Advanced in vitro models for studying drug induced toxicity

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CHAPTER 6

DISCUSSION AND CONCLUSION

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With an estimated cost of $1.8 billion and 10 -15 years development time for a single drug [1-3], drug attrition at late stage development or withdrawal from the market is a serious concern for both industry and society. Recent technical advances have revolutionized pharmaceutical research, giving rise to new methodologies and systems biology approaches to interpret the mechanistic biological response. Application of these technologies in the drug development pipeline is expected to have a major positive impact on the discovery of novel drug targets and the development of safe medicines.

Drug attrition due to toxicity and efficacy have increased in recent years [4]. Though there were several scientific advances that helped in discovering new therapeutic targets, the methods used to test toxicity had little improvements. A principal reason for this is lack of physiologically relevant test systems that can be used for pre-clinical screening. We mainly rely on animal models to predict human specific responses. Though animal models will give biological responses involving a whole organism, often the drug metabolism, pharmacokinetics and pharmacodynamics are quite different to humans, for example nifedipine, a calcium channel blocker is metabolized by CYP3A4 in humans but not in rats [5]. Also CYP isoforms are different in each species making it difficult and dangerous to extrapolate animal data to humans [6]. To overcome this, human cell based *in vitro* models could be used. However, the current gold standards – primary human cells - are difficult to obtain and rapidly lose tissue specific functions. Assays using immortalized cell lines have also been developed, but these poorly represent human tissue due to loss of tissue specific functions in a non-physiological environment. Often, the cells used are of cancer origin and host extensive genomic alterations. Though several models have been proposed for long-term culture of differentiated cells, a thorough validation of these models is required to assess their efficiency and practical applicability in high-throughput screening assays. A combination of human cell-based organotypic systems with mechanism-based toxicity profiling and high content screening methods to study dynamic cellular perturbations will be promising future tools in reducing drug attrition rates and developing safer drugs.

In this thesis we discuss a novel high-throughput screening (HTS) compatible organotypic *in vitro* model that was developed to study drug induced liver injury (DILI). This simple static 3D-cell culture model induces morphological and functional differentiation of hepatocytes into polarized epithelial spheroids. An in-depth analysis of these spheroids was performed to assess the functional similarity to *in vivo* hepatocytes. Microarray gene expression analysis was performed in time during hepatocyte differentiation to study various elements involved in the formation of polarized spheroids. A comparative transcriptomic study showed similarities in the

mechanistic responses between this model and higher *in vivo* models. In this thesis I also describe a high content screening assay that was developed to study dynamic cell stress responses to toxicants.

Overall, the main focus of this thesis it to address current problems in the pre-clinical drug safety assessment in areas related to advancing current *in vitro* models, methodologies used to study toxicity and application of advanced 'omics approaches to predict drug induced toxicity.

3D cell culture systems in pre-clinical drug development

Ever since the invention of 2D cell culture systems there has been little progress in developing *in vitro* models that mimic the tissue-like microenvironment. Cells in our body reside in a three-dimensional space surrounded by extracellular matrix. This facilitates cell-cell and cell-matrix interactions that regulate gene and protein expression essential for proper tissue function [7]. Several models have been developed to simulate a 3D environment, but to successfully implement them in a drug development pipeline, the model should be suitable for high-throughput testing, retain a tissue like architecture and polarity for an extended period to allow chronic drug exposures and most importantly is sensitive in identifying toxic compounds. In chapter 2 we described a hydrogel-based static 3D cell culture model for inducing and maintaining differentiated spheroids from liver hepatoma cell line HepG2. Spheroids are also formed from HepG2 cells grown on peptide gels [8] and scaffold-free hanging drops [9]. However, due to the presence of native extracellular matrix ligands, the morphological complexity and functional similarity to *in vivo* hepatocytes is much more pronounced in our model. HepG2 spheroids in our hydrogel system show well-defined apical and baso-lateral domains resembling polarized hepatocytes. In chapter 2 transcriptomic changes in time during 3D culture were analysed. This gave us an overview of the genes associated with differentiation and regulation of functional pathways in HepG2 spheroid culture. A recent study showed formation of liver buds with co-cultures of iPSC's with mesenchymal stem cells and endothelial cells (HUVEC's); when these liver buds are transplanted into mice they vascularized and showed functional properties of a liver [10]. 83 genes involved in development of liver were analyzed and shown to increase in these liver buds; these 83 genes were also serially upregulated in HepG2 spheroids (Chapter 2) indicating a degree of restoration of the complex ontogenic profile of human liver in HepG2 spheroids.

