CYP450 activity (CYP450, monooxygenase activity, unspecific monooxygenase activity, heme binding, and iron ion binding). Furthermore, rat hepatocytes cultured in modified medium and rats *in vivo* shared processes occurring in the nucleus, cholesterol biosynthesis and tryptophane and butanoate metabolism. Response to unfolded protein was predominantly up-regulated by APAP in both human hepatocytes and rats *in vivo*. Protein biosynthesis, a process over-represented in human hepatocytes, rat hepatocytes in standard medium, and rats *in vivo*, is down-regulated in both hepatocyte systems and up-regulated in rat livers *in vivo*. Another process, ribonucleoprotein complex, is also down-regulated in human hepatocytes and up-regulated in rats *in vivo*.

DISCUSSION

Recent toxicogenomics studies successfully identified differences and similarities between chemical-specific profiles of APAP and other compounds [13], different and similar signatures between subtoxic and overtly toxic doses of APAP [10], (in-)consistencies between APAP-induced profiles in primary human hepatocytes and HepG2 cells [14], and robust genomic signatures of APAP-induced toxicity in mice in a multicenter study [12]. The present study further contributes to the information on acute toxicity of APAP as it compares *in vitro* and *in vivo* toxicogenomics data as well as interspecies toxicogenomics results in an attempt to unravel mechanisms of action across species of liver injury due to APAP intake. To this purpose, sandwich-cultured human and rat hepatocytes were treated with APAP. Resulting gene expression profiles were compared with gene expression profiles in rat livers of rats following exposure to APAP *in vivo* using data published elsewhere (Heinloth *et al*., 2006, see methods section).

The concentrations and doses selected for the present study to enable comparison were based on results from the previous studies comparing APAP profiles [10, 13, 14]. *In vitro*, no toxicity has been observed by treatment of rat hepatocytes in sandwich configuration with 1 mM APAP [32]. Only moderate effects on the MTT reduction were observed upon treatment of rat hepatocytes with 10 mM APAP [13]. Therefore, in the present study, two concentrations were included, namely, 10 mM and a concentration in between 1 and 10 mM, 5 mM. Cytotoxicity was only observed after treatment of human hepatocytes and rat hepatocytes cultured in modified medium with 10 mM APAP. For comparing APAP-induced gene expression modifications between *in vitro* and

in vivo, we selected data generated from the *in vivo* dose at which acetaminophen in rats was maximally hepatotoxic, as indicated with histopathology and clinical chemistry data, namely, 1.5 g/kg b.wt. [10].

The present study showed that more genes were similarly modulated in rat hepatocytes cultured in modified medium and rats *in vivo* as compared to rat hepatocytes cultured in standard medium and rats *in vivo*. These results are concordant with previous findings which already showed that the modified medium increased the metabolic competence of the rat hepatocyte system [23], resulting in a more *vivo*-like response to compounds compared to the response in rat hepatocytes cultured in standard medium [22].

In vitro-in vivo as well as interspecies extrapolation of APAP toxicogenomics data is complicated based upon the outcome of listings of differentially expressed single genes, even though these genes may share similar biology. Pathway analysis instead offers translation of these genes to a common and therefore easy to compare vocabulary [6, 33, 34]. In particular, interspecies comparison between rats and humans at the modulated gene level is problematic due to poor annotation of the rat genome; for only ca. 30% of the rat genes human orthologues are present on the Agilent microarrays used here. Therefore, in the present study, GO terms, pathway, and gene set analysis, all ways to compile individual gene information into biological categories, were used to compare biology between *in vitro* and *in vivo* (rat hepatocytes *in vitro* and rats *in vivo*), and between organisms (human and rat hepatocytes).

In the present study, MAPPFinder [29] was used to characterize GO terms, GenMAPP contained pathways and gene sets over-represented in the subsets of significantly modulated genes per system. Although the number of significantly modulated genes was high and did not differ considerably between human hepatocytes, rat hepatocytes cultured in modified medium, and rats *in vivo*, only in human hepatocytes significantly modified biochemical pathways and biological processes could be identified, using this pathway mapping approach based on preselected genes. While some pathways and processes were identified in rat hepatocytes treated with 10 mM APAP the number of differentially expressed pathways and processes, at least with the threshold set as outlined in the methods section, was too low to enable comparison between systems.

T-profiler analysis includes not only genes that were categorized as differentially expressed based on statistics, but uses all other genes within the complete data set to statistically evaluate the distribution of gene expression of predefined groups of genes (GO terms, KEGG pathways, and gene sets retrieved from GenMAPP, Biocarta, etc.), in comparison to the distribution of expression of all genes on the array. With this tool, a group of genes can be scored as significantly up- or down-regulated even if none of its individual member genes are significantly modulated [31]. In the present study, the advantage of the continuous approach by T-profiler analysis above the discrete approach with which MAPPFinder analysis was performed in the present study is reflected in the large number of pathways and processes identified as predominant in each model system.

According to the parallelogram approach, T-profiler results were thus compared between rat and human hepatocytes *in vitro* and between rat hepatocytes *in vitro* and rats *in vivo*. Equal to the gene expression results, similarity between rat *in vitro* and human *in vitro* systems was higher than similarity between rat hepatocytes *in vitro* and rats *in vivo*. Still, in contrast to results based on single gene comparison, pathway analysis indicates that APAP effects in rat hepatocytes cultured in modified medium are quite similar to effects in rats *in vivo*, at least concerning those pathways differentially expressed.

