Advanced in vitro models for studying drug induced toxicity

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CHAPTER 1

GENERAL INTRODUCTION AND SCOPE OF THIS THESIS

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Toxicogenomics-based cellular models -
Alternatives to current animal testing for safety assessment

INTRODUCTION

Driven by the emergence of new diseases, increased drug resistance and an unmet need for therapies for existing diseases, there is an ever-increasing demand for new medicines. The remarkable advances in science and technology have supported the discovery of new chemical entities but the pharmaceutical industry is struggling to translate this into approved drugs for the clinic. Considering the enormous investments associated with the development of a new drug, attrition at later stages of drug development or after release into the market is a major concern for pharmaceutical companies, not to mention patients. Toxicity is the major reason for drug attrition with 40% of new chemical entities failing after pre-clinical safety studies in animals and 89% before they enter clinical trails [1], with hepatotoxicity and cardiotoxicity being the main reasons followed by nephrotoxicity, neurotoxicity and gastro-intestinal toxicity [2]. It is therefore important to efficiently screen new chemical entities for their adverse effects, excluding toxic drugs at an earlier stage, and selecting the most promising drug candidates for further development.

Predicting safety of a chemical entity at the preclinical stage has remained a major challenge. Recent years have seen an increase in attrition rates - even after rigorous testing in both *in vitro* and *in vivo* test models. This is partly due to poor prediction of human-specific responses in these models. The drug metabolism of animals differs from those of humans and may not accurately predict a human specific stress response. For example nifedipine, a calcium channel blocker, is metabolized by CYP3A4 in humans but not in rats [3] [4]. Human derived cell lines might offer an advantage in drug screening at the pre-clinical stage; currently primary human hepatocytes are considered as the gold standard [5] for toxicity testing but they too lose liver cell properties within hours after *in vitro* culture and show a high donor specific variability in gene expression [6, 7]. In addition to primary cells, immortalized cell lines and hepatocarcinoma cell lines can be used. These cells have either undergone mutations or lost a majority of liver specific functions under non-physiological culture conditions. The unnaturally high rate of proliferation of these cell lines also compromises their suitability for testing. Therefore there is a lack of *in vitro* models that can stably maintain human liver specific functions. To address this, development of a physiologically relevant human organotypic *in vitro* model is essential for safety assessment. In such models hepatocytes should remain differentiated and acquire many of the growth and metabolism characteristics of functional liver, thereby offering a promising tool to improve *in vitro* testing by both reducing and complementing pre-clinical animal testing.

Drug discovery and development

Drug discovery and development is a precarious process taking up to 12-16 years with costs exceeding $1.8 Billion and increasing every year [8, 9]. The process of drug development is mainly divided into the discovery phase, clinical development phase and approval phases. In the early discovery phase once a druggable target is selected for a disease compounds are screened with a battery of *in vitro* and *in vivo* tests to identify hits [10]. Later, the identified hits or lead compounds go through a series of tests to characterize the pharmacokinetic and toxicological properties of the drug. Successful lead candidates are further optimized and rigorously tested in both *in vitro* and *in vivo* models before they are tested in humans. Accurate screening of safe druggable targets plays a key role in the success of the drug.

After a thorough investigation at the discovery phase, drug candidates are selected for human clinical trials by filing an application with regulatory agencies. Both EMEA and FDA have a similar evaluation process with preclinical testing followed by a clinical development phase [11]. On approval, phase I clinical studies are conducted, in which the compound is tested in about 20 to 80 volunteers looking mainly at pharmacokinetics and pharmacodynamics of the drug to evaluate a safe dosing range. In phase II, effectiveness of the drug is analyzed in about 100 to 300 patients with a disease condition that is intended for treatment and studying any side effects caused by the drug. After a positive evaluation the drug candidate is ready for phase III testing on a large number of patients (100 – 3000) to further evaluate safety and efficacy of the drug candidate [12]. Different populations are analyzed at this stage to find dosage, drug reactions, and drug-drug interactions [12]. After successful clinical trials and approval by regulatory authorities the drug is released into the market.

Even after this conscientious drug development process there are several drug withdrawals from the market due to adverse drug reactions. When we look from the early discovery phase 40% of the drugs are withdrawn due to toxicity after animal testing [1]. Between 1997-2005, forty-five of the approved drugs had black box warnings with 10 related to hepatotoxicity and 16 drugs were withdrawn out of which 5 (31%) were due to hepatotoxicity [13, 14]. In another survey on post-marketed drug withdrawals between 1998 to 2008 liver toxicity was the major reason for drug withdrawal [2]. After liver toxicity renal injury is the second most leading cause of drug attrition [15]. 25% of the marketed drugs with potential nephrotoxicity warnings lead to acute kidney injury increasing mortality and morbidity in patients [15, 16]. Development of advanced alternative methods to identify the liver and kidney organ toxicities may therefore aid in decreasing drug attrition.

The liver: a major organ for drug metabolism and a main target for drug-induced toxicity.

The liver is the major organ involved in drug metabolism and disposition. Liver parenchymal cells (hepatocytes) carry out this function and possess the majority of the enzymes involved in metabolism, which are housed in smooth endoplasmic reticulum. Of all cell types in the body, hepatocytes have the most extensive network of smooth endoplasmic reticulum. The expression of the xenobiotic metabolizing enzymes is a coordinately regulated and controlled mechanism. Drug metabolism by Phase I enzymes involves catalysis of the drug either by oxidation, reduction and hydrolysis steps leading a water-soluble or toxic reactive metabolites, which are further, modified by phase II enzymes for excretion. Phase II enzymes are mainly involved in conjugation steps, where a charged group is added to make a reactive metabolite less toxic to hepatocytes either by glucuronidation, sulphation, acetylation, methylation. Phase I and II steps render drugs into less toxic water soluble molecules that could be excreted through various routes, either blood, bile or renal elimination.

Approximately 70% of drugs are eliminated by CYP450 metabolism [17]. CYP450 metabolism can also lead to toxic reactive metabolites, which can cause hepatocellular necrosis [18]. These enzymes can also convert certain chemicals to carcinogenic metabolites; electrophilic metabolites produced by the CYP450 enzymes may bind to nucleophilic cellular components such as DNA leading to mutations and cancer in humans [19]. Some drugs have the ability to induce or reduce the expression levels of CYP450 enzymes, which can be detrimental to patients who are on multiple medications, as the co-administered drug may change the pharmacokinetics of a second drug leading to adverse drug reactions [20, 21]. It is estimated that out of two million serious adverse drug reactions that occur per year in United States, 26% of them are caused due to such drug-drug interactions, [17, 22] emphasizing a need for creating a complete safety profile of all the drugs. Positive evaluation of hepatotoxic drugs may decrease the incidence of liver toxicity and therefore drug attrition and huge costs involved with it.

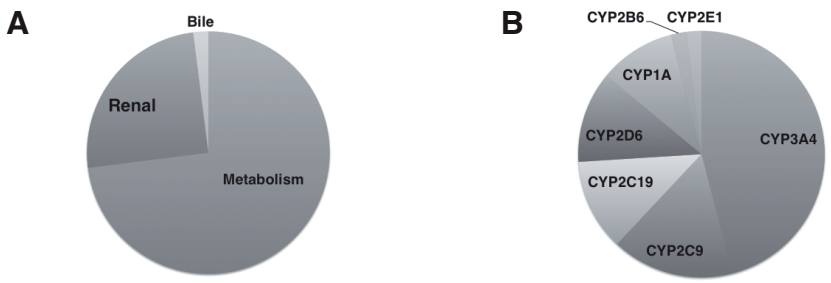

Figure 1. Process of metabolism and excretion of 200 drugs prescribed in 2002 (A) and CYP450 enzymes associated in their metabolism (B) (adapted from Wienkers *et al* (2005) ([17]).

Renal drug clearance and toxicity

After the liver, the kidneys play a major role in the clearance of drugs [17]. The primary functions of the kidney are filtration, secretion, reabsorption of body fluids, but importantly, excretion of drugs and drug metabolites. When the filtrates or drug metabolites reach the nephron, the functional unit of the kidney, they are highly concentrated to up to 100–fold in the tubular regions of the nephron [23, 24], making the kidney vulnerable to toxic injury leading to acute renal failure or loss of kidney function. In a study of patients admitted to intensive care due to kidney failure, 19% were caused by drug-induced toxicity [23, 25].