The metabolism of drugs to toxic intermediates frequently underlies hepatotoxicity [11]. The rapid loss of specific CYP450 enzymes in primary human hepatocytes [12, 13] or overall low levels in immortalized cell lines including HepG2 cells [14] compromise accurate prediction of metabolite induced toxicity. Stable metabolic

competence that can enable chronic drug exposures is an ultimate goal for toxicity screening assays. Microarray transcript analysis showed serial upregulation of several phase I, II, and III drug metabolic enzymes in 3D cultured HepG2 spheroids up to 28 days with stable higher expression between day 21 and day 28 (Chapter 3), some of these genes were also validated by qPCR analysis which complemented the microarray analysis (Chapter 2). HPLC/LC-MS analysis showed higher metabolite levels of phase I, phase II metabolites (glucuronidation, sulphation) in 3D HepG2 spheroids from midazolam (CYP3A4), diclofenac (CYP2C9 and glucuronidation), bufuralol (CYP2D6), testosterone (substrate for multiple CYP's) and paracetamol (sulphation) substrates indicating increased metabolic competence compared to monolayer HepG2 cells. With increased metabolic competence and the ability to sustain functional stability for longer periods in microplates, HepG2 spheroids may be attractive tools in pre-clinical drug safety testing.

Current *in vitro* models used in conventional screening assays are not compatible for repeated drug exposure studies. This is mainly due to their continued growth in tissue culture plates requiring more space with time. HepG2 spheroids overcome this limitation as spheroid differentiation leads to a pronounced reduction of proliferation, such that spheroid size remains stable after day 14. Indeed, protein and mRNA levels of Ki-67, a marker for cell proliferation were undetectable in differentiated HepG2 spheroids (Chapter 2). HepG2 cultured on synthetic hydrogel in combination with integrin binding peptide domains did not result in inhibition of cell proliferation (in house observations) highlighting the importance of ECM with native tissue composition. Microarray analysis also showed strong inhibition of the cell cycle pathway in HepG2 spheroids (Chapter 2). Low levels of cell proliferation were also observed in differentiated HepaRG spheroids and in hanging drops [15] enabling long-term drug exposure studies. The sensitivity to known DILI compounds [16, 17] was also significantly higher in HepG2 spheroids with repeated dosing regimens (Chapter 2). Previous studies applying high-content imaging approaches to primary human hepatocyte cultures measuring mitochondrial function, oxidative stress and GSH content could differentiate toxic and non-toxic compounds at a dose range of 100-fold cMax [17]. Similarly, scoring based on mitochondrial membrane potential, DNA content, intracellular calcium levels and plasma membrane permeability on HepG2 cells identified DILI compounds with 93% sensitivity at 30-fold cMax concentrations [18]. In our study the repeated dosing of HepG2 spheroids showed sensitivity in identifying all the DILI compounds tested (8). For example, the withdrawn drug trovafloxacin mesylate was toxic at its maximum plasma concentration (cMax) with TC50 5.6 µM, whereas the TC50 of trovafloxacin mesylate was around 23.7 µM and 35.7 µM in a study on micro-patterned primary human hepatocytes from 2 different donors [19]. In the same study another withdrawn drug, troglitazone was toxic in