In the following section, the relevance of the biochemical pathways and biological processes, which in the present study have shown to be similar between systems, will be discussed shortly in terms of known mechanisms of APAP-induced liver injury. Although detailed study of the toxicogenomics results per system might aid in unraveling the mechanistic response of APAP in human hepatocytes, rat hepatocytes, and rats *in vivo*, this is beyond the scope of the present study.

Recently, a multicenter study was performed in order to reveal robust genomic signatures of APAP-induced toxicity [12]. Therefore, several laboratories performed a toxicogenomics study in which mice were treated with acute toxic doses of APAP *in vivo*, all adhering to a standard protocol. Robust biological endpoints included major energy-consuming pathways like lipid metabolism, electron transport, and cofactor metabolism which were repressed by APAP at all time points (6h, 12h, and 24h) [12]. Additionally, loss of mitochondrial function and concomitant generation of oxidative stress was observed in rodent studies upon exposure to toxic doses of acetaminophen [8, 10, 12, 35]. These responses are proposed mechanisms of acetaminophen-induced hepatotoxicity following toxic doses in humans as well [36-38]. In the present study, these mechanisms, repression of energy-consuming biochemical pathways lipid metabolism, fatty acid metabolism, and electron transport, were repressed upon APAP treatment in rat-human hepatocytes *in vitro* and rat *in vitro-in vivo*. Furthermore, mitochondrial function was clearly impaired in rat *in vitro* and human *in vitro* and oxidoreductase activity and related pathways including CYP450 activity were down-regulated in rat-human hepatocytes *in vitro* and rat *in vitro-in vivo*. These observations which, unlike other studies, include effects on human hepatocytes *in vitro* within a parallelogram approach, support the hypothesis that APAP-induced effects on energy-consuming biochemical pathways, mitochondria, and oxidoreductase activity are likely to drive injury in humans and therefore contribute to further unraveling the mechanism of acetaminophen hepatotoxicity. Results in the present study, however, provide no quantitative assessment of human hepatotoxic risks, as doses of acetaminophen used in rats *in vivo* represent a major overdose for humans *in vivo*, far beyond the maximum therapeutic dose of acetaminophen for an average adult within 24h, which is 4 grams.

In conclusion, the present study is the first to reveal that robust APAP-induced toxicogenomics results which were previously found in rats and mice are relevant to the situation in humans as well. In a parallelogram approach, toxicogenomics including T-profiler pathway analysis uncovered impairment of energy-consuming biochemical pathways and biological processes,

Table 3. T-profiler results on GO terms significant in at least two systems.

	Human hepatocytes		Rat hepatocytes standard		Rat hepatocytes modified		Rat *in vivo*
Ontology	**5 mM**	**10 mM**	**5 mM**	**10 mM**	**5 mM**	**10 mM**	**1.5 g/kg b.wt.**
Biological process							
metabolism	-6.22* (0.00)	-4.94* (0.00)	-2.39 (1.00)	-2.88 (0.97)	-5.30* (0.00)	-8.72* (0.00)	-5.42* (0.00)
lipid metabolism	-4.90* (0.00)	-4.31* (0.02)	1.48 (1.00)	0.32 (1.00)	-4.64* (0.00)	-6.64* (0.00)	-4.67* (0.00)
fatty acid metabolism	-5.36* (0.00)	-4.96* (0.00)	-0.23 (1.00)	-0.40 (1.00)	-3.95 (0.06)	-6.23* (0.00)	-4.32* (0.01)
cholesterol biosynthesis	-2.87 (0.99)	-2.33 (1.00)	-3.01 (0.89)	-3.94 (0.07)	-3.04 (0.87)	-4.53* (0.01)	-6.97* (0.00)
Protein biosynthesis	-4.44* (0.01)	-2.67 (1.00)	-3.1 (0.82)	-5.07* (0.00)	-1.63 (1.00)	-0.4 (1.00)	11.14* (0.00)
response to unfolded protein	4.29* (0.02)	3.75 (0.20)	1.16 (1.00)	0.31 (1.00)	1.09 (1.00)	1.5 (1.00)	5.08* (0.00)
L-phenylalanine catabolism	-4.54* (0.01)	-3.37 (0.60)	-3.35 (0.50)	-3.31 (0.55)	-4.02* (0.05)	-4.66* (0.00)	-1.15 (1.00)
electron transport	-3.16 (0.86)	-4.15* (0.04)	-0.23 (1.00)	-1.31 (1.00)	-6.56* (0.00)	-7.36* (0.00)	-6.38* (0.00)
ion transport	-0.07 (1.00)	1.89 (1.00)	2.56 (1.00)	4.96* (0.00)	0.30 (1.00)	1.46 (1.00)	-4.89* (0.00)
Molecular function							
oxidoreductase activity	-5.54* (0.00)	-5.90* (0.00)	-2.40 (1.00)	-2.95 (0.94)	-6.87* (0.00)	-8.92* (0.00)	-3.68 (0.18)
monooxygenase activity	-2.42 (1.00)	-1.98 (1.00)	-2.73 (1.00)	-4.09* (0.04)	-6.50* (0.00)	-5.65* (0.00)	-4.83* (0.00)
unspecific monooxygenase activity	-2.62 (1.00)	-2.56 (1.00)	-3.24 (0.64)	-3.27 (0.60)	-7.04* (0.00)	-5.40* (0.00)	-4.60* (0.00)
heme binding	-2.67 (1.00)	-1.68 (1.00)	-2.75 (0.99)	-3.26 (0.61)	-7.86* (0.00)	-6.81* (0.00)	-7.228* (0.00)
iron ion binding	-2.44 (1.00)	-2.89 (0.99)	-3.90 (0.08)	-4.29* (0.02)	-8.32* (0.00)	-8.74* (0.00)	-5.99* (0.00)
RNA binding	-3.86 (0.13)	-3.98 (0.08)	-4.46* (0.01)	-5.02* (0.00)	-0.75 (1.00)	-0.61 (1.00)	6.50* (0.00)
Receptor activity	-0.19 (1.00)	2.15 (1.00)	3.06 (0.85)	5.09* (0.00)	0.87 (1.00)	1.42 (1.00)	-6.19* (0.00)
Cellular component							
Nucleus	4.07 (0.06)	1.53 (1.00)	1.59 (1.00)	-1.14 (1.00)	3.29 (0.58)	4.15* (0.03)	5.63* (0.00)
mitochondrion	-10.18* (0.00)	-9.73* (0.00)	-1.28 (1.00)	-3.32 (0.54)	-5.09* (0.00)	-7.37* (0.00)	-0.16 (1.00)
Peroxisome	-6.92* (0.00)	-6.19* (0.00)	-2.31 (1.00)	-2.12 (1.00)	-6.78* (0.00)	-8.01* (0.00)	-5.05* (0.00)
ribonucleoprotein complex	-3.73 (0.21)	-4.14* (0.04)	-2.66 (1.00)	-3.18 (0.72)	-0.25 (1.00)	-0.89 (1.00)	4.69* (0.00)

