Minireview

In vitro platforms for evaluating liver toxicity

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Abstract

The liver is a heterogeneous organ with many vital functions, including metabolism of pharmaceutical drugs and is highly susceptible to injury from these substances. The etiology of drug-induced liver disease is still debated although generally regarded as a continuum between an activated immune response and hepatocyte metabolic dysfunction, most often resulting from an intermediate reactive metabolite. This debate stems from the fact that current animal and *in vitro* models provide limited physiologically relevant information, and their shortcomings have resulted in ''silent'' hepatotoxic drugs being introduced into clinical trials, garnering huge financial losses for drug companies through withdrawals and late stage clinical failures. As we advance our understanding into the molecular processes leading to liver injury, it is increasingly clear that (a) the pathologic lesion is not only due to liver parenchyma but is also due to the interactions between the hepatocytes and the resident liver immune cells, stellate cells, and endothelial cells; and (b) animal models do not reflect the human cell interactions. Therefore, a predictive human, *in vitro* model must address the interactions between the major human liver cell types and measure key determinants of injury such as the dosage and metabolism of the drug, the stress response, cholestatic effect, and the immune and fibrotic response. In this mini-review, we first discuss the current state of macro-scale *in vitro* liver culture systems with examples that have been commercialized. We then introduce the paradigm of microfluidic culture systems that aim to mimic the liver with physiologically relevant dimensions, cellular structure, perfusion, and mass transport by taking advantage of micro and nanofabrication technologies. We review the most prominent liver-on-a-chip platforms in terms of their physiological relevance and drug response. We conclude with a commentary on other critical advances such as the deployment of fluorescence-based biosensors to identify relevant toxicity pathways, as well as computational models to create a predictive tool.

Keywords: Drug-induced liver injury, liver on chip, hepatotoxicity, high content screening, predictive modeling

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Introduction

The liver is a central metabolizing organ and is susceptible to damage by chemicals and/or their metabolites that enter the body. Pharmaceuticals pose a particular risk leading to drug-induced liver injury (DILI), the cause of which is still debated. Hepatotoxicity is a major cause for drug withdrawals from the market, resulting in huge financial losses for pharmaceutical companies.[1–4] Several drugs, including troglitazone, nefadazone, and trovafloxacin have been withdrawn from the market due to their hepatotoxicity, while some drugs such as diclofenac and the over the counter drug acetaminophen are still in the market but pose a significant risk.[5,6] Current techniques for DILI assessment prior to pre-clinical trials include animal models and *in vitro* models using primary hepatocytes alone or in co-culture with other cell types – in 2D and 3D formats.[7–9] Though critical in providing initial assessment of drug toxicity, they are limited in some capacity to fully assess the broader responses leading to compound failure during clinical trials, or in the worst case, upon market release as a ''silent'' hepatotoxin forcing withdrawal. In order to reduce the attrition of compound failure due to DILI, it is essential to create *in vitro* models that can effectively

recapitulate liver response to evaluate predictable and unpredictable hepatotoxins over the breadth of genetically diverse human population.

Designing a liver platform for identifying DILI

The spectrum of DILI can be categorized by several classification methods, although liver injury often is noted simply in the clinic as hepatocellular jaundice or cholestatic liver disease (Table 1). DILI can manifest as all forms of acute and chronic liver disease, be dose related and predictable from animal preclinical studies, or, more often, not be dose related and unpredictable from animal trials. It is the latter type of compound that passes though animal safety studies as a ''silent'' hepatotoxin.[10] It is now hypothesized that infrequent hepatotoxicities are likely associated with an idiosyncratic immune response originating from the generation of reactive drug metabolites.[2,11]