one donor and negative in other donor, underscoring the problem of donor variability with primary human hepatocytes. Troglitazone was toxic in our study with a TC50 of 100 µM (Chapter 2). Though we used a small set of compounds to test in this study (4 negative and 8 DILI compounds), we emphasized that the repeated dosing is critical to accurately predict compound toxicity. Repeated (lower concentration) dosing might actually represent a more accurate representation of the mechanism of toxicity in humans compared to single high dose exposures on 2D *in vitro* models, which have a high incidence of false positives due to parent compound accumulation or other off-target effects. Further validation with a large set of compounds is required to more comprehensively and accurately assess the sensitivity of HepG2 spheroids in identifying DILI.

Toxicogenomics in drug development

Toxicogenomics could play a major role in lead discovery and reducing attrition of compounds at later stages of drug development. Mechanistic understanding of how molecules are involved in toxic injury could lead to identification of biomarkers that could serve as a reference to test new chemical entities. Reliable human *in vitro* models would be ideal for predictive toxicogenomic studies, allowing increased throughput, reduced animal testing and reduced costs. Though primary human hepatocytes are considered as 'gold standards' in *in vitro* screening assays [20], large inter-individual variability has also been seen in toxicogenomic-transcript profiling [21, 22]. Transcriptomic profiling has shown that HepG2 cells are better in discriminating genotoxic and non-genotoxic compounds compared to HepaRG cells [23]. With its stable non-proliferating phenotype HepG2 spheroids could be ideal for toxicogenomic studies. We have seen significant upregulation of many pathways associated with functional hepatocytes in HepG2 spheroids (Chapter 3). Xenobiotic metabolism pathways such as PXR/RXR, PPARα/RXRα are significantly activated in HepG2 spheroids. PXR induces the expression of CYP3A4, which is involved in metabolism of an estimated 60% of xenobiotics [24] other target genes include phase I enzymes CYP - 2B6, 2B9, SC8, 2C9, 2C19 and drug transporters MRP2, OATP2 [25]. PPARα involve in activation CYP4A family and CYP 1A, 2A, 2C, 2E sub families. Thus presence of active xenobiotic metabolism pathways may help in identifying genes associated with mechanistic toxic response in predictive toxicogenomic studies. Chapter 4 describes microarray gene expression profiles of HepG2 spheroids treated with diclofenac compared with *in vivo* mouse and rat models, 2D HepG2 cells, primary human hepatocytes and human liver slices. Molecular pathways associated with diclofenac induced hepatocyte injury such as mitochondrial dysfunction were activated in HepG2 spheroids, human liver slices and rat liver models, but not in other models. Previous studies have shown that the Nrf2 signaling pathway plays

an important role in diclofenac injury. Genes such as HO-1 in the Nrf2 pathway are important markers for hepatocellular injury. HO-1 gene up regulation was seen in *in vivo* models and HepG2 spheroids but not in HepG2 cells grown in monolayer cultures (Chapter 4) indicating enhanced functional activities of HepG2 spheroids similar to higher models. Cholestasis is observed in patients taking diclofenac [26]. Genes related to cholestatic liver disease were also significantly activated in HepG2 spheroids. In this study we have investigated transcript profiles from HepG2 spheroids exposed to a single dose of diclofenac. A detailed investigation with increasing dose and time points would likely provide further insight into the mechanisms leading to diclofenac-induced hepatocellular injury. The transcriptomic responses observed with a single dose of diclofenac indicate that, in many aspects, HepG2 spheroids show similarities in the stress response compared to human liver slices, primary human hepatocytes and *in vivo* models than its 2D counterpart.

Identification of key events and stress pathways involved in response to a toxic injury will provide information about important genes and proteins that could be used as indicators of a cytotoxic cascade. Early screening assays to identify these stress reporters would be valuable in drug discovery. Imaging based approaches such as High Content Screening (HCS) assays may complement compound prioritization strategies in pre-clinical drug screening.