*Significant GO terms with an *E* value below 0.05 (between brackets); positive and negative *T* values indicate predominant up- and down-regulation of genes within the term, respectively.

Table 4. T-profiler results on KEGG pathways significant in at least two systems.

	Human hepatocytes		Rat hepatocytes standard		Rat hepatocytes modified		Rat *in vivo*
KEGG Pathway	**5 mM**	**10 mM**	**5 mM**	**10 mM**	**5 mM**	**10 mM**	**1.5 g/kg b.wt.**
Fatty acid metabolism	-8.19* (0.00)	-5.66* (0.00)	0.23 (1.00)	-0.62 (1.00)	-4.31* (0.00)	-7.03* (0.00)	-3.28 (0.14)
Bile acid biosynthesis	-6.64* (0.00)	-4.68* (0.00)	0.59 (1.00)	-0.56 (1.00)	-3.44 (0.08)	-4.37* (0.00)	-1.52 (1.00)
Glycolysis/ Gluconeogenesis	-4.83* (0.00)	-3.45 (0.09)	1.28 (1.00)	-0.70 (1.00)	-1.94 (1.00)	-4.99* (0.00)	-0.29 (1.00)
Glycine, serine and threonine metabolism	-4.18* (0.01)	-4.18* (0.01)	-2.80 (0.52)	-2.76 (0.57)	-5.82* (0.00)	-5.38* (0.00)	-2.42 (0.89)
Tryptophan metabolism	-2.88 (0.51)	-1.99 (1.00)	-1.44 (1.00)	-1.48 (1.00)	-5.88* (0.00)	-5.63* (0.00)	-3.76* (0.02)
Tyrosine metabolism	-6.62* (0.00)	-4.87* (0.00)	-1.63 (1.00)	-1.45 (1.00)	-4.52* (0.00)	-5.63* (0.00)	-1.38 (1.00)
Butanoate metabolism	-2.56 (0.85)	-2.59 (0.82)	-0.15 (1.00)	0.17 (1.00)	-3.17 (0.20)	-4.24* (0.00)	-4.87* (0.00)
Valine, leucine and isoleucine degradation	-5.22* (0.00)	-4.01* (0.01)	0.96 (1.00)	-0.05 (1.00)	-4.25* (0.00)	-6.16* (0.00)	-4.76* (0.00)
Lysine degradation	-3.81* (0.02)	-4.10* (0.01)	0.80 (1.00)	0.08 (1.00)	-2.13 (0.99)	-3.93* (0.01)	-3.37 (0.10)
Cytochrome P450	-3.13 (0.27)	-3.06 (0.32)	-2.84 (0.48)	-3.54 (0.06)	-8.40* (0.00)	-7.04* (0.00)	-6.10* (0.00)
Metabolism of xenobiotics by cytochrome P450	-7.57* (0.00)	-4.96* (0.00)	-2.30 (0.96)	-2.82 (0.50)	-8.19* (0.00)	-8.12* (0.00)	-1.83 (1.00)

*Significant KEGG pathways have an *E* value below 0.05 (between brackets); positive and negative *T* values indicate predominant up- and down-regulation of genes within the KEGG pathway, respectively.

mitochondrial function, and oxidoreductase activity, which are known to be involved in APAP-induced acute hepatotoxicity, in rat hepatocytes *in vitro*, rats *in vivo* and in human hepatocytes. Completion of the toxicogenomics-based parallelogram supports the hypothesis that the biochemical pathways and biological processes observed in the present study in human hepatocytes and rats *in vitro-in vivo* might have a role in prognosis for human acute APAP-induced liver injury.