A critical component of any *in vitro* model is the ability to evaluate and identify negative compounds. New molecular entities (NME) which have positive *in vitro* findings would be followed up with clinical investigation and further analysis using traditional methods; however, it is important that *in vitro* models provide information regarding the NMEs with a negative effect, where their full potential will be realized. A significant failure of most *in vitro* models is an inability to evaluate and predict all aspects of DILI. Historically, the goal of toxicity testing has been to simply rank order drugs/toxins as acute toxins within a large set of compounds, using simple cytotoxicity assays (live/dead dyes, LDH leakage, ATP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) in established cells lines such as HepG2, HepaRG, or rodent primary hepatocytes. However, this approach fails to meet the need to identify the idiosyncratic hepatotoxins that slip undetected past these simpler *in vitro* assays. As many forms of DILI originate from reactive metabolites generated by human specific metabolism, any platform must be constructed with metabolically competent human hepatocytes and non-parenchymal cells. This rules out the use of human cell lines which generally lack relevant levels of phase I metabolic activity, phase II conjugation, transporter functions related to drug clearance, or readily available rodent primary hepatocytes which may have different rates or routes of metabolic drug clearance.[12–14]

Hepatotoxicity can also be classed as predictable, for example, in cases of acute acetaminophen toxicity, or often as unpredictable, as exemplified by diverse

Table 1 Classifications of drug-induced human liver injury. (A color version of this table is available in the online journal.)

Classification	Pattern of liver injury	Example drug
Simple classification		
Intrinsic	Dose dependent, predicable, reproducible in animals at sub-lethal doses	Super therapeutic acetaminophen
Non-intrinsic allergic	No dose-dependency, unpredictable, not reproducible in animals	Phenytoin
Non-intrinsic non-allergic	Adaptive immune response, short latency period, delayed latency, absence of hypersensitivity response	Isoniazid
Clinical laboratory classification		
Autoimmune	Circulating antibodies	Tienilic acid
Hepatocellular	↑AST, ↑ALT, ↑Bilirubin	Acetaminophen
Cholestatic	↑ALP, ↑ Bilirubin	Chlorpromazine
Infiltrative	↑ALP	Tamoxifen
Histopathologic classification		
Acute hepatocellular injury	Spotty necrosis to fulminant liver failure (massive necrosis)	Acetaminophen, ketoconazole, diclofenac, nefazodone
Chronic hepatocellular injury	Pigment accumulation, steatosis, steatohepatitis, phospholipidosis, fibrosis, cirrhosis	Phenacetin, aspirin, valproic acid, amiodarone, methotrexate, tamoxifen
Acute cholestasis	Reduction in bile flow resulting from reduced secretion or obstruction	Amiodarone, chloroquine, methotrexate, vitamin A, cyclosporine, troglitozone, amoxicillin-clavulanate
Chronic cholestasis	Portal inflammation with degeneration of the bile duct (vanishing bile duct syndrome)	Tolbutamide, imipramine
Granulomatous hepatitis	Macrophage accumulation without necrosis located in periportal or portal areas.	Chlorpropamide, amoxicillin-clavulanate, cabbanazepine, diltiazem
Autoimmune hepatitis	Necroinflammatory lesions	Methyldopa, minocycline
Vascular lesions	Injury to sinusoids, hepatic veins, and hepatic arteries	Dacarbazine vincristine, azathioprine
Neoplastic lesions	Focal nodular hyperplasia and hepatocellular adenomas	Floxuridine, danazol

↑: increase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphotase.

compounds such as diclofenac, erythromycin, and ibuprofen to name a few. These latter examples, referred to as idiosyncratic toxins, have commonalities: (a) they are undetected in animal studies; (b) require human specific metabolism; and (c) often require a latency period from treatment to the appearance of the injury.[10] Many of the current 2-D and 3-D liver models in general can segment toxic from non-toxic compounds that act by direct toxicity to the hepatocyte, but often lack the necessary organization and cell types to properly address the idiosyncratic type response.