Proximal tubular epithelial cells in the nephron have the highest level of transporters compared to any other segment in the nephron playing a major role in reabsorption [26, 27]. These are the first cells that are exposed to the glomerular filtrate containing high levels of toxins, which makes them susceptible to toxic injury causing mitochondrial dysfunction, increased oxidative stress and decreased excretion leading to kidney injury [24, 28, 29]. Upon injury by a nephrotoxic agent proximal tubule cells die either by apoptosis or necrosis [30]. Nephrotoxic drugs have either a direct toxic effect on various segments of the nephron or they induce inflammation of the renal interstitium leading to acute renal failure. Many antibiotics, chemotherapeutics and immunosuppressant's induce direct injury to the tubules [31]. The intact tight junction complex, which is essential for proper reabsorption, may also be disrupted causing a leaky epithelium ultimately leading to proximal tubular disruption [31, 32].

Renal Inflammation in nephrotoxicity

Several nephrotoxic compounds induce an inflammatory response, which can aggravate renal injury. At the early stages of injury morphological and functional changes in tubular epithelium and vascular endothelial cells are observed [33]. This leads to infiltration of leukocytes to the injured site where renal tubular epithelial cells produce inflammatory cytokines TNFα, IL-6, IL-1β and TGF-β [34, 35] which induce an inflammatory cascade and can enhance cytotoxicity.

Nephrotoxic drugs such as cisplatin can increase the expression of various pro-inflammatory cytokines and chemokines, with TNFα being identified as a key factor in enhancing the inflammatory response. Inhibition of cisplatin-induced TNFα production led to downregulation of other cytokines and decreased renal injury [36-38]. Several mechanisms have been proposed for cisplatin-induced TNFα production, involving ERK and p38 MAPK mediated activation, [37, 39] imbalance in NF-κB and JNK/c-Jun signaling [40]. Therefore, inflammation plays a major role in the pathophysiology of kidney injury, which needs to be thoroughly investigated during early phases of drug discovery. Nephrotoxicity is mainly detected at late stages of

drug development with only 2% of attrition at preclinical testing and 19% during clinical trials [41]. Besides the current conventional end points, use of advanced screening methods such as live cell imaging for early apoptotic events [42] might help in identifying safe compounds thereby decreasing attrition rates due to nephrotoxicity.

Pre-clinical safety testing: where do we stand?

With a high incidence of drug-induced organ toxicity it is very important to make an accurate estimation of pharmacokinetic and pharmacodynamics properties of a new chemical entity during pre-clinical assessment. Currently various *in vivo* and *in vitro* studies are performed to validate the safety of chemical entities and their targeted use for a human disease, mainly relying on animal models for long-term effects of the drug. But only 50% of the chemicals that caused hepatotoxicity in clinical developmental phases had concordance with toxicity in animal models [43]. This may be attributed to species difference, interbred lab animals, disease state vs. healthy animals, genetic and environmental difference that exist in human population [14]. This emphasizes a need for efficient models that could accurately predict the safety profile of the compounds. Human cell based models offer several advantages in terms of relevance to humans and increased throughput compared to animal models. Current standard *in vitro* models used for screening assays include hepatocytes for liver toxicity, renal proximal tubule epithelial cells for nephrotoxicity, vascular endothelial cells for vascular toxicity, neuronal and glial cells for neurotoxicity, cardiomyocytes for cardiotoxicity, skeletal myocytes for rhabdomyolysis [44].

	Sensitivity	Specificity
DNA synthesis	10	92
Protein synthesis	4	97
Glutathione depletion	19	85
Superoxide induction	1	97
Caspase-3 induction	5	95
Membrane integrity	2	99
Cell viability	10	92
Cell viability or GSH or DNA synthesis	25	83
Regulatory animal toxicity tests	52	N/A

Table 1. Percentage predictivity of *in vitro* toxicity assays and regulatory animal studies for 611 compounds with hepatotoxicity warnings as described by Xu *et al* in [14].

However, *in vitro* models fail to preserve organ specific functions in an artificial environment and are not reliable for making accurate predictions. For example, *in vitro* testing on hepatic cell lines measuring various indicators of cell stress and toxicity

did not improve predictivity compared to animal studies alone [14, 45]. All the *in vitro* tests combined together had only half the sensitivity to that of regulatory animal testing (as shown in table 1, sensitivity and specificity of 611 hepatotoxic compounds tested [14]).

The main focus of this thesis work is to explore improved *in vitro* models and methods for organ toxicity, with a principal focus on *in vitro* liver toxicity, which will be discussed in detail.

Predicting drug induced liver injury (DILI) at preclinical stages

There are several possible reasons for poor prediction of human toxicity in pre-clinical studies. The current methods used to assess toxicity at the preclinical level were introduced in 1970's and have not kept pace with technological developments [46]. Furthermore, test models may not give a toxic response due to loss of organ specific cell functionality or there is a difference in the mechanism of drug metabolism between humans and the cells/tissues of the model. The implementation of improved toxicological approaches and exploring the mechanistic toxicity on human-relevant test models may improve the early safety prediction.

Preclinical hepatotoxicity assessment is done in a tiered approach. In the tier 1 studies normal animal models (rodent and non-rodent) are exposed with multiple doses and durations of the drug at much higher levels than those used in clinical studies to estimate the 'dose-limiting toxicity' or no effect level (NOEL) and no adverse effect level (NOAEL) [47]. Various parameters are measured to evaluate toxicity and identify the safety level of the drug that could be administered in the clinical phases. If hepatotoxicity was found to be the limiting factor, then various *in vitro* screening assays, including covalent binding assays are considered to indicate specific toxicity issues and their potential severity [48, 49]. Though the main reason for poor prediction lies with a lack of physiological relevance of the models, human genetics and other underlying disease conditions might lead to idiosyncratic reactions, which are hard to identify at the pre-clinical and clinical phases. Of 28 compounds developed by Rhone-Poulenc Rorer (now a subsidiary of Sanofi-Aventis) between 1988 and 1994, 10 of them showed signs of liver toxicity in animal models; 7 of these compounds were tested in humans of which only 1 compound showed human liver toxicity [50]. There are many other studies, which show a lack of correlation with animal toxicity [51]. In this respect, human cell based models with a stable liver specific function could make an important contribution to predicting drug safety.

***in vitro* cell models for studying hepatotoxicity**

Precision cut liver slices (PCLS): Tissue slices contain all the cell types and tis-

sue microenvironment [52], which may enable them to respond better to a chemical stimulus. Precision cut human liver slices have shown to retain drug metabolism enzymes [53-55] and they have been used for various drug metabolism and toxicity studies [56, 57]. Microarray transcriptomic studies on rat PCLS showed similar mechanistic gene expression profile as *in vivo* liver tissue [58]. Recently, proteomic analysis after compound exposure on mouse, rat and human PCLS was shown to demonstrate *in vivo* like responses [59]. Though the model is unique in its composition and similarity to *in vivo* liver responses, a rapid decline in liver specific functions [54] is a limitation for chronic drug exposure studies. Recent advances in slicing and cryo-preservation techniques were shown to maintain the viability of liver slices [52], which may help to overcome laborious extraction procedures for test samples and low-throughput.

Primary hepatocytes (PHH): Primary hepatocytes isolated from human or animal tissue largely retain liver specific enzymes and are widely used for evaluating drug metabolism. Freshly isolated primary human hepatocytes are considered the 'gold standard' for *in vitro* drug assessment [5, 60]. Isolation of primary human hepatocytes is a complex process, which can lead to poor retention of liver enzyme activity [61]. Recent advances in cryopreservation techniques have helped to maintain the differentiated status of primary hepatocytes without any major loss of functions due to cryoinjury [62, 63]. But once cultured *in vitro*, primary hepatocytes rapidly lose their liver specific functions [5, 64]. Sandwich culturing of primary hepatocytes (between layers of collagen gel) has been shown to improve stability of expression of metabolic enzymes and support formation of bile canaliculi [65-68]. Although human primary hepatocytes are most promising for *in vitro* assessment in terms of physiological relevance, the limited availability of human donors, donor specific variability and cost have made this impractical for routine assessment [6, 61]. Primary hepatocytes from rat and mouse were also investigated and similar limitations were observed. Compared to rat hepatocytes, mouse hepatocytes are better in maintaining liver specific functions and discriminating carcinogens from non-carcinogens in a toxicogenomics study [69-71].