High Content Screening (HCS) methods in preclinical drug development

Current *in vitro* assays measure cell death as an endpoint to assess the safety of compounds. Cell death may provide an estimation of a lethal dose, but it cannot represent complex molecular perturbations that occur during cell stress and injury. High content screening will allow us to visualize dynamic cellular responses upon xenobiotic stress and may provide an efficient evaluation of the compound's safety profile. With the advancement in automated imaging systems, HCS is now becoming compatible with HTS. Imaging systems are integrated with robotics and automated live cell imaging can be performed. In chapter 5, we describe the use of such an imaging platform to study the dynamic apoptotic and necrotic responses of mouse kidney proximal tubular epithelial cells (PTEC's) in real time after exposure to toxicants. PTEC's are susceptible to drug induced injury due to their re-absorption and secretory functions. Pro-inflammatory cytokine such as TNFα are induced as a result of nephrotoxicant insult to the kidney, which in turn aggravates the toxic injury [27, 28]. In this study, the dynamic role of TNFα in increasing apoptosis (Annexin-V) and necrosis (Propidium iodide) in mouse PTEC's was studied in real time. Cisplatin, cyclosporine A, tacrolimus and azidothymidine showed a synergistic apoptotic response to TNFα (Chapter 5). Though we could identify nephrotoxic compounds, 50% of the tested drugs did not show any signs of toxicity in this study. This may

largely be attributed to the lack of organotypic physiology in monolayer cultures, as most nephrotoxic drugs cause kidney injury by complex pathogenic mechanisms such as changes in intraglomerular hemodynamics, inflammation and crystalline nephropathy caused by pro-urine concentration [29], which will demand complex organotypic models for their detection at the very least. Additionally, lack of metabolic competence and species differences in these monolayer-cultured mouse PTEC's could also be a reason for decreased sensitivity. Utilization of complex human cell based 3D cell culture models, simulating physiological structure and function together with immune components are expected to improve the prediction of nephrotoxicity, but given the complexity of this organ, there is a long way to go before animal models can be completely replaced.

HCS is a new tool that was introduced to toxicology in recent years and none of the drugs that are currently on the market have passed through this type of screening assay. The pharmaceutical industry is now utilizing these assays at pre-clinical screening assays [30] and analyzing its potential to overcome current limitations in drug screening. With current improvements in integrating HCS with HTS, and feasibility to analyze multiple indicators of cell stress simultaneously, HCS would be a valuable tool in prioritizing new chemical entities.

CONCLUSION AND FUTURE PERSPECTIVES

To address the need for an *in vitro* model with improved functionality and stability for a longer period, we have developed a hydrogel based static 3D *in vitro* model using HepG2 cells. This model induces robust morphological and functional differentiation, with a strong induction of metabolic enzymes and transporters, many of which are poorly expressed in monolayer cultures. Functional bile canaliculi are formed in 3D HepG2 spheroid cultures. Comparative microarray gene expression analysis of our 3D HepG2 model with primary human hepatocytes, HepaRG and human liver revealed its close resemblance to human liver at the pathway level. The metabolic competence of HepG2 spheroids in hydrogels could be retained for up to 4 weeks, making repeated drug exposures feasible and repeated dosing on these spheroids was sensitive in identifying DILI compounds. Comparative transcriptomic profiling of diclofenac induced stress response mechanisms were similar in higher *in vivo* models and *in vitro* HepG2 spheroids. The assay we have developed is implemented in a 384 well format for low cost and increased throughput. This assay represents a novel and more physiologically relevant method for studying drug-induced liver injury.