ACKNOWLEDGEMENTS

We would wish to thank Dr. R. de Ligt, Dr. C. Krul, and M. Schut for their help in obtaining primary rat hepatocytes. We are thankful to Dr. N. Treijtel and K. Mathijs for their assistance in isolation of human hepatocytes.

Table 5. T-profiler results on gene sets significant in at least two systems.

	Human hepatocytes		Rat hepatocytes standard		Rat hepatocytes modified		Rat *in vivo*
Gene set	**5 mM**	**10 mM**	**5 mM**	**10 mM**	**5 mM**	**10 mM**	**1.5 g/kg b.wt.**
Gene sets from GenMAPP							
Fatty acid metabolism	-8.39* (0.00)	-6.4* (0.00)	1.46 (1.00)	0.97 (1.00)	-3.71 (0.08)	-5.27* (0.00)	-2.14 (1.00)
Glycolysis Gluconeogenesis	-4.64* (0.00)	-3.64 (0.12)	1.61 (1.00)	-0.19 (1.00)	-1.94 (1.00)	-4.42* (0.00)	-0.09 (1.00)
Gamma Hexachlorocyclohexane degradation	-4.31* (0.01)	-3.53 (0.18)	-1.40 (1.00)	-1.21 (1.00)	-4.43* (0.00)	-4.13* (0.02)	-1.73 (1.00)
Glycine serine and threonine metabolism	-4.45* (0.00)	-3.64 (0.12)	-2.11 (1.00)	-2.65 (0.97)	-5.04* (0.00)	-4.38* (0.01)	-0.61 (1.00)
Tyrosine metabolism	-6.93* (0.00)	-4.57* (0.00)	-1.70 (1.00)	-1.36 (1.00)	-5.09* (0.00)	-4.95* (0.00)	-2.03 (1.00)
Tryptophane metabolism	-6.70* (0.00)	-5.18* (0.00)	-1.93 (1.00)	-2.41 (1.00)	-6.59* (0.00)	6.06* (0.00)	-3.36 (0.28)
Gene sets from BioCarta							
Electron transporter activity	-3.74 (0.08)	-3.88* (0.05)	-1.64 (1.00)	-1.96 (1.00)	-4.79* (0.00)	-4.35* (0.01)	-0.01 (1.00)
Gene sets from GO							
GO_0005739 (mitochondrion)	-4.39* (0.01)	-4.29* (0.01)	-0.42 (1.00)	-2.24 (1.00)	-3.45 (0.21)	-5.70* (0.00)	-1.43 (1.00)
Manually Curated gene sets							
Human mitoDB_6_2002	-7.13* (0.00)	-7.11* (0.00)	-0.95 (1.00)	-3.36 (0.28)	-4.20* (0.01)	-6.45* (0.00)	2.35 (1.00)
Mitochondr	-7.38* (0.00)	-7.23* (0.00)	-1.08 (1.00)	-3.33 (0.31)	-4.34* (0.01)	-6.01* (0.00)	1.79 (1.00)
ADULT LIVER vs FETAL LIVER GNF2	-6.71* (0.00)	-4.71* (0.00)	-1.70 (1.00)	-2.57 (0.99)	-5.30* (0.00)	-5.04* (0.00)	-3.52 (0.61)
Gene sets from Peng et al 2002							
RAP DOWN	-2.44 (1.00)	-1.62 (1.00)	-2.1 (1.00)	-3.90* (0.04)	0.41 (1.00)	1.23 (1.00)	8.51* (0.00)
GLUT DOWN	-1.76 (1.00)	-1.62 (1.00)	-3.21 (0.43)	-5.22* (0.00)	-0.81 (1.00)	-1.35 (1.00)	7.90* (0.00)

*Significant gene sets have an *E* value below 0.05 (between brackets); positive and negative *T* values indicate predominant up- and down-regulation of genes within the gene set, respectively.

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Chapter VIII

Summary and general discussion

This thesis aims to more reliably predict toxic responses which would occur in livers of humans exposed to well-known hepatotoxicants using a toxicogenomics-based parallelogram approach, ultimately contributing to the reduction, refinement, and replacement of animal experimentation. To that purpose, in the studies described in this thesis, which focussed on the liver as the target organ for toxicity, sandwich-cultured primary rat and human hepatocytes, and rats *in vivo* were exposed to the well-known liver toxicants coumarin, thioacetamide, and acetaminophen. Using microarray analysis, differentially expressed genes, biochemical pathways, and biological processes induced in the rat/human in vitro models and in rats *in vivo* were identified upon exposure to these hepatotoxicants. Toxicity of the observed responses was verified by traditional toxicology measures and by the well-documented toxic properties of the model compounds. From these findings, possible hazards towards man where inferred.

TOXICOGENOMICS-BASED PARALLELOGRAM APPROACH

The classical and well-accepted route to extrapolate rat toxicity data to humans is by the parallelogram approach. This involves comparison of rat data obtained in in vitro models with rat *in vivo* and application of the forthcoming rules to extrapolate human *in vitro* effects to human *in vivo* effects. In a similar way, comparison of rat and human *in vitro* data allows for the extrapolation of the effects in rats *in vivo* to humans *in vivo*. For the hepatotoxicants used in the toxicogenomics experiments described in this thesis, namely, coumarin, thioacetamide, and acetaminophen, the parallelogram approach (Fig. 1) was used to theoretically predict hepatotoxicant-induced gene expression responses in humans.