Immortalized cell lines: Cell immortalization either due to mutations in growth regulating genes or certain gene insertions provide a valuable and limitless source of material for studying the biological responses of the organs. Due to their unlimited growth, ease of availability and use they are convenient for high-throughput screening assays without large variation between experiments. Among the various immortalized cell lines available, Fa2N4 and HepG2 are 'first alternatives' after primary human hepatocytes [72] and the recently introduced HepaRG is a promising addition

with their high levels of metabolic enzymes [73].

Fa2N4: Fa2N-4 cells are derived from primary human hepatocytes and immortalized with SV40 large T-antigen [60, 74, 75]. These cells were shown to posses various CYP450 enzymes and transporters [75] but at the same time they lack certain xenobiotic nuclear receptors like CAR and important drug transporters classes like OATPs [60, 76] which limits their use for assessing hepatotoxicity.

HepaRG: HepaRG cell line is derived from female carcinoma patient. It is a bi-potent progenitor cell line, which differentiates into biliary and hepatocyte-like cells in the presence of DMSO [77-79]. Once differentiated in the presence of DMSO, HepaRG cells express high levels of metabolic enzymes, transporters and xenobiotic nuclear receptors [73, 80]. The culture of HepaRG cells in high concentration of DMSO in the medium has seen certain drawbacks such as increased cell death and LDH and AST enzymes levels [81]. Earlier observations in primary hepatocytes and our observations with HepG2 cells have shown that concentrations of DMSO at 0.1% or above can induce phase 1 and 2 enzyme expression [5, 6, 82]. Therefore high levels of DMSO might give an inappropriate estimation of enzyme induction. It was also observed that prototypical inducers of CYP3A4 like rifampin and phenobarbital did not induce CYP3A4 induction in HepaRG cells [73, 80]. Though promising with high levels of drug detoxifying enzymes, the media formulation and proprietary status of these cells may complicate their use for routine toxicological assessments.

HepG2 cells: HepG2 is a well-differentiated hepato-carcinoma cell line. HepG2 cells are best characterized and are widely used for various toxicological and pharmacological studies [83-87]. They express various liver-specific enzymes and drug metabolizing enzymes [88]. HepG2 cells express functionally active p53 protein, which can activate the DNA damage response and induce apoptosis, making it a desirable model for toxicity studies, especially genotoxic studies [60, 89]. These cells also show the presence of active nuclear transcription factor E2-related factor-2 (Nrf2) system, which is essential for induction and the expression of various phase II drug metabolizing enzymes and transporters for detoxification [90].

HepG2 cells are widely used in various high-throughput studies [86, 91-95] including EPA's ToxCast™ and Tox21™ programs for predicting and prioritizing chemicals [95-98]. The cells were also used in the pharmaceutical industry for lead identification [99]. A recent study combining a toxicogenomics approach and Ames test demonstrated that HepG2 cells could accurately predict *in vivo* genotoxicity [100]. In a high content screening approach these cells showed 93% sensitivity in identifying hepatotoxic compounds [86]. Comparative toxicogenomics analysis be-

tween HepG2 and HepaRG showed that HepG2 cells are better in discriminating genotoxic compounds and non-genotoxic compounds than HepaRG [101]. Earlier studies also indicated that these cells had high sensitivity and specificity in identifying genotoxic compounds [94, 102, 103].

With several advantages in ease of use, availability and considerable biological responses, HepG2 cells could be an ideal replacement for primary hepatocytes. However, the major concern with HepG2 cells and other hepatocyte cell lines is the lack of metabolic competence compared to primary hepatocytes [7, 14] [104]. This may be partly due to their hepatocarcinoma origin and oncogenic transformation, but also because they have been passaged extensively, resulting in drift from the original hepatocyte genetic profile. Furthermore, the absence of an *in vivo* like environment in a tissue culture dish results in a dedifferentiated phenotype with inevitable loss of function. A high proliferation rate associated with immortalized cells is a major limitation in identifying compounds that inhibit cell growth and induce apoptosis.

3D cell culture

In vivo, hepatocytes are highly polarized with distinct basal-lateral sinusoids and apical canalicular domains [105] which are essential for proper functioning of the liver. This highly polarized morphology is lost when cells are cultured under non-physiological conditions [106]. Hepatocytes cultured in a 3D environment using bioreactors [107, 108], hanging drop methods [109], collagen sandwich cultures [110], micro-space cultures [111], micro-patterned systems [112], microfluidic perfusion systems [113, 114], collagen and Matrigel cultures [115, 116] and other synthetic biomaterials [68, 117] have shown to re-acquire tissue specific properties and possess many hallmarks of *in vivo* epithelial cells.

Besides an increased physiological relevance, 3D models could also support long-term culture of the micro-tissues [108, 118, 119], representing a new dimension in *in vitro* toxicity assessment for repeated drug exposure studies. Maintaining robust tissue-like properties and balancing this with a high-throughput methodology is a big challenge. Some of the current 3D platforms cannot offer flexibility for high-throughput toxicity studies. A preferred choice for high-throughput screening assays would be to grow micro-tissues in a 384 or 1536 well formats. Bioreactors or microfluidic devices might emulate a tissue-like environment with respect to gaseous exchange and flow of nutrients but the throughput may be challenging with these models requiring complex equipment. High content imaging is increasingly used for toxicological screening assays [86]. Such imaging based approaches are technically challenging to apply to 3D culture systems. Although these challenges will inevitably be resolved as the technology develops, the use of biochemical end-points for 3D cultures are more feasible for routine high-throughput assays.

HepG2 cells in 3D culture

HepG2 cells show a spheroid morphology when cultured in micro-space cultures [111], bioreactors [107], peptide gels [68] and Matrigel [119] with distinctive characteristics of polarized epithelial hepatocytes. The gene expression of metabolic enzymes is also higher in HepG2 spheroids cultured on micro-space cultures [111] or ECM gels, in contrast to low levels with conventional 2D cultures. Increased expression of CYP450 enzymes might offer a great improvement to safety assessment studies and studying drug-drug interactions where the activation of a xenobiotic response by one (not necessarily toxic) compound may increase the metabolism of a second compound into toxic intermediates [120]. The absence or impairment of CYP450 enzymes most likely accounts for the failure to identify some hepatotoxic compounds *in vitro*.

Some functional activities of polarized hepatocytes were also recapitulated in 3D cell culture models. The formation of bile canaliculi is an important feature that was shown in HepG2 cells cultured on peptide hydrogels [68] and even more prominently with Matrigel cultures in our lab as described in chapter 2. The restoration of excretory function is likely due to the improved morphological differentiation of the hepatocytes, in particular, the establishment of apical-basal polarity - but also the restored expression of transporters and other components of the excretory machinery. Many drugs disrupt excretion pathways, for example rifampicin inhibits activity of OATP1B3, a transporter essential for bile acid flow [121]. HepG2 3D culture models may therefore allow the evaluation of the effects of new chemical entities on transporter function at the *in vitro* screening stage.

Earlier reports have shown that HepG2 cells have higher gene expression associated with cell cycle regulation, DNA, RNA nucleotide metabolism, transcription, transport and signal transduction and lower transcription levels associated with cell death, lipid metabolism and xenobiotic metabolism. In this thesis, we demonstrate that in HepG2 spheroids, genes associated with cell cycle were strongly downregulated and most of the xenobiotic metabolism pathways and stress signaling pathways such as the Nrf2 system were highly upregulated. HepG2 cells were also known to be inherently lacking in important nuclear xenobiotic receptors, [122, 123] but were re-expressed in the differentiated HepG2 spheroids as described in chapter 2 of this thesis.

An improved metabolic competence in 3D HepG2 spheroids and a functional similarity to *in vivo* hepatocytes in a micro plate setup would be promising for high-throughput toxicity screening assays. The ability to maintain a functionally stable phenotype for an extended period is an important feature that would allow the study of chronic drug exposure. A thorough investigation on low-dose, long-term effects on the gene expression, metabolite formation and morphological perturbations

will provide more insight into the mechanisms of toxicity, and should be taken into consideration when validating and evaluating the potential of 3D cell culture systems as surrogates to human liver tissue or a replacement for animal models. Modern sensitive approaches such as toxicogenomics to identify the mechanistic information and high content screening (HCS) to identify subtle morphological and physiological changes that occur before cell death on 3D organotypic models will provide further detailed insight into the mechanisms of toxicity and will help identify biomarkers of liver injury.