Liver platform cell types

Liver injury is a collective response between all or many of the resident liver cells, suggesting a minimal number of cell types must be present on the liver platform to fully elucidate most forms of DILI. In addition to hepatocytes, non-parenchymal cells in the liver associated with DILI pathology include Kupffer, sinusoidal endothelial, and stellate cells. The incorporation of primary cells or functionally responsive cell lines of these three important liver cells into liver platforms is an area of active investigation. The success of any liver platform depends critically on the use of fully competent hepatocytes. Despite recent improvements in coculturing methodology to prolong viability and functionality, and the availability of fresh and cryopreserved primary hepatocytes, their use is hampered by the finite supply from a single donor source. The best alternative to the single donor would be having cells from a renewable source. By far, the brightest hope for renewable cells is the embryonic stem cells (ESCs) or the adult-induced pluripotent stem cells (iPSCs) that can be matured into functional hepatocytes.[15] The iPSC-derived hepatocytes offer a unique opportunity to revolutionize pharmacological and toxicological assessment because of the potential to test cells from normal and diseased tissue, as well as from genetic and environmentally diverse adult humans. Despite the promise of renewable human hepatocytes from iPSC and ESC, the current protocols yield inefficient differentiation and maturation with low yields and heterogeneous cell populations retaining immature fetal liver characteristics.[16,17]

Current commercial *in vitro* approaches to liver toxicity testing

In vitro models and preclinical trials are essential tools for drug assessment required by regulatory agencies. However, the lack of human specific metabolism ultimately can lead to their failure to predict human DILI.[7–9] Human-based *in vitro* models comprising of microsomes, cell lines, primary hepatocytes, and liver slices[18–31] provide additional information to the existing animal models. However, they can be limited by poor stability, and, with the exception of precision cut liver slices, lack the hierarchy and structural components of liver. Monolayer cultures of primary hepatocytes are the most commonly used format for toxicity assessment and provide a suitable model for initial assessment, but are severely hindered by the lack of 3D organization, non-parenchymal cells, and thus cell–cell interactions via contact or paracrine effects. Nonetheless, isolated primary hepatocytes continue to be the most relevant system to study *in vitro* drug metabolism and hepatotoxicity and provide an initial assessment of drug toxicity and enzyme function. Important hepatic functions decrease rapidly after isolation, so only acute and short-term studies are possible. In addition to the use for drug toxicity assessment, and by virtue of having competent/relevant CYP 450 expression, mono cultures/monolayer cultures of primary hepatocytes are widely used for ''first pass'' liver clearance assessment as part of the pharmacokinetic (PK) evaluation.[32] A major innovation for primary hepatocyte cultures was the introduction of a matrix sandwich for hepatocytes which provides a platform to stabilize and increase the culture time of hepatocytes to up to seven weeks.[33–35] The presence of matrix on top and bottom of hepatocytes stabilizes the cells, acting as a scaffold which allows soluble factor secretion by hepatocytes into the local environment. Several ECM matrices, such as collagen (Type 1), Matrigel™, and poly-electrolyte layers have demonstrated the ability to stabilize hepatocytes for long-term culture.[36–39] In the last decade, several new approaches and advances have been introduced to improve the functional stability of long-term culture of hepatic cultures. In addition to the various *in vitro* models proposed so far, there is a growing interest in the pharmaceutical community, and Pharmaceutical Research and Manufacturers of America (PhRMA) and other regulatory agencies. Several recommendations have been made to compare the *in vitro* and *in vivo* studies, which need to be considered while developing and validating any *in vitro* platform.[40–42] Several design parameters, such as stability, CYP activity, metabolite formation, and reaction velocities should be validated for any *in vitro* platform.[40,43] In addition, the interpretation of *in vitro* data and *in vivo* extrapolation is very critical for the success of any platform, most of which have been described as per the recommendations of PhRMA.[40,42,44–49] Herein, we specifically review commercial macroscale and liver on a chip approaches for long-term culture of hepatocytes. A consistent theme of these novel commercial systems is the 3D organization of hepatocytes and support cells extend the hepatic-tissue cultures as a primary screening tool from several days to weeks.

Recently, Regenemed (San Diego, CA, USA) has demonstrated a liver tissue culture using transwell approach, culturing hepatocytes and non-parenchymal cells in a near-physiological ratio.[50] Initially, non-parenchymal cells are seeded in a nylon screen sandwich on a transwell insert (12 μm pore size) and stabilized for a week, followed by inoculation with hepatocytes allowing the formation of a 3D liver tissue (Figure 1a). Albumin, transferrin, fibrinogen secretion, and urea synthesis in both rat and human liver models were stable in 3D culture up to three months; additionally, the cultures exhibited stable CYP 3A4, 1A1, and 2C9 activity. Inflammatory response of the liver tissue was demonstrated by exposing cultures to LPS and measuring release of pro-inflammatory cytokines: IL-6, IL-8, TNF-α, IL-1β, and others. Finally, their human 3D liver tissue has been used to test drug toxicity (Table 2).