In this model we used Matrigel, for re-differentiation of HepG2 cells into mature hepatocytes acquiring many specialized functions. Matrigel is a basement membrane matrix containing laminin (~60%), type IV collagen (~30%), entactin (~8%) and various quantities of decorin, transforming growth factor β1 and other

growth factors that help in cell attachment and differentiation [31]. Collagen type IV and laminin are present in developing and adult liver and are known to promote hepatocyte differentiation [32]. The exact mechanism involved in differentiation of HepG2 cells in Matrigel has to be further investigated. Cell surface integrins bind to laminins and collagens and induce differentiation [33] and we think this may be the first step in the differentiation of HepG2 cells in 3D culture. In this study we have seen a strong expression of β1-integrin on the basal surface of the polarized HepG2 spheroids. β1 integrin plays a crucial role in the early phases of development, [34] adhesion of β1 inegrin to Matrigel is evident in our observations. Further investigation on expression and role of other integrin binding sites on HepG2 spheroids might help in designing synthetic gels with tailored integrin binding domains for hepatocyte adhesion, growth and differentiation. If successful, this may circumvent the need of animal-derived Matrigel that has an undefined composition. Several studies have reported batch-to-batch experimental variation with Matrigel, but in our studies we did not see a significant difference in the spheroid morphology, gene expression (qPCR validation) or cytotoxic readouts with different batches.

With a defined cell density we were able to achieve a spheroid size of ~100 µm, which is important for proper gaseous exchange as spheroids above >200 µm cause hypoxia [35]. H&E staining of HepG2 spheroids (Chapter 2) show a cavity in the center of the spheroid. Though we did not investigate the presence of apoptotic or necrotic cells, histological examination suggests that the cavity may have some debris from extracellular matrix or apoptotic cells. Apoptosis is an intrinsic part of tissue remodeling. In 3D cultures of mammary epithelial cells, cavitation was shown to occur by apoptosis of cells in the interior of the spheroid [36]. Normally, apoptotic cells are cleared by macrophages, which are not present in our *in vitro* model. Necrosis can be induced by a lack of oxygen or nutrients. However, we believe that this is unlikely in HepG2 spheroids with optimal spheroid size.

The functional unit of the liver, the acinus is divided into 3 zones based on the distance from the arterial blood supply [37]. Zone 1, which is closest to the portal vein, receives the most oxygenated blood and hepatocytes in this region have functions that include gluconeogenesis, β-oxidation, amino acid catabolism and cholesterol synthesis. CYP450 metabolism, glycolysis, lipogenesis are mainly carried by zone 3 hepatocytes [37-39]. The increased expression of drug metabolism enzymes and genes involved in glycolysis, cholesterol synthesis etc. may indicate the presence of zonal gradients and functions in HepG2 spheroids. Oxygen levels in cell culture incubators have a significant impact on the functional activity of hepatocytes [40]. Normal monolayer cell cultures are exposed to 21% oxygen, whereas hepatocytes *in vivo* have oxygen levels of 8-9% in zone 1 and 3-5% in zone 3 [41]. Though we

did not measure the oxygen levels in HepG2 spheroids, the presence of ECM gel may have resulted in physiological oxygen concentrations and thereby increased functionality.

The successful application of an organotypic *in vitro* model in an industrial setup depends on its simplicity and compatibility to HTS assays. This HepG2 spheroid model stands out with several advantages compared to other spheroid models. The advantages include compatibility with regular microplates, use of easily available well-characterized HepG2 cells, which differentiate into a non-proliferating spheroid with a stable phenotype for longer periods. Spheroids from bioreactors or hanging drops need to be transferred to plates before performing an assay and they are no longer in an environment that induced the formation of spheroids, whereas in our model HepG2 spheroids are stably attached to the hydrogel for any downstream applications.