Toxicogenomics approaches in DILI

The availability of complete genome sequences led to the successful evolution of functional genomics which helped to discern biological responses at a whole new level. Toxicogenomics, a combination of transcriptomic, proteomic and metabolomic analysis with conventional toxicology investigates the effects of compounds on overall changes giving comprehensive mechanistic information on mode of action of a toxic response.

Transcriptomics analysis with microarray technology allow us to identify tens of thousands of genes that change upon xenobiotic exposure, allowing us to quickly interpret genes and stress signaling pathways associated with chemical toxicity. Also, recent advances in proteomic technologies either by two-dimensional gel electrophoresis 2-DE and gel-free LC-MS techniques are available for high-throughput protein analysis [124, 125]. Many toxicogenomic studies have been conducted to assess hepatotoxicity in rodent species, especially in rats, as it is a preferred choice due to ease of manipulation and breeding characteristics [126-132]. These studies have demonstrated that specific liver pathologies can be predicted by toxicogenomic approaches [133-137].

A repository of toxicogenomics data is available in public databases such as Gene Expression Omnibus (GEO) [138], Array Express [139], Comparative Toxicogenomics Database (CTD) [140] or EDGE [141, 142], Chemical Effects in Biological Systems (CEBS) [129]. Additionally, the InnoMed PredTox consortium developed large-scale toxicogenomics databases, aimed at assessing the value of toxicogenomics by combining the results with conventional readouts [143]. TG-GATES (Genomics Assisted Toxicity Evaluation System) has a database of *in vivo* and *in vitro* gene expression profiles of liver and kidney upon exposure to 150 chemicals, mainly including drugs that are currently used for patients [144]. These databases will help us to perform a comparative analysis of prototypical compounds in various models.

Availability of large 'omics' data sets requires complex analysis algorithms to interpret the biological response. Availability of open source bioinformatics tools, like Bioconductor are helpful for data normalization of transcriptomics data. Several

commercial applications like ingenuity pathway analysis (IPA ®), Metacore™ (and other tools as discussed in [145]) are valuable for interpreting complex genomic data. These programs are built on a knowledge base developed from scientific literature databases on genes, proteins and chemicals providing relationships between changes in the gene expression to biological pathways.

Toxicogenomics aims to discern biological responses upon toxic insult and develop biomarkers that could predict a toxic outcome. The discovery of biomarker signatures will not only help in accurate prediction of toxicity, but also greatly reduces the use of animals for toxicity studies or may even replace animal testing if an *in vitro* model out-performs animal test models. A thorough comparative investigation of *in vitro* models with their *in vivo* counter parts may weigh the similarities between them. Kienhuis *et al* compared rat hepatocytes and rat *in vivo* gene expression profiles, which showed only a very minor overlap between the models, although the overlap increased with modification of the cell culture medium [146]. Additional improvement in the cell culture conditions - either media or using 3D cell culture models might further improve the predictive power of *in vitro* assays. *In vitro* toxicogenomics studies using human cell lines might be valuable to predict human specific responses; a number of studies from human derived hepatocytes - either primary or cell lines - are currently available [101, 147-151] and are promising. Our observations of gene expression profiles with diclofenac exposed *in vitro* 3D HepG2 cells showed a similar pathway profile to that of *in vivo* models. A number of stress signaling pathways that are not activated in 2D HepG2 cells were seen in 3D HepG2 cells which were in common with human liver slices exposed to diclofenac.

High-content screening for studying drug induced organ toxicity

High content imaging is a valuable methodology, which has the capacity to identify sensitive biological changes that are otherwise impossible with end point cytotoxicity assays. It will allow us to visualize dynamics of stress induced biological perturbations inside a cell in real time. Recent technical advances in fully automated microscopy stations and image data analysis methodologies have further improved the power to HCS for high-throughput screening assays.

Availability of various cell-permeable fluorescent molecular probes allowed us to study the kinetics of stress responses in real time. Automated measurements of live-cell apoptosis using Annexin-V, which binds to phosphotidyl-serine during early events of apoptosis is an efficient way to measure the kinetics of apoptosis upon compound exposure [152]. HCS has an advantage that multiple parameters can be measured. In a study to identify DILI compounds, primary human hepatocytes were challenged with 300 compounds measuring mitochondrial damage, oxidative stress and intracellular glutathione levels [153]. This analysis had a true-positive rate of 50-

60% and very low false-positive rate of 0-5%. In another study using HepG2 cells, four parameters were used; these measured intracellular calcium (Fluo-4AM), DNA content (Hoechst), mitochondrial membrane potential (TMRM) and plasma membrane permeability (TOTO3) and were compared with 7 conventional readouts that are used to assess toxicity. The high-content image analysis showed much higher sensitivity of 93% and specificity of 98 % compared to 25% and 90% of conventional assay readouts [86].

The application of toxicogenomics and high content screening in drug safety testing on human relevant *in vitro* models, may increase the predictive power of *in vitro* toxicity screening assays and provide sufficient functional data to reduce the reliance on animal models. The near *in vivo* properties of 3D cultures, their ease of use, low cost and availability suggest that these models offer great promise and are likely to play a significant part in animal-free toxicity testing in the future.

Aim and outline of this thesis:

Currently there is a dearth of *in vitro* models that could preserve the functional properties of a tissue for an extended period and offer compatibility to high-throughput screening assays. The key aim of this thesis is to develop an organotypic *in vitro* model for toxicity studies that can be used in pre-clinical drug safety testing and the *in vitro* study of liver biology. To this end we have developed a robust *in vitro* model, which show many hallmarks of *in vivo* hepatocytes, is applied in a 384-micro-well format and is compatible with standard medium- and high-throughput lab infrastructure for routine drug screening.

Chapter 2 describes the development and validation of a 3D cell culture methodology, which enables HepG2 cells to reacquire lost functional hepatocytes properties. The cells differentiated and formed spheroids. Spheroids were analyzed for their polarized morphology, expression of functional differentiation markers, presence of functional activities of hepatocytes and their sensitivity to identify hepatotoxic compounds.

Chapter 3 further characterizes the 3D HepG2 spheroid model and compares them to other *in vitro* liver models by analyzing gene transcription. Microarray analysis gene expression data was acquired during the differentiation process of HepG2 cells in 3D culture. Biologically significant pathways that are altered in differentiated 3D HepG2 cells compared to conventional 2D cultured HepG2 cells were thoroughly investigated. A detailed comparison of gene expression profiles was made between 3D HepG2 spheroids and other hepatocyte models (PHH and HepaRG) to human

liver. Common pathways between human liver and 3D HepG2 spheroids were highlighted.

Chapter 4 describes a comparative transcriptomic study between various *in vitro* and *in vivo* models in response to hepatotoxicant exposure. Gene expression profiles from *in vitro* HepG2 spheroids, primary human hepatocytes, primary rat and mouse hepatocytes, human liver slices and *in vivo* rat and mouse models exposed with diclofenac were compared. Various stress-signaling pathways that are activated upon diclofenac exposure were analyzed.

Chapter 5 describes a novel live-cell HCS assay for measuring nephrotoxicity. Toxicity caused by nephrotoxic compounds was analyzed in real time on proximal tubular kidney cells. The role of inflammation in conferring sensitivity to nephrotoxicants was investigated using the pro-inflammatory cytokine TNFα.

Chapter 6 provides a summary and general discussion of the findings and implications of the work in this thesis.

REFERENCES

1 McKim, J. M., Jr. Building a tiered approach to in vitro predictive toxicity screening: a focus on assays with in vivo relevance. Comb Chem High Throughput Screen 13, 188-206 (2010).

2 MacDonald, J. S. & Robertson, R. T. Toxicity testing in the 21st century: a view from the pharmaceutical industry. Toxicol Sci 110, 40-46, doi:10.1093/toxsci/kfp088 (2009).