A new liver culture strategy commercialized by Insphero (Schlieren, Switzerland) is a 3D microtissue spheroid

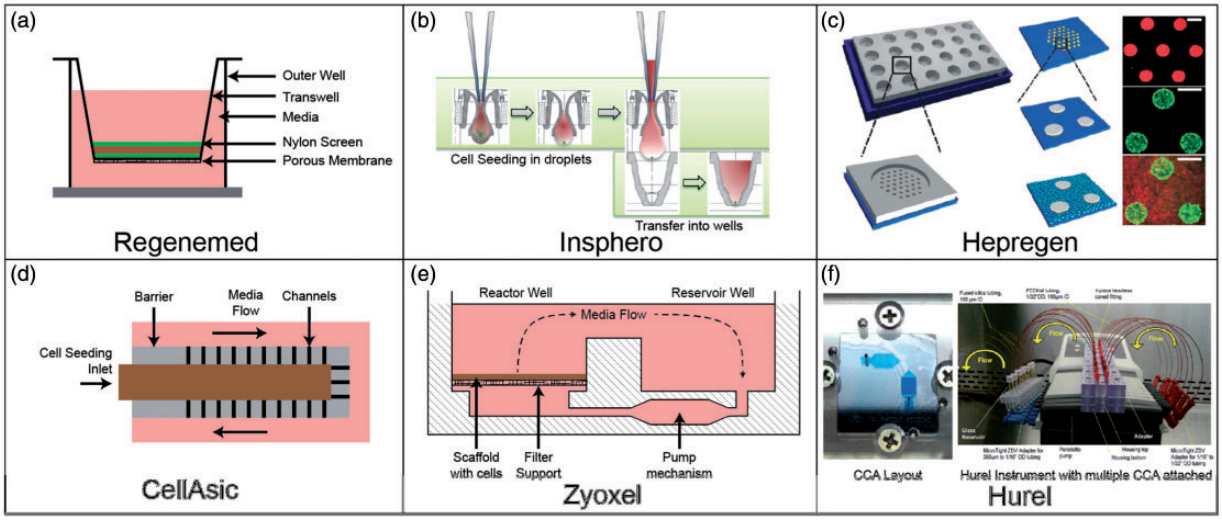

Figure 1 (a) Regenemed strategy for liver tissue culture. Non-parenchymal cells are introduced into the nylon scaffold followed by inoculation with hepatocytes (adapted from Kostadinova et al.[50]). (b) Hanging drop strategy by Insphero. Hepatocytes and non-parenchymal cells are introduced into the drop and allowed to form microtissue that is transferred into a 96 well plate and cultured. (c) Hepregen micropattern strategy used elastomeric molds to pattern hepatocytes followed by addition of 3T3-J2 cells. (d) CellAsic device structure uses a microstructure pattern to shield the hepatocytes from flow, mimicking an endothelial-like layer (adapted from Lee et al.[62] and Zhang et al.[63]). (e) The Zyoxel platform consists of two reservoirs, with cells in one and media for re-circulation in the other (adapted from Domansky et al.[70]). (f) The Hurel Platform incorporates a Cell Culture Analog (CCA) with cell seeding area with multiple devices in parallel. (A color version of this figure is available in the online journal.)