Toxicogenomics responses of hepatotoxicants in *in vitro* as well as *in vivo* systems were presented in this thesis as differentially expressed genes (gene lists and Venn diagrams), expression patterns as visualized using principal component analysis, and in the context of biochemical pathways and biological processes. These pathways and processes were identified using two pathway analysis tools: MAPPFinder [1], which was used to identify over-represented pathways based on pre-selected genes, and T-profiler, for which no pre-selection of subsets is needed [2].

Parallelogram comparisons as presented in this thesis benefit from toxicogenomics approaches, in particular whenever the toxicogenomics data are combined with pathway mapping tools. Additional to increasing the biological information, focusing on the affected biochemical pathways and biological processes instead of the gene lists per se improves the comparison of rat *in vitro*-to-*in vivo* and of rat-human interspecies. Between experiments, *in vitro*-*in vivo* or interspecies, slightly different listings of genes which expression changes upon compound treatment might be obtained which in fact are components of similar pathways or processes. Furthermore, gene lists differ for a large part in nomenclature between species, genetic pathways and processes, however, use common vocabularies [3-5].

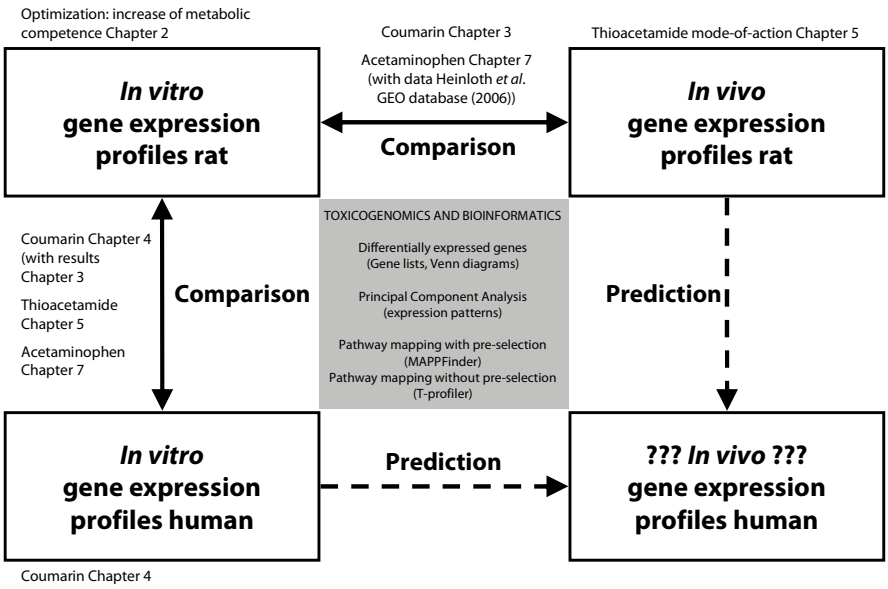

Figure 1. Toxicogenomics-based parallelogram approach.

RAT *IN VITRO*-TO-*IN VIVO* COMPARISONS

In this paragraph, experiments conducted in this thesis will be discussed along the upper axis of the parallelogram approach (Fig. 1): the relevance of gene expression responses generated upon exposure to hepatotoxicants in rat hepatocyte-based sandwich cultures *in vitro* for the situation in rats *in vivo*.

The experiments described in **Chapter II** of this thesis specifically aimed at increasing the relevance of the conventional rat hepatocyte-based sandwich model for rats *in vivo* at the level of metabolic competence, since conventional sandwich cultures of rat hepatocytes have shown rapid decline of metabolic enzymes, in particular cytochrome P450s (CYP450). Sandwich-cultured primary rat hepatocytes, maintained as described by Beken *et al.* [6], were therefore cultured in presence of the enzyme inducers phenobarbital, dexamethasone, and β-napththoflavone, known to induce Cyp2b1/2, Cyp3a and Cyp1a1/2, respectively. Gene expression and enzyme activity analysis showed increased metabolic competence in this modified *in vitro* system. As a result, the basal gene expression profile in the modified system was also less distinct from the gene expression profile observed in rats *in vivo*, in comparison to the gene expression profile observed in hepatocytes cultured under standard conditions. Thus, for toxico- and pharmacological studies, toxicogenomics indicated that the sandwich cultured rat hepatocytes which are maintained in modified medium offered an improvement over existing hepatic models with respect to sustaining metabolic competence *in vitro*.

In **Chapter III** of this thesis, rat *in vitro*-to-*in vivo* comparisons of gene expression profiles and toxicity endpoints were performed using the model compound coumarin. As toxicity of coumarin in rats depends on metabolic activation by the CYP450 enzyme system, it was hypothesized that the rat *in vitro* model in which hepatocytes are cultured in modified medium would be more relevant for the situation in the rat *in vivo* as compared to the conventional rat *in vitro* model, due to its increased metabolic competence. Indeed, the experiments described in **Chapter III** showed that metabolism of coumarin into active metabolites, coumarin-induced toxicity, gene expression profiles, and consequently, biological pathways in the sandwich-cultured rat hepatocytes in modified medium better represent the situation *in vivo* as compared to the conventional *in vitro* model.