3 Patki, K. C., Von Moltke, L. L. & Greenblatt, D. J. In vitro metabolism of midazolam, triazolam, nifedipine, and testosterone by human liver microsomes and recombinant cytochromes p450: role of cyp3a4 and cyp3a5. Drug metabolism and disposition: the biological fate of chemicals 31, 938-944, doi:10.1124/dmd.31.7.938 (2003).

4 Martignoni, M., Groothuis, G. M. & de Kanter, R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert opinion on drug metabolism & toxicology 2, 875-894, doi:10.1517/17425255.2.6.875 (2006).

5 LeCluyse, E. L. Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 13, 343-368 (2001).

6 LeCluyse, E. et al. Expression and regulation of cytochrome P450 enzymes in primary cultures of human hepatocytes. Journal of biochemical and molecular toxicology 14, 177-188 (2000).

7 Westerink, W. M. a. & Schoonen, W. G. E. J. Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. Toxicology in vitro : an international journal published in association with BIBRA 21, 1581-1591, doi:10.1016/j.tiv.2007.05.014 (2007).

8 DiMasi, J. a., Hansen, R. W. & Grabowski, H. G. The price of innovation: new estimates of drug development costs. Journal of health economics 22, 151-185, doi:10.1016/s0167-6296(02)00126-1 (2003).

9 Paul, S. M. et al. How to improve R&D productivity: the pharmaceutical industry's grand challenge. Nat Rev Drug Discov 9, 203-214, doi:10.1038/nrd3078 (2010).

10 Hughes, J. P., Rees, S., Kalindjian, S. B. & Philpott, K. L. Principles of early drug discovery. Br J Pharmacol 162, 1239-1249, doi:10.1111/j.1476-5381.2010.01127.x (2011).

11 San Miguel, M. T. & Vargas, E. Drug evaluation and approval process in the European Union. Arthritis and rheumatism 55, 12-14, doi:10.1002/art.21712 (2006).

12 Meadows, M. The FDA's drug review process: ensuring drugs are safe and effective. FDA consumer 36, 19-24 (2002).

13 Lasser, K. E. et al. Timing of new black box warnings and withdrawals for prescription medications. JAMA : the journal of the American Medical Association 287, 2215-2220 (2002).

14 Xu, J. J., Diaz, D. & O'Brien, P. J. Applications of cytotoxicity assays and pre-lethal mechanistic assays for assessment of human hepatotoxicity potential. Chemico-biological interactions 150, 115-128, doi:10.1016/j.cbi.2004.09.011 (2004).

15 in Accelerating the Development of Biomarkers for Drug Safety: Workshop Summary The National Academies Collection: Reports funded by National Institutes of Health (2009).

16 Pannu, N. & Nadim, M. K. An overview of drug-induced acute kidney injury. Critical care medicine 36, S216-223, doi:10.1097/CCM.0b013e318168e375 (2008).

17 Wienkers, L. C. & Heath, T. G. Predicting in vivo drug interactions from in vitro drug discovery data. Nat Rev Drug Discov 4, 825-833, doi:10.1038/nrd1851 (2005).

18 Park, B. K., Pirmohamed, M. & Kitteringham, N. R. The role of cytochrome P450 enzymes in hepatic and extrahepatic human drug toxicity. Pharmacology & therapeutics 68, 385-424 (1995).

19 Gonzalez, F. J. Overview of experimental approaches for study of drug metabolism and drug-drug interactions. Adv Pharmacol 43, 255-277 (1997).

20 Saito, M., Hirata-Koizumi, M., Urano, T., Miyake, S. & Hasegawa, R. A literature search on pharmacokinetic drug interactions of statins and analysis of how such interactions are reflected in package inserts in Japan. Journal of clinical pharmacy and therapeutics 30, 21-37, doi:10.1111/j.1365-2710.2004.00605.x (2005).

21 Sproule, B. A., Naranjo, C. A., Brenmer, K. E. & Hassan, P. C. Selective serotonin reuptake inhibitors and CNS drug interactions. A critical review of the evidence. Clinical pharmacokinetics 33, 454-471 (1997).

22 Lazarou, J., Pomeranz, B. H. & Corey, P. N. Incidence of adverse drug reactions in hospitalized

patients: a meta-analysis of prospective studies. JAMA : the journal of the American Medical Association 279, 1200-1205 (1998).
23 Loghman-Adham, M., Kiu Weber, C. I., Ciorciaro, C., Mann, J. & Meier, M. Detection and management of nephrotoxicity during drug development. Expert opinion on drug safety 11, 581-596, doi:10.1517/14740338.2012.691964 (2012).
24 Naughton, C. A. Drug-induced nephrotoxicity. American family physician 78, 743-750 (2008).
25 Uchino, S. et al. Continuous renal replacement therapy: a worldwide practice survey. The beginning and ending supportive therapy for the kidney (B.E.S.T. kidney) investigators. Intensive care medicine 33, 1563-1570, doi:10.1007/s00134-007-0754-4 (2007).
26 Lee, A. Pharmacokinetics, bioavailability, (hpmpc). Metabolism Clinical And Experimental 24 (1996).
27 Schlatter, P., Gutmann, H. & Drewe, J. Primary porcine proximal tubular cells as a model for transepithelial drug transport in human kidney. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 28, 141-154, doi:10.1016/j.ejps.2006.01.009 (2006).
28 Zager, R. A. Pathogenetic mechanisms in nephrotoxic acute renal failure. Seminars in nephrology 17, 3-14 (1997).
29 Markowitz, G. S. & Perazella, M. A. Drug-induced renal failure: a focus on tubulointerstitial disease. Clinica chimica acta; international journal of clinical chemistry 351, 31-47, doi:10.1016/j.cccn.2004.09.005 (2005).
30 Nony, P. A. & Schnellmann, R. G. Mechanisms of renal cell repair and regeneration after acute renal failure. J Pharmacol Exp Ther 304, 905-912, doi:10.1124/jpet.102.035022 (2003).
31 Choudhury, D. & Ahmed, Z. Drug-associated renal dysfunction and injury. Nature clinical practice. Nephrology 2, 80-91, doi:10.1038/ncpneph0076 (2006).
32 Lameire, N., Van Biesen, W. & Vanholder, R. Acute renal failure. Lancet 365, 417-430, doi:10.1016/S0140-6736(05)17831-3 (2005).
33 Becker, E. L. Acute Renal Failure: Diagnosis and Management. Archives of internal Medicine 126, 168-169 (1970).
34 Daha, M. R. & van Kooten, C. Is the proximal tubular cell a proinflammatory cell? Nephrol Dial Transplant 15 Suppl 6, 41-43 (2000).
35 Bonventre, J. V. & Zuk, A. Ischemic acute renal failure: an inflammatory disease? Kidney Int 66, 480-485, doi:10.1111/j.1523-1755.2004.761_2.x (2004).
36 Ramesh, G. & Reeves, W. B. TNF-alpha mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity. The Journal of clinical investigation 110, 835-842, doi:10.1172/JCI15606 (2002).
37 Ramesh, G. & Brian Reeves, W. Cisplatin increases TNF-alpha mRNA stability in kidney proximal tubule cells. Renal failure 28, 583-592, doi:10.1080/08860220600843839 (2006).
38 Tsuruya, K. et al. Direct involvement of the receptor-mediated apoptotic pathways in cisplatin-induced renal tubular cell death. Kidney Int 63, 72-82, doi:10.1046/j.1523-1755.2003.00709.x (2003).
39 Dumitru, C. D. et al. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell 103, 1071-1083 (2000).
40 Benedetti, G. et al. TNF-alpha-mediated NF-kappaB survival signaling impairment by cisplatin enhances JNK activation allowing synergistic apoptosis of renal proximal tubular cells. Biochemical pharmacology 85, 274-286, doi:10.1016/j.bcp.2012.10.012 (2013).
41 Izzedine, H., Harris, M. & Perazella, M. A. The nephrotoxic effects of HAART. Nature reviews. Nephrology 5, 563-573, doi:10.1038/nrneph.2009.142 (2009).
42 Benedetti, G. et al. A screen for apoptotic synergism between clinical relevant nephrotoxicant and the cytokine TNF-alpha. Toxicol In Vitro, doi:10.1016/j.tiv.2013.09.004 (2013).
43 Olson, H. et al. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regulatory toxicology and pharmacology : RTP 32, 56-67, doi:10.1006/rtph.2000.1399 (2000).
44 P.Li, A. in vitro approaches to evaluate ADMET drug properties. Current topics in Medicinal Chemistry 4 (2004).
45 Lee, W. M. Drug-induced hepatotoxicity. The New England journal of medicine 349, 474-485, doi:10.1056/NEJMra021844 (2003).
46 Hartung, T. Look back in anger - what clinical studies tell us about preclinical work. Altex 30, 275-291 (2013).