Table 2 Assays and markers reported in primary literature to address physiological mechanisms of toxicity in multi-component liver platforms that include human hepatocytes

MOT	InSphero 3D Insight Human Liver 3D Microtissues Hepatocytes, primary NPC*	Hepregen Micropatterned attachment: Hepatocytes, 3T3 fibroblasts	CellAsic HepG2, endothelial cell-like barrier	Hurel Hepatocyte, primary human lung microvascular endothelial cells	Regenemed
Oxidative stress		Glutathione levels[64]			Glutathione[50,65]
Macromolecular interactions (reactive metabolites, covalent binding)		Clearance[64] Metabolites[55,56]		Clearance, Metabolism[66,67]	Clearance, cyp induction/inhibition[65]
Mitochondrial function, respiration, permeability, Calcium Flux	Intra-tissue ATP[68†]	ATP[54†], MTT[54⋆]			ATP[65†]
BSEP – canalicular flow	IHC (BSEP)[68]	IHC (MRP2, Zo1)[54] CMFDA[55]		CMFDA[66]	
Immune stress	IL-6 release after LPS stimulation[68]				LPS stimulation, cytokine profile[65]
Protein synthesis inhibition (multiple levels)	Albumin[68]	Albumin, urea secretion[55]	Albumin[69]		Albumin, urea, fibrinogen, transferrin[65]
Drugs evaluated	Diclofenac, Acetaminophen, Trovafloxacin	Alprazolam, Atazanavir, Atomoxetine, Diazepam, Diclofenac, Flecainide, Glimepiride, Lidocaine, Meloxicam,	Diclofenac	Caffeine, Buspirone, Imipramine, Timodol, Sildenafil, Metoprolol, Carbamazepine, Antipyrine	Fenofibrate, Troglitazone, Trovafloxacin, Lovafloxacin, Pioglitazone, Acetaminophen

(continued)

Table 2 Continued

MOT	InSphero 3D Insight Human Liver 3D Microtissues Hepatocytes, primary NPC*	Hepregen Micropatterned attachment: Hepatocytes, 3T3 fibroblasts	CellAsic HepG2, endothelial cell-like barrier	Hurel Hepatocyte, primary human lung microvascular endothelial cells	Regenemed
		Midazolam, Prednisolone, Riluzole, Risperidone, Theophylline, Tolbutamide, Atomoxetine, Trypan Blue, Voriconazole, others[3,4,8]			

*NPC – non-parenchymal liver cell.
†ATP used as marker of cell viability.

culture using a gravity-enforced cellular assembly, enabling formation of cellular contacts.[51] Briefly, hepatocytes and non-parenchymal cells are introduced into a hanging drop in a specifically designed multiwell plate which forms a microtissue spheroid in three days (Figure 1b). After formation, the spheroids can be transferred into a spheroid-specific 96 well plate and cultured for up to five weeks with stable functions. Further, staining the microtissues revealed maintenance of cellular phenotype of endothelial and Kupffer cells within the spheroids. Toxicity assays with acetaminophen and diclofenac show better TC_{50} values when compared with 2D cultures.

Another approach is the co-culture of discrete micropatterned hepatocyte islands surrounded and stabilized by stromal cells (3T3-J2 fibroblasts) and this approach is commercially available as Hepatopac (Hepregen, Medford, MA, USA).[52–56] The system uses a standard 24 well plate format with reusable elastomeric molds to pattern 500 μm diameter hepatocyte islands surrounded by the stromal cells (Figure 1c). The co-culture system maintained its function for up to six weeks, and had exhibited stable albumin secretion, urea synthesis, Phase I and II drug metabolism, and formation of canaliculi networks.

Liver on a chip approaches

Microfabrication techniques enable design of bio-mimetic liver systems with physiological hepatocyte density and architecture, which allow precise control of media flow rates, mass transport, and oxygen gradients (zonation).[14,57–59] Soft lithography allows the creation of *in vivo*-like geometries enabling hepatic cultures with (a) 3D tissue microarchitecture and (b) cell/nutrient ratio similar to the liver. Furthermore, these dynamic microsystems allow precise control of media flow for supplying fresh nutrients and removal of waste products. A striking feature of the liver is the variation of metabolic activity of hepatocytes depending on oxygen availability along the liver acinus (which lies between two adjacent portal triads and portal veins).[60,61] Precise control of architecture and media flow offers the opportunity to create oxygen gradient in hepatic cultures, enabling the development of *in vitro* platforms to study zonation.