Toxicogenomics responses of thioacetamide were described separately for rat liver *in vivo* in **Chapter V** and for rat liver cells *in vitro* in **Chapter VI**. Although different microarray platforms were used which hampered direct comparison of gene expression profiles due to different gene lists and/or nomenclature inherent to platform differences, comparisons at the biochemical pathway and biological process level were made. In **Chapter V** the aim was to identify the mode-of-action of thioacetamide rats *in vivo* using the toxicogenomics and pathway mapping approach. *In vivo* toxicity as determined by traditional clinical chemistry and histopathology as well as thioacetamide affected genetic pathways and biological processes by means of microarray analysis were identified. Experiments proposed from a mechanistic angle the onset of necrosis, response to wounding, inflammatory response and blood coagulation as consequences of thioacetamide-induced liver injury. Furthermore, cholesterol biosynthesis and pathways related to lipid metabolism were predominant toxic responses to thioacetamide exposure. Although thioacetamide is a very potent hepatotoxicant *in vivo*, the toxic potency was less in rat hepatocytes *in vitro*, even in the modified system as has been shown in **Chapter VI**. Unlike the results described for coumarin in **Chapter III**, increase of the metabolic competence of the rat *in vitro* system did not result in more pronounced effects of thioacetamide on cytotoxicity and gene expression patterns. The low toxic potency of thioacetamide in rat hepatocyte-based *in vitro* systems has been reported by others as well [7]. Still, pathway mapping showed thioacetamide-induced effects on cholesterol biosynthesis and cholesterol related pathways and processes, which could be indicative for non-toxicity related physiological changes as a consequence of compound exposure. However, genes involved in cholesterol metabolism were down-regulated in rats *in vivo* and up-regulated in sandwich-cultured rat hepatocytes *in vitro*, showing an important discordance between the results observed in rats *in vitro* and *in vivo*.

Acetaminophen-induced gene expression profiles were compared between rat hepatocytes *in vitro* and rats *in vivo* in the experiments described in **Chapter VII**. Similar to coumarin (**Chapter III**), the rat hepatocytes cultured in modified medium showed a more *in vivo*-like response compared to rat hepatocytes cultured in standard medium, as reflected by cytotoxicity as well as by the number of significant genes and pathways induced by acetaminophen. Pathway

mapping resulted in similar biochemical pathways and biological processes in metabolically more competent rat hepatocytes *in vitro* and rats *in vivo*, showing repression CYP450 activity and impairment of the energy-consuming biochemical pathways lipid metabolism, fatty acid metabolism, and electron transport.

In summary, for coumarin and acetaminophen, sandwich-cultured rat hepatocytes with increased metabolic competence as presented in this thesis show the ability to generate toxicogenomics responses upon hepatotoxicant exposure which demonstrated partial overlap with toxicogenomics responses observed in livers obtained from rats exposed *in vivo*. These results could however not be obtained for thioacetamide. Despite increased metabolic competence, the modified rat hepatocyte *in vitro* system is not optimal for reflecting all potential toxicities.

RAT-HUMAN INTERSPECIES COMPARISONS

Secondly, the outcome of experiments described in this thesis is discussed which have been performed with the objective to compare toxicogenomics results from rat to human *in vitro* liver models, along the left axis of the parallelogram (Fig. 1). The *in vitro* systems used in these experiments include conventional sandwich-cultured primary rat hepatocytes as described by Beken *et al*. 6, modified sandwich-cultured rat hepatocytes with increased metabolic competence (**Chapter II**), and sandwich-cultured primary human hepatocytes as described by Hamilton *et al*. [8].

Since in cultured human hepatocytes the functional alterations affecting the liver-specific properties upon prolonged culturing, particular the CYP450 enzymes, are much less pronounced [9], in this thesis no experiments were performed to optimize the sandwich-cultured human hepatocytes. Comparison of metabolic activity between rat and human hepatocytes in sandwich culture showed that enzyme levels of rat hepatocytes fluctuated significantly after isolation, whereas there was only a slight decrease in the enzyme activities of human hepatocytes, suggesting that human cells might be more suitable for *in vitro* studies [10]. A major complication of using human hepatocytes to investigate hepatotoxicity is the tremendous inter-individual variability caused by phenotypic variability in the human population [11] and the influence of life style factors on gene expression [12, 13]. This inter-individual variability observed in human hepatocytes *in vitro* reflects the genuine phenotypic variability in the human population. However, it might be suggested that variability resulting in differences in metabolism represents the real situation opposed to that for hepatocytes from genetically identical rats.

In **Chapter IV**, gene expression profiling was performed on human hepatocytes treated with coumarin. Results were compared to the coumarin-induced toxicogenomics responses in rat hepatocytes described in **Chapter III**. Other than rats, humans metabolize coumarin to water soluble metabolites, readily excreted in the urine [14-16]. Therefore, no cytotoxicity of coumarin was observed in sandwich-cultured human hepatocytes, not even at the highest concentration

level employed. Still, coumarin induced differential expression of genes which could be mapped to biochemical pathways and biological processes. Interspecies results were concordant with respect to the therapeutic/beneficial effects of coumarin on blood coagulation. Furthermore, results were similar for fatty acid metabolism and urea cycle and metabolism of aminogroups. Impairment of pathways reflective of hepatotoxicity were observed in human hepatocytes treated with the highest concentration of coumarin and showed the sensitivity of the microarray approach compared to traditional measures which did not indicate cytotoxicity. However, the highest concentration applied in these experiments is unlikely to be relevant to exposure of humans *in vivo*. Still, clear hepatotoxic responses at the biochemical pathway and biological process level which were observed in rat hepatocytes cultured in modified medium were not observed in human hepatocytes upon coumarin treatment, probably due to interspecies differences in metabolism. Although similarities were observed in gene expression responses to coumarin between rat and human hepatocytes, the experiments conducted in **Chapter IV** underscore the need to use human hepatocytes to predict human effects.