47 FDA, C. f. D. E. a. R. C. Guidance for the industry: Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers, <http://www.fda.gov/downloads/Drugs/Guidances/UCM078932.pdf> (2005).

48 Kapeghian, J. C. & Traina, V. M. The role of experimental toxicology in safety evaluation: challenges facing the pharmaceutical industry. Medicinal research reviews 10, 271-280 (1990).

49 Dean, J. H. & Olson, H. M. The integration of investigative toxicology in the drug discovery process. Biology of the cell / under the auspices of the European Cell Biology Organization 77, 3-8 (1993).

50 Ballet, F. Hepatotoxicity in drug development: detection, significance and solutions. Journal of hepatology 26 Suppl 2, 26-36 (1997).

51 FDA, U. S. F. a. D. A. Nonclinical Assessment of Potential Hepatotoxicity in Man, 2000).

52 de Graaf, I. A. et al. Preparation and incubation of precision-cut liver and intestinal slices for application in drug metabolism and toxicity studies. Nat Protoc 5, 1540-1551, doi:10.1038/nprot.2010.111 (2010).

53 de Kanter, R. et al. A rapid and simple method for cryopreservation of human liver slices. Xenobiotica 28, 225-234, doi:10.1080/004982598239533 (1998).

54 Graaf, I. A., Groothuis, G. M. & Olinga, P. Precision-cut tissue slices as a tool to predict metabolism of novel drugs. Expert opinion on drug metabolism & toxicology 3, 879-898, doi:10.1517/17425255.3.6.879 (2007).

55 Vickers, A. E. & Fisher, R. L. Organ slices for the evaluation of human drug toxicity. Chemico-biological interactions 150, 87-96, doi:10.1016/j.cbi.2004.09.005 (2004).

56 Klassen, L. W. et al. An in vitro method of alcoholic liver injury using precision-cut liver slices from rats. Biochemical pharmacology 76, 426-436, doi:10.1016/j.bcp.2008.05.012 (2008).

57 Hadi, M. et al. Human precision-cut liver slices as an ex vivo model to study idiosyncratic drug-induced liver injury. Chemical research in toxicology 26, 710-720, doi:10.1021/tx300519p (2013).

58 Elferink, M. G. et al. Microarray analysis in rat liver slices correctly predicts in vivo hepatotoxicity. Toxicol Appl Pharmacol 229, 300-309, doi:10.1016/j.taap.2008.01.037 (2008).

59 van Swelm, R. P. et al. Proteomic profiling in incubation medium of mouse, rat and human precision-cut liver slices for biomarker detection regarding acute drug-induced liver injury. Journal of applied toxicology : JAT, doi:10.1002/jat.2917 (2013).

60 LeCluyse, E. L., Witek, R. P., Andersen, M. E. & Powers, M. J. Organotypic liver culture models: meeting current challenges in toxicity testing. Critical reviews in toxicology 42, 501-548, doi:10.3109/10408444.2012.682115 (2012).

61 Pless, G., Sauer, I. M. & Rauen, U. Improvement of the cold storage of isolated human hepatocytes. Cell transplantation 21, 23-37, doi:10.3727/096368911X580509 (2012).

62 Li, A. P. Evaluation of drug metabolism, drug-drug interactions, and in vitro hepatotoxicity with cryopreserved human hepatocytes. Methods Mol Biol 640, 281-294, doi:10.1007/978-1-60761-688-7_15 (2010).

63 McGinnity, D. F., Soars, M. G., Urbanowicz, R. A. & Riley, R. J. Evaluation of fresh and cryopreserved hepatocytes as in vitro drug metabolism tools for the prediction of metabolic clearance. Drug metabolism and disposition: the biological fate of chemicals 32, 1247-1253, doi:10.1124/dmd.104.000026 (2004).

64 Wilkening, S. & Bader, A. Influence of culture time on the expression of drug-metabolizing enzymes in primary human hepatocytes and hepatoma cell line HepG2. Journal of biochemical and molecular toxicology 17, 207-213, doi:10.1002/jbt.10085 (2003).

65 LeCluyse, E. L., Fix, J. A., Audus, K. L. & Hochman, J. H. Regeneration and maintenance of bile canalicular networks in collagen-sandwiched hepatocytes. Toxicol In Vitro 14, 117-132 (2000).

66 LeCluyse, E. L., Ahlgren-Beckendorf, J. A., Carroll, K., Parkinson, A. & Johnson, J. Regulation of glutathione S-transferase enzymes in primary cultures of rat hepatocytes maintained under various matrix configurations. Toxicol In Vitro 14, 101-115 (2000).

67 LeCluyse, E. L., Audus, K. L. & Hochman, J. H. Formation of extensive canalicular networks by rat hepatocytes cultured in collagen-sandwich configuration. Am J Physiol 266, C1764-1774 (1994).

68 Malinen, M. M., Palokangas, H., Yliperttula, M. & Urtti, A. Peptide Nanofiber Hydrogel Induces Formation of Bile Canaliculi Structures in Three-Dimensional Hepatic Cell Culture. Tissue Eng Part A, doi:10.1089/ten.TEA.2012.0046 (2012).

69 Mathijs, K. et al. Discrimination for genotoxic and nongenotoxic carcinogens by gene expression profiling in primary mouse hepatocytes improves with exposure time. Toxicol Sci 112, 374-384, doi:10.1093/toxsci/kfp229 (2009).
70 Mathijs, K. et al. Gene expression profiling in primary mouse hepatocytes discriminates true from false-positive genotoxic compounds. Mutagenesis 25, 561-568, doi:10.1093/mutage/geq040 (2010).
71 Mathijs, K. et al. Assessing the metabolic competence of sandwich-cultured mouse primary hepatocytes. Drug metabolism and disposition: the biological fate of chemicals 37, 1305-1311, doi:10.1124/dmd.108.025775 (2009).
72 Szabo, M., Veres, Z., Baranyai, Z., Jakab, F. & Jemnitz, K. Comparison of human hepatoma HepaRG cells with human and rat hepatocytes in uptake transport assays in order to predict a risk of drug induced hepatotoxicity. PLoS One 8, e59432, doi:10.1371/journal.pone.0059432 (2013).
73 Aninat, C. et al. Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. Drug metabolism and disposition: the biological fate of chemicals 34, 75-83, doi:10.1124/dmd.105.006759 (2006).
74 Ripp, S. L. et al. Use of immortalized human hepatocytes to predict the magnitude of clinical drug-drug interactions caused by CYP3A4 induction. Drug metabolism and disposition: the biological fate of chemicals 34, 1742-1748, doi:10.1124/dmd.106.010132 (2006).
75 Mills, J. B., Rose, K. A., Sadagopan, N., Sahi, J. & de Morais, S. M. Induction of drug metabolism enzymes and MDR1 using a novel human hepatocyte cell line. J Pharmacol Exp Ther 309, 303-309, doi:10.1124/jpet.103.061713 (2004).
76 Hariparsad, N., Carr, B. A., Evers, R. & Chu, X. Comparison of immortalized Fa2N-4 cells and human hepatocytes as in vitro models for cytochrome P450 induction. Drug metabolism and disposition: the biological fate of chemicals 36, 1046-1055, doi:10.1124/dmd.108.020677 (2008).
77 Gripon, P. et al. Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci U S A 99, 15655-15660, doi:10.1073/pnas.232137699 (2002).
78 Cerec, V. et al. Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. Hepatology 45, 957-967, doi:10.1002/hep.21536 (2007).
79 Guillouzo, A. et al. The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. Chemico-biological interactions 168, 66-73, doi:10.1016/j.cbi.2006.12.003 (2007).
80 Kanebratt, K. P. & Andersson, T. B. Evaluation of HepaRG cells as an in vitro model for human drug metabolism studies. Drug metabolism and disposition: the biological fate of chemicals 36, 1444-1452, doi:10.1124/dmd.107.020016 (2008).
81 Hoekstra, R. et al. The HepaRG cell line is suitable for bioartificial liver application. Int J Biochem Cell Biol 43, 1483-1489, doi:10.1016/j.biocel.2011.06.011 (2011).
82 Nishimura, M., Ueda, N. & Naito, S. Effects of dimethyl sulfoxide on the gene induction of cytochrome P450 isoforms, UGT-dependent glucuronosyl transferase isoforms, and ABCB1 in primary culture of human hepatocytes. Biological & pharmaceutical bulletin 26, 1052-1056 (2003).
83 Hockley, S. L., Arlt, V. M., Brewer, D., Giddings, I. & Phillips, D. H. Time- and concentration-dependent changes in gene expression induced by benzo(a)pyrene in two human cell lines, MCF-7 and HepG2. BMC genomics 7, 260, doi:10.1186/1471-2164-7-260 (2006).
84 van Delft, J. H. et al. Discrimination of genotoxic from non-genotoxic carcinogens by gene expression profiling. Carcinogenesis 25, 1265-1276, doi:10.1093/carcin/bgh108 (2004).
85 Olsavsky, K. M. et al. Gene expression profiling and differentiation assessment in primary human hepatocyte cultures, established hepatoma cell lines, and human liver tissues. Toxicol Appl Pharmacol 222, 42-56, doi:10.1016/j.taap.2007.03.032 (2007).
86 O'Brien, P. J. et al. High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. Archives of toxicology 80, 580-604, doi:10.1007/s00204-006-0091-3 (2006).
87 Fredriksson, L. et al. Diclofenac inhibits tumor necrosis factor-Œ±-induced nuclear factor-Œ∫B activation causing synergistic hepatocyte apoptosis. Hepatology (Baltimore, Md.) 53, 2027-2041, doi:10.1002/hep.24314 (2011).
88 Hewitt, N. J. & Hewitt, P. Phase I and II enzyme characterization of two sources of HepG2 cell