A microfluidic liver sinusoid model by CellAsic (Hayward, CA, USA) uses lithography techniques to create an artificial endothelial-like barrier to mimic the porous liver sinusoid.[62,63] The device eliminates the need for endothelial cells by constructing a structural barrier (with posts) that shields hepatocytes from media stress and simultaneously allows nutrient exchange (Figure 1d). The design allows a nutrient flow of ~100 pL/s and can support ~250 cells. The device demonstrates high cell viability up to seven days under perfusion conditions, and response to drugs (Table 2). This design is multiplexed into convenient 96 well plate formats containing 32 devices, with gravity-based flow for ease of use. Although the device is effective in mimicking the sinusoidal barrier function of the liver, other cell functions, such as synthesis, detoxification, and drug metabolism need to be established.

A multi-well plate platform by Zyoxel (Oxfordshire, UK)[70] incorporates hepatocytes with non-parenchymal cells and media flow induced by a pneumatic controlled underlay. The bioreactor is made of polystyrene with two connected chambers, a media reservoir and a reactor chamber, with poly-carbonate scaffolds for cell culture (Figure 1e). The design aims to create an environment similar to the liver in terms of fluid flow, oxygen gradient, and shear stress. Perfusion up to 1.2 mL/min is achieved by pumping the media between the reservoirs using pneumatic inputs at the bottom of the chambers; achieving an oxygen concentration similar to a sinusoid (145 μM to 50 μM at a flow of 0.25 mL/min). Hepatocytes, in co-culture with LSEC enriched non-parenchymal fractions, are seeded on the scaffolds within the reactor well which are maintained for up to 13 days with high viability and phenotype retention. In spite of mimicking liver environment, the Zyoxel system is not conducive to imaging (due to scaffold), and the non-parenchymal cells are a LSEC-enriched fraction which needs further characterization.

Another approach by Hurel (Beverly Hills, CA, USA)[71] adapts a microfluidic microscale cell culture analog (μCCA)

that can incorporate multiple tissues to interact in a physiologically based pharmacokinetic model. The Hurel platform can accommodate multiple CCA units each consisting of a Hurel plastic biochip with connections to a fluid reservoir and a pump to complete the circuit (Figure 1f), offering the ability to run multiple CCAs in parallel. Initial short duration studies with hepatocytes in the devices have shown high-density cell seeding, viability, and metabolic functionality under flow conditions for 24 h. Hepatic co-culture system, with non-parenchymal cells within the device, shows high-viability after seeding and *in vivo* like clearance for various drugs up to eight days.[72]

The commercial liver-on-a-chip platforms described earlier provide interesting solutions for culturing hepatocytes under flow conditions for 1–2 weeks. Although these on-chip models provide useful insights for creating liver-mimics, additional studies to evaluate important liver functions (albumin secretion, urea excretion, drug toxicity) are needed to realize their full potential.

Tools for evaluating liver platform toxicity responses

The ultimate implementation of a human predictive cell-based liver platform must be capable of identifying all or most DILI pathology. Platforms that achieve this will most likely have several commonalities including the use of a microfluidic-based chamber with as many competent resident liver cell types as necessary to reproduce the adaptive or adverse injury response, support generation of human specific reactive metabolites, and have the ability to measure time and dose-dependent multi-cellular stresses, either by non-invasive image-based mechanism of toxicity (MOT) pathway analysis or by analysis of secretion products. The platforms will need to be tested and validated against clinical drugs at relevant concentrations. Finally, the results must be linked to a database annotated with pre-clinical, clinical, molecular, and additional cell-based data for modeling a predictive DILI signature.