The experiments described in **Chapter VI** aimed to compare the toxicogenomics response to thioacetamide between rat and human hepatocytes. Similar to the results in rat hepatocytes cultured in either standard or modified medium, the toxic potency of thioacetamide was low in human hepatocytes, as was shown at the level of cytotoxicity and gene modulation. Therefore, it appears that for thioacetamide-induced toxicity *in vivo* factors are present, that are absent *in vitro*. These factors might include involvement of other organs in the thioacetamide response, e.g. blood providing the liver with cytokines and inflammatory cells, which have shown to be involved in thioacetamide toxicity [17]. Furthermore, livers of rats *in vivo*, thioacetamide might affect the other cell types, Kupffer cells and stellate cells, which are absent or less present in hepatocyte cultures *in vitro*. Both pathway analysis approaches, MAPPFinder and T-profiler, identified low numbers of similar biochemical pathways and biological processes in human hepatocytes as well as in rat hepatocytes: induction of genes involved in cholesterol biosynthesis. Along the left axis of the parallelogram approach, results in **Chapter VI** confirm interspecies comparison of the low (toxic) response of thioacetamide between rat and human hepatocytes.

Rat-to-human interspecies comparisons were made based upon acetaminophen-induced responses on gene expression in the experiments described in **Chapter VII**. Similarities between human hepatocytes and rat hepatocytes cultured in modified medium at the level of differentially expressed genes as well as at the biochemical pathway and biological process level were higher compared to similarities between rat *in vitro- in vivo*. Concordant effects at the pathway and process level as determined by T-profiler analysis showed repression of lipid metabolism, electron transport, and CYP450 enzyme activity related pathways. Furthermore, known acetaminophen-induced responses on mitochondria were observed in both human and rat hepatocyte *in vitro* models. The results in **Chapter VI** confirm that interspecies extrapo-

lations between rat and human hepatocytes are feasible for acetaminophen-induced gene expression responses.

Thus, especially the toxicogenomics responses of acetaminophen, but also the low (toxicity) profiles induced by thioacetamide could very well be extrapolated from rat hepatocytes cultured in modified medium *in vitro* to human hepatocytes *in vitro*. However, interspecies differences in metabolism of compounds and consequently toxicity, underscore the value of using human hepatocyte-based models for future hepatocyte-based *in vitro* studies for risk assessment and identification of modes-of-action of compounds.

COMPLETION OF THE PARALLELOGRAM APPROACH: RELEVANCE TO HUMAN HAZARD

Combination of the results for coumarin, thioacetamide, and acetaminophen along the upper axis of the parallelogram approach: rat *in vitro*-to-*in vivo* comparisons, with the results along the left axis of the parallelogram approach: rat-human interspecies comparisons, enable prediction of hepatoxicant-induced (qualitative) responses in humans *in vivo*.

For coumarin, strong concordance is observed between rats *in vitro* and *in vivo* and between rat and human hepatocytes *in vitro* for the coumarin-induced effects on complement and coagulation cascades, already at an *in vivo* achievable plasma concentration. Only the highest concentration of coumarin, to which humans are unlikely to be exposed *in vivo*, resulted in repression of fatty acid metabolism in human hepatocytes. This effect was already observed in rats *in vitro* and *in vivo* at lower concentrations/doses. The hepatotoxic responses of coumarin on methionine metabolism in rats *in vitro* and *in vivo*, however, were not observed in human hepatocytes and are thought to be metabolism dependent. Furthermore, the highest concentration resulted in impairment of mitochondrial function in human hepatocytes, which was not observed in rat *in vitro*/*in vivo* at any concentration/dose. Thus, the parallelogram approach shows the therapeutic activity of coumarin along all axes, suggesting that this response would occur in humans *in vivo*. Coumarin-specific toxic responses relevant for humans could not be identified.

The combination of rat *in vitro*-*in vivo* results and rat-human interspecies results within the parallelogram approach implicates that thioacetamide-induced impairment of cholesterol metabolism is likely to occur in humans *in vivo*. However, genes involved in cholesterol metabolism are down-regulated *in vivo* and up-regulated in both rat and human hepatocytes *in vitro*, showing an important discordance between the results observed *in vitro* and *in vivo*. Due to this, the relevance of each model for the situation in humans *in vivo* is unclear and no prediction can be made.

Rat *in vitro*-to-*in vivo* extrapolations as well as *in vitro* rat-to-human interspecies extrapolations could very well be made between toxicogenomics responses observed upon acetaminophen

exposure. Although mitochondrial dysfunction is a well-described effect of acetaminophen-induced *in vivo* hepatotoxicity, this was not confirmed by analysis of gene expression data in rats *in vivo* in the present study. This effect, however, did occur in rat hepatocytes cultured in modified medium as well as in human hepatocytes. Repression of other biochemical pathways and biological processes known to be involved in acetaminophen-induced acute hepatotoxicity, including lipid metabolism related pathways, electron transport, and CYP450 enzyme activity in rat and human hepatocytes *in vitro* as well as in rats *in vivo* suggest these effects are likely to occur in humans *in vivo*.