lines. Xenobiotica 34, 243-256, doi:10.1080/00498250310001657568 (2004).
89 Vollmer, C. M. et al. p53 selective and nonselective replication of an E1B-deleted adenovirus in hepatocellular carcinoma. Cancer Res 59, 4369-4374 (1999).
90 Adachi, T. et al. Nrf2-dependent and -independent induction of ABC transporters ABCC1, ABCC2, and ABCG2 in HepG2 cells under oxidative stress. Journal of experimental therapeutics & oncology 6, 335-348 (2007).
91 O'Brien, P. & Haskins, J. R. In vitro cytotoxicity assessment. Methods Mol Biol 356, 415-425 (2007).
92 Schoonen, W. G., Westerink, W. M. & Horbach, G. J. High-throughput screening for analysis of in vitro toxicity. EXS. 99, 401-452 (2009).
93 Abraham, V. C., Towne, D. L., Waring, J. F., Warrior, U. & Burns, D. J. Application of a high-content multiparameter cytotoxicity assay to prioritize compounds based on toxicity potential in humans. J Biomol Screen 13, 527-537 (2008).
94 Westerink, W. M., Stevenson, J. C., Horbach, G. J. & Schoonen, W. G. The development of RAD51C, Cystatin A, p53 and Nrf2 luciferase-reporter assays in metabolically competent HepG2 cells for the assessment of mechanism-based genotoxicity and of oxidative stress in the early research phase of drug development. Mutat Res 696, 21-40, doi:10.1016/j.mrgentox.2009.12.007 (2010).
95 Judson, R. S. et al. In Vitro Screening of Environmental Chemicals for Targeted Testing Prioritization: The ToxCast Project. Environ Health Perspect 118 (2009).
96 Martin, M. T., Judson, R. S., Reif, D. M., Kavlock, R. J. & Dix, D. J. Profiling chemicals based on chronic toxicity results from the U.S. EPA ToxRef Database. Environ Health Perspect 117, 392-399 (2009).
97 Shukla, S. J., Huang, R., Austin, C. P. & Xia, M. The future of toxicity testing: a focus on in vitro methods using a quantitative high-throughput screening platform. Drug Discov Today, doi:S1359-6446(10)00261-8 [pii]10.1016/j.drudis.2010.07.007 (2010).
98 delft, J. v. Toxicogenomics-Based Cellular Models :Alternatives to Animal Testing for Safety Assessment. (Elsevier, 2014).
99 Gamo, F. J. et al. Thousands of chemical starting points for antimalarial lead identification. Nature 465, 305-310, doi:10.1038/nature09107 (2010).
100 Magkoufopoulou, C. et al. A transcriptomics-based in vitro assay for predicting chemical genotoxicity in vivo. Carcinogenesis 33, 1421-1429, doi:http://dx.doi.org/10.1093/carcin/bgs182 (2012).
101 Jennen, D. G. et al. Comparison of HepG2 and HepaRG by whole-genome gene expression analysis for the purpose of chemical hazard identification. Toxicol Sci 115, 66-79 (2010).
102 Uhl, M. et al. Effect of chrysin, a flavonoid compound, on the mutagenic activity of 2-amino-1-methyl-6-phenylimidazo[4,5- b]pyridine (PhIP) and benzo(a)pyrene (B(a)P) in bacterial and human hepatoma (HepG2) cells. Arch Toxicol 77, 477-484, doi:10.1007/s00204-003-0469-4 (2003).
103 Uhl, M., Helma, C. & Knasmuller, S. Single-cell gel electrophoresis assays with human-derived hepatoma (Hep G2) cells. Mutat Res 441, 215-224 (1999).
104 Gerets, H. H. J. et al. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. Cell biology and toxicology, doi:10.1007/s10565-011-9208-4 (2012).
105 Wang, L. & Boyer, J. L. The maintenance and generation of membrane polarity in hepatocytes. Hepatology 39, 892-899, doi:10.1002/hep.20039 (2004).
106 Maurice, M., Rogier, E., Cassio, D. & Feldmann, G. Formation of plasma membrane domains in rat hepatocytes and hepatoma cell lines in culture. J Cell Sci 90 (Pt 1), 79-92 (1988).
107 Fey, S. J. & Wrzesinski, K. Determination of drug toxicity using 3D spheroids constructed from an immortal human hepatocyte cell line. Toxicol Sci 127, 403-411, doi:10.1093/toxsci/kfs122 (2012).
108 Leite, S. B. et al. 3D HepaRG Model as an attractive tool for toxicity testing. Toxicol Sci, doi:10.1093/toxsci/kfs232 (2012).
109 Tung, Y.-C. et al. High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. The Analyst 136, 473-478, doi:10.1039/c0an00609b (2011).
110 Dunn, J. C., Tompkins, R. G. & Yarmush, M. L. Hepatocytes in collagen sandwich: evidence for