Mechanisms of toxicity

Drugs or their reactive metabolites have been linked to liver injury through a variety of MOTs including oxidative stress, covalent binding of reactive metabolites to macromolecules, changes in intracellular calcium flux, mitochondrial respiration dysfunction, inhibition of the Bile Salt Exporter Protein (BSEP), and other transport proteins, stimulation of autoimmunity, protein synthesis inhibition, and fluid or ion imbalance.[73,74] Table 2 presents a comparison of the toxicity and cell viability parameters of selected commercial platforms. We now know that measurements of MOTs have proven to be only partly successful in our ability to predict the full expression of liver injury, but there is a consensus as to the reason for the modest success. Drug-induced injury can end in two possible fates. The first is the development and progression in severity and duration to a pathologically significant or even potentially fatal lesion, whereas, the second is adaptation, a not-well understood process whereby the liver injury abruptly disappears, even though drug treatment continues.[75] It is the adaptive response to injury that provides a reasonable explanation for the modest level of concordance between MOT-based analysis and DILI prediction. More sophisticated liver models including a full complement of liver non-parenchymal cells may provide deeper insight into the mechanisms behind the adaptive response.[76]

In recent years, led by the case for BSEP inhibition produced liver injury gaining significant clinical concordance,[77] our understanding to detect any drug-inhibited transport of drugs on other transport proteins such as MRP1, MRP2, and the link to inflammatory responses is gaining significance.[74,78] The more useful *in vitro* liver platforms would allow evaluation of drug effect on multiple transport protein function in addition to BSEP which should contribute to the understanding of mechanism and prevention of drug-produced liver injury. Drug metabolism-produced reactive metabolites that lead to liver injury has been noted with compounds such as acetaminophen, chloramphenicol, danazol, diclofenac, flutamide, ibuprofen, imipramine, indomethacin, isoniazid, hydralazine, nitrofurantoin, piroxicam, procainamide, sulphamethoxazole, tacrine, and tamoxifen.[79] In consideration that over 60% of the drugs that have been taken off the market for hepatotoxicity have been shown to produce reactive metabolites, the metabolic activity of the liver is a necessity.[80,81]

Finally, the liver models should be capable to measure basic first pass drug clearance of compounds as part of the pharmacokinetic (PK) predictions. However, most current models are stand-alone, consisting of hepatocytes and liver support cells, and, lacking the gastrointestinal transport/metabolism systems, use of the stand-alone liver as a PK model should be approached cautiously. Orally administered drugs must pass first through the gut and liver before reaching the systemic circulation, so criteria such as bioavailability, transporter function, GI metabolism, and even the gut biome impact systemic drug availability ahead of any additional first pass hepatic loss.[50,82]

Fluorescent probes and biosensors

An important element of the liver platform approach is the ability to collect and interpret physiological changes in response to drugs, toxins, or environmental cues in real-time as well as over time to capture acute and chronic effects. The ability to monitor intracellular changes and cell–cell interactions in a quantitative, real-time method is predicted to improve determination of cell viability and early toxicity signatures of individual cells.[83,84] Fluorescent molecules (probes) and protein-based fluorescent biosensors are powerful tools for the reporting of spatiotemporal dynamics.[85] Fluorescence-based probes that are targeted to particular substrates and subcellular compartments are widely used for live cell studies.[85] Protein-based fluorescent biosensors aim to detect real-time and molecular specific changes in time and space by combining fluorescent dyes or fluorescent proteins to peptides/proteins that sense chemical/molecular changes.[25,26,85–88] The initial protein-based fluorescent biosensors were native proteins