Generally, the model hepatotoxicants to which rat and human hepatocytes *in vitro* and rats *in vivo* were exposed as described in this thesis to predict human effects using a toxicogenomics-based parallelogram approach did not result in an unequivocal conclusion. Rat *in vitro*-to-*in vivo* extrapolations could be made for coumarin, however, for prediction of human toxic effects, hepatocyte-based *in vitro* models of human origin should be used. For thioacetamide, rat *in vitro*-to-*in vivo* comparisons were problematic, whereas rat-to-human interspecies extrapolation was successful (although gene expression responses were low and responses did not indicate toxicity). Acetaminophen enabled full completion of the parallelogram approach, showing comparable responses between rat *in vitro*-to-*in vivo* as well as between rat and human hepatocytes *in vitro*. These responses are therefore likely to occur in humans as well.

CONCLUSION

The research presented in this thesis showed the applicability of a toxicogenomics-based parallelogram approach to estimate the molecular toxicological effects of the well-known hepatotoxicants coumarin, thioacetamide, and acetaminophen in humans. The present studies highlight the added value of pathway analysis approaches in toxicogenomics, in particular those approaches that do not require pre-selection of differentially expressed genes, to facilitate *in vitro*-to-*in vivo* and interspecies extrapolation. Results for coumarin and acetaminophen, compounds which need metabolic activation by the CYP450 enzyme system, showed that *in vitro*-to-*in vivo* extrapolations of hepatotoxic responses in rats benefit from a metabolically competent, toxicogenomics-based, hepatocyte *in vitro* system, as is represented in the modified *in vitro* model in this thesis. Furthermore, gene expression profiles could very well be extrapolated from rat to human hepatocytes *in vitro* for thioacetamide and acetaminophen. The toxicogenomics-based parallelogram approach was successfully completed for acetaminophen, implicating that responses observed in rat and human hepatocytes *in vitro* as well as in rats *in vivo* are likely to occur in humans as well.

LIMITATIONS AND RECOMMENDATIONS

The *in vitro* models used in this thesis obviously have limitations which might contribute to the differences regarding *in vitro*-to-*in vivo* responses to thioacetamide and to compounds in general. The hepatocyte-based *in vitro* systems used in the present studies only rely on one liver cell type, namely hepatocytes, whereas other liver cells like Kupffer cells are absent. These cells may affect drug metabolizing enzyme expression and function via cytokines. Furthermore, the hepatocyte *in vitro* model is a 'static' system, by contrast to the dynamic *in vivo* situation where the blood flow makes a critical contribution to the exchanges between cells and their environment [9]. Further research should continue to optimize *in vitro* systems, increasing the relevance to the situation *in vivo*.

Hepatocyte-based *in vitro* systems in general should be customized to the particular applications of interest [18]. One option is presented in this thesis in which conventional rat hepatocyte-based sandwich cultures were optimized by increasing the metabolic competence with addition of enzyme inducers to culture medium, creating a customized *in vitro* model for investigation of compounds which need metabolic activation by the CYP450 enzyme system. Implementation of standardized, optimized, and customized *in vitro* systems, like the ones presented in this thesis, which precede *in vivo* animal testing, can significantly reduce the number of animals needed.

Due to metabolism-mediated species differences in response to compounds, *in vitro* cultures of human hepatocytes would be the system that probably best predicts toxic effects in humans [9, 10, 13]. These results are confirmed in this thesis for coumarin. Recent advances in isolation and culture techniques enabled obtaining primary human hepatocytes of high quality and viability for long term culture [8, 19, 20], and now increasingly enable the use of human hepatocytes in hazard and risk assessment of chemical compounds and drugs. Furthermore, recent initiatives focus on development of differentiated human hepatocytes from embryonic stem cells. In the future, these approaches will work towards solving problems of availability of human hepatocytes and their inter-individual variability.

The toxicogenomics-based parallelogram approach to predict hepatotoxicant-induced gene expression responses in humans *in vivo* should be combined with pathway analysis approaches to increase biological information and facilitate *in vitro*-to-*in vivo* and interspecies comparison. Pathway analysis approaches for which no pre-selection of subsets of (differentially expressed) genes is needed, e.g. T-profiler, enable identification of biochemical pathways and biological processes even at low level modulation as has been shown by studies presented in this thesis and are therefore preferable to methods which need predefinition of subsets of genes.

Although the model hepatotoxicants coumarin, thioacetamide, and acetaminophen are known to induce hepatocellular necrosis, no generic gene expression profile for necrosis could be identified based on results presented in this thesis. To identify the status of the observed responses, homeostatic adaptations, non-specific (toxic) responses, or responses unique for

centrilobular necrosis, in future studies, the number of compounds under investigation should be increased and should include both necrotizing and non-necrotizing hepatotoxicants.

Research described in this thesis focused on the extrapolation of mechanistic, qualitative, responses identified using microarray analysis and biochemical pathways and biological process analysis. Therefore, in the present studies, no quantitative assessment of human hepatotoxic risks can be achieved. For example, doses of acetaminophen used in rats *in vivo* represent a major overdose for humans *in vivo*, far beyond the maximum therapeutic dose of acetaminophen for an average adult within 24h, which is 4 grams. As the toxicogenomics-based parallelogram approach showed to be applicable for acetaminophen, future studies should focus on larger dose ranges, including doses relevant for normal human exposure.

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