With a stable non-proliferating phenotype of HepG2 spheroids may answer the need for an *in vitro* model for sub-chronic toxicity studies. Our repeated dosing study for 7-days was sensitive in identifying all the hepatotoxic compounds tested, highlighting the importance of repeated dosing in toxicity screening assays. The increased sensitivity could be due to mechanistic toxic injury, for example bosentan, which induces cholestatic liver injury, was not toxic in a single exposure study in spheroids, but upon repeated dosing the toxicity was observed. Further studies with cholestatic endpoints are required to confirm our hypothesis. We have observed that the spheroids are viable with a polarized phenotype at least until 45 days (data not shown), which provides an opportunity for further long-term toxicity studies. The synthetic fluorescent bile acid uptake assay (chapter 2) that we have developed to assess inhibition of OATP1B3 transporter activity may also be used to screen drugs that obstruct bile flow leading to cholestatic liver injury. HepG2 spheroids could have a multitude of applications, which needs to be further explored.

Co-culturing HepG2 spheroids with other liver cell types may further improve its physiological relevance and identification of chemical entities where immune mediators or other factors secreted by non-parenchymal cells play a key role in aggravating liver injury. Co-culture of iPSC's (with human MSC's/HUVEC's) on Matrigel led to formation of liver buds *in vitro* [10]; it would be interesting to investigate if a similar level of differentiation and phenotypic characteristics could be achieved with HepG2 cells. iPSC derived hepatocytes were recently tested for safety assessment studies. iPSC's on 3D (nanopillar plate) were shown to perform better than 2D iPSC's and HepG2 cells in identifying hepatotoxic compounds [42], but the metabolic competence and sensitivity to hepatotoxic compounds was still significantly lower than PHH. iPSC's cells require special differentiation and selection methods, their ease of use and strengths are yet to be evaluated for successful application in the

pharmaceutical industry. The use of simple culture methods (10% FBS in DMEM) for HepG2 spheroid culture without the need of a proprietary or special media formulations as required for HepaRG and iPSC's culture will inevitably increase reliability and reproducibility and reduce cost of this model. In our experience, complex media formulations that included several growth factors to induce differentiation showed poor assay reproducibility (Kidney proximal tubule 3D Cell cultures, data not shown). DMSO a key factor in differentiation of HepaRG cells was also shown to induce 3-4 fold higher LDH and AST levels, reduction in proliferation and decreased hepatic functions [43]. Though this is a limitation for drug screening assays, previous studies and our observations have shown that HepaRG cell gene expression is more similar to PHH than HepG2 cells which may increase its predictive power in drug screening assays. But, it has to be also noted that HepG2 cells performed better in discriminating genotoxic compounds than HepaRG cells [21, 23].

Dis-advantages of our HepG2 spheroid model may include the use of animal origin ECM product, with undefined growth factors and batch-to-batch variability. The levels of CYP450 enzymes though higher are still significantly lower to those of PHH. There is an increased susceptibility to contamination during long-term maintenance of spheroids, which demands a careful attention in maintaining sterile conditions. Contamination during late stages of differentiation would cost significant amount of time in a scientific study. Future studies with improved synthetic biomaterials that could mediate cellular differentiation and serve as better 3D matrices have to be tested. Co-culturing HepG2 cells with other hepatic cells and studying their role in improving physiological characteristics would be interesting to analyze.

With several advantages and dis-advantages in each model a large validation screen on these models would help in identifying a better model in pre-clinical safety testing assays, which may help to decreasing drug attrition rates.

The pressure to develop predictive *in vitro* models not only comes from the pharmaceutical industry to decrease drug attrition but also from legislative pressure to ban animal testing. Recently, in March 2013, animal testing to evaluate human safety of cosmetic products and their ingredients was banned in the EU [44]. HepG2 spheroid model and other organotypic models could be used as an alternative to animal models after a thorough validation of their performance in various fields. Combination of –omic approaches (protein, miRNA, metabolomic) together with transcriptomic study would provide more insights into mode of action of a drug and discovering biomarkers. New assays that we have developed for testing ADME properties of new chemical entities and high-content screening methods to evaluate dynamic cellular stress responses are promising new tools for assessment of toxicity.

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