transcriptional and translational regulation. The Journal of cell biology 116, 1043-1053 (1992).
111 Nakamura, K. et al. Evaluation of drug toxicity with hepatocytes cultured in a micro-space cell culture system. Journal of bioscience and bioengineering 111, 78-84, doi:10.1016/j.jbiosc.2010.08.008 (2011).
112 Khetani, S. R. et al. Use of micropatterned cocultures to detect compounds that cause drug-induced liver injury in humans. Toxicol Sci 132, 107-117, doi:10.1093/toxsci/kfs326 (2013).
113 Lee, J., Kim, S. H., Kim, Y. C., Choi, I. & Sung, J. H. Fabrication and characterization of microfluidic liver-on-a-chip using microsomal enzymes. Enzyme and microbial technology 53, 159-164, doi:10.1016/j.enzmictec.2013.02.015 (2013).
114 Trietsch, S. J., Israels, G. D., Joore, J., Hankemeier, T. & Vulto, P. Microfluidic titer plate for stratified 3D cell culture. Lab on a chip 13, 3548-3554, doi:10.1039/c3lc50210d (2013).
115 Zhang, F., Xu, R. & Zhao, M.-j. QSG-7701 human hepatocytes form polarized acini in three-dimensional culture. Journal of cellular biochemistry 110, 1175-1186, doi:10.1002/jcb.22632 (2010).
116 Matsui, H., Takeuchi, S., Osada, T., Fujii, T. & Sakai, Y. Enhanced bile canaliculi formation enabling direct recovery of biliary metabolites of hepatocytes in 3D collagen gel microcavities. Lab on a chip 12, 1857-1864, doi:10.1039/c2lc40046d (2012).
117 Kim, Y. & Rajagopalan, P. 3D hepatic cultures simultaneously maintain primary hepatocyte and liver sinusoidal endothelial cell phenotypes. PloS one 5, e15456-e15456, doi:10.1371/journal.pone.0015456 (2010).
118 Gunness, P. et al. 3D organotypic cultures of human HepaRG cells: a tool for in vitro toxicity studies. Toxicol Sci 133, 67-78, doi:10.1093/toxsci/kft021 (2013).
119 Ramaiahgari et al. A 3D in vitro model of differentiated HepG2 cell spheroids with improved liver-like properties for repeated dose high-throughput toxicity studies. Archives of toxicology (2014).
120 Lin, J. H. CYP induction-mediated drug interactions: in vitro assessment and clinical implications. Pharm Res 23, 1089-1116, doi:10.1007/s11095-006-0277-7 (2006).
121 Hirano, M., Maeda, K., Shitara, Y. & Sugiyama, Y. Drug-drug interaction between pitavastatin and various drugs via OATP1B1. Drug metabolism and disposition: the biological fate of chemicals 34, 1229-1236, doi:10.1124/dmd.106.009290 (2006).
122 Kanno, Y. & Inouye, Y. A consecutive three alanine residue insertion mutant of human CAR: a novel CAR ligand screening system in HepG2 cells. The Journal of toxicological sciences 35, 515-525 (2010).
123 Naspinski, C. et al. Pregnane X receptor protects HepG2 cells from BaP-induced DNA damage. Toxicological sciences : an official journal of the Society of Toxicology 104, 67-73, doi:10.1093/toxsci/kfn058 (2008).
124 Barrier, M. & Mirkes, P. E. Proteomics in developmental toxicology. Reprod Toxicol 19, 291-304, doi:10.1016/j.reprotox.2004.09.001 (2005).
125 Van Summeren, A., Renes, J., van Delft, J. H., Kleinjans, J. C. & Mariman, E. C. Proteomics in the search for mechanisms and biomarkers of drug-induced hepatotoxicity. Toxicol In Vitro 26, 373-385, doi:10.1016/j.tiv.2012.01.012 (2012).
126 Ruepp, S. et al. Assessment of hepatotoxic liabilities by transcript profiling. Toxicol Appl Pharmacol 207, 161-170 (2005).
127 Natsoulis, G. et al. The liver pharmacological and xenobiotic gene response repertoire. Mol Syst Biol 4, 175 (2008).
128 Hirode, M. et al. Gene expression profiling in rat liver treated with compounds inducing phospholipidosis. Toxicol Appl Pharmacol 229, 290-299 (2008).
129 Waters, M. et al. CEBS--Chemical Effects in Biological Systems: a public data repository integrating study design and toxicity data with microarray and proteomics data. Nucleic Acids Res 36, D892-900, doi:10.1093/nar/gkm755 (2008).
130 McMillian, M. et al. A gene expression signature for oxidant stress/reactive metabolites in rat liver. Biochem Pharmacol 68, 2249-2261 (2004).
131 McMillian, M. et al. Inverse gene expression patterns for macrophage activating hepatotoxicants and peroxisome proliferators in rat liver. Biochem Pharmacol 67, 2141-2165 (2004).
132 Aitman, T. J. et al. Progress and prospects in rat genetics: a community view. Nature genetics 40, 516-522, doi:10.1038/ng.147 (2008).
133 Ellinger-Ziegelbauer, H. et al. The enhanced value of combining conventional and “omics” anal-

yses in early assessment of drug-induced hepatobiliary injury. Toxicol Appl Pharmacol 252, 97-111 (2011).
134 Steiner, G. et al. Discriminating different classes of toxicants by transcript profiling. Environ Health Perspect 112, 1236-1248 (2004).
135 van Ravenzwaay, B. et al. The use of metabolomics for the discovery of new biomarkers of effect. Toxicol Lett 172, 21-28, doi:S0378-4274(07)00159-2 [pii]10.1016/j.toxlet.2007.05.021 (2007).
136 Boitier, E. et al. A comparative integrated transcript analysis and functional characterization of differential mechanisms for induction of liver hypertrophy in the rat. Toxicol Appl Pharmacol 252, 85-96 (2011).
137 Suter, L. et al. EU framework 6 project: predictive toxicology (PredTox)--overview and outcome. Toxicol Appl Pharmacol 252, 73-84 (2011).
138 Edgar, R., Domrachev, M. & Lash, A. E. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 30, 207-210 (2002).
139 Brazma, A. et al. ArrayExpress--a public repository for microarray gene expression data at the EBI. Nucleic Acids Res 31, 68-71 (2003).
140 Mattingly, C. J., Rosenstein, M. C., Colby, G. T., Forrest, J. N., Jr. & Boyer, J. L. The Comparative Toxicogenomics Database (CTD): a resource for comparative toxicological studies. Journal of experimental zoology. Part A, Comparative experimental biology 305, 689-692, doi:10.1002/jez.a.307 (2006).
141 Hayes, K. R. et al. EDGE: a centralized resource for the comparison, analysis, and distribution of toxicogenomic information. Molecular pharmacology 67, 1360-1368, doi:10.1124/mol.104.009175 (2005).
142 Kiyosawa, N., Manabe, S., Yamoto, T. & Sanbuissho, A. Practical application of toxicogenomics for profiling toxicant-induced biological perturbations. International journal of molecular sciences 11, 3397-3412, doi:10.3390/ijms11093397 (2010).
143 Mulrane, L. et al. Creation of a digital slide and tissue microarray resource from a multi-institutional predictive toxicology study in the rat: an initial report from the PredTox group. Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie 60, 235-245, doi:10.1016/j.etp.2007.12.004 (2008).
144 Uehara, T. et al. The Japanese toxicogenomics project: application of toxicogenomics. Molecular nutrition & food research 54, 218-227, doi:10.1002/mnfr.200900169 (2010).
145 Ganter, B., Zidek, N., Hewitt, P. R., Muller, D. & Vladimirova, A. Pathway analysis tools and toxicogenomics reference databases for risk assessment. Pharmacogenomics 9, 35-54, doi:10.2217/14622416.9.1.35 (2008).
146 Kienhuis, A. S. et al. Parallelogram approach using rat-human in vitro and rat in vivo toxicogenomics predicts acetaminophen-induced hepatotoxicity in humans. Toxicol Sci 107, 544-552, doi:10.1093/toxsci/kfn237 (2009).
147 Hart, S. N. et al. A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. Drug Metab Dispos 38, 988-994 (2010).
148 Hockley, S. L. et al. Interlaboratory and interplatform comparison of microarray gene expression analysis of HepG2 cells exposed to benzo(a)pyrene. OMICS 13, 115-125 (2009).
149 Lambert, C. B., Spire, C., Claude, N. & Guillouzo, A. Dose- and time-dependent effects of phenobarbital on gene expression profiling in human hepatoma HepaRG cells. Toxicol Appl Pharmacol 234, 345-360 (2009).
150 Dere, E., Lee, A. W., Burgoon, L. D. & Zacharewski, T. R. Differences in TCDD-elicited gene expression profiles in human HepG2, mouse Hepa1c1c7 and rat H4IIE hepatoma cells. BMC Genomics 12, 193, doi:1471-2164-12-193 [pii]10.1186/1471-2164-12-193 (2011).
151 Horiuchi, S. et al. Global gene expression changes including drug metabolism and disposition induced by three-dimensional culture of HepG2 cells-Involvement of microtubules. Biochem Biophys Res Commun 378, 558-562, doi:S0006-291X(08)02282-1 [pii]10.1016/j.bbrc.2008.11.088 (2009).
152 Puigvert, J. C., de Bont, H., van de Water, B. & Danen, E. H. High-throughput live cell imaging of apoptosis. Current protocols in cell biology / editorial board, Juan S. Bonifacino [et al.] Chapter 18, Unit 18 10 11-13, doi:10.1002/0471143030.cb1810s47 (2010).
153 Xu, J. J. et al. Cellular imaging predictions of clinical drug-induced liver injury. Toxicol Sci 105, 97-105, doi:10.1093/toxsci/kfn109 (2008).