covalently labeled with fluorescent dyes and incorporated into living cells. This original method was named ''fluorescent analog cytochemistry''[27] and paved the way to using genetically engineered biosensors.[25,28,89] Protein-based fluorescent biosensors are key tools for light microscopy and high content screening (HCS)[25,26] and enable monitoring and measuring changes in the intracellular distribution, as wells as protein modifications such as conformational change, translocation, ligand binding, analyte changes, and post-translational modifications.[89–91] The first use of GFP spawned a large supply of derived fluorescent proteins that span the spectrum of visible to far-red wavelengths, all of which are genetically encodable.[28,92] The abundant research efforts to understand and further improve fluorescent proteins led to the extension of their applications into fluorescence-based protein biosensors.[92,93] Biosensors have been built from a wide range of proteins with inherent fluorescent chromophores (XFPs) and extrinsic chromophores such as small molecule sensors such as aptamers[94] and fluorophore-binding proteins.[95,96] Intrinsic fluorescent biosensors based on fluorescent proteins have a variety of architectures and design. They are used to monitor enzymatic activities such as protein complementation,[97] translocation,[89] protein modification,[98,99] and the presence of intracellular ions.[100] Enzymatic and post-translational modifications have used two different covalently linked fluorescent proteins to monitor Förster resonance energy transfer (FRET), such that the loss or gain of energy transfer is ratiometrically related to the presence of modified proteins. Notably was the co-discovery of the first fluorescent protein calcium sensors.[101,102] Protein engineering approaches and biophysical studies have also contributed to improvements in spectral variants of GFP and modified structures and new ways to use a chromophore.[103,104] Novel engineering via artificial truncation of GFP near the chromophore that are then fused, resulting in new N and C termini, are known as circular permutations (cpGFP).[105] The insertion of protein domains near the chromophore allow for conformation dependent and ratiometric signaling changes. The basis of this rational design concept has resulted in biosensors that can monitor calcium flux, signaling ions, and reactive molecules such as reactive oxygen species.[106,107] Biosensors to identify MOT and other indicators of liver injury can be inserted into the resident liver cells on the liver platforms constructed with HCS measurement capacity.

HCS approach to measuring hepatotoxicity

HCS permitted the moderate throughput of cell-based assays in drug discovery and development.[89,108] Evaluation of DILI using multi-parameter cell feature analysis and measured by fluorescence imaging was introduced by Haskins et al.[109] and was implemented by O'Brien et al.[110] and later extended by Xu et al.[84] using HCS. The first commercial assay to predict hepatotoxicity was introduced in 2007 as CellCiphr™ Profiling (introduced by Cellumen, now offered by Cyprotex, Macclesfield, UK). The latter is a predictive toxicity analysis built on multi-parametric HCS-based fluorescent probe measurements collected in a hepatoma cell line and primary rodent hepatocytes and validated against a large number of hepatotoxic and non-hepatotoxic compounds. A predictive risk assessment is calculated using a classifier model comparing the *in vitro* cell signatures against a database of animal pre-clinical toxicity information.[111] Overall, the combined use of multi-parametric *in vitro* assays from monolayer liver cell cultures and classification raised the predictive success of taking any compound into clinical development from a random 50%, which is the predictivity of animal pre-clinical studies, to better than 70%.[83] Collectively, these efforts established that HCS multiplexed data measurements coupled to computer driven analysis can translate to useful predicitve models.

''Omics – Measurements''

Ever since the introduction of microarray genomics technology over two decades ago, ''omics'' platforms have expanded to include three core technologies applied to toxicology: genomics (also referred to as toxicogenomics or transcriptomics); proteomics; and metabolomics, to assess biomolecule changes in tissue or blood and urine. The ''genomics'' array platforms include mRNA transcripts, DNA methylation patterns, single nucleotide polymorphism levels in tissues and cells, and microRNA (miRNA) in tissues, cells, or body fluids. Proteomics is capable of finding changes in protein expression in tissues or body fluids while metabolomics evaluates the changes in endogenous and xenobiotic metabolites secreted into blood or urine. The pioneering studies in toxicogenomics[112] demonstrated a link between specific gene-expression profiles/signatures and specific MOTs, which led to the research strategy of the National Center for Toxicogenomics (NCT) at the NIEHS to relate gene expression fingerprints to specific adverse effects demonstrated by conventional clinical chemistry and histopathology of toxicity markers.[113] In 2011, the EU Framework Project published the results and conclusions of a consortium of 15 pharmaceutical companies, 2 small companies, and 3 universities which evaluated the transcriptomics, proteomics, and metabolomics results from 16 compounds dosed in two-week rat studies.[114] The project concluded that whole organ total RNA extractions for transcriptomic analysis could generate mechanistic hypotheses when a histopathologic lesion was evident, but that proteomics and metabolomics were limited to being supportive of these findings. As it was not the goal of the study to determine if any of the ''omics'' platforms could be used as an independent predictive tool, and, indeed, a 16 compound study is too small for such determination, it was evident from the study that the use of invasive tissue transcriptomics still requires traditional histopathology to deliver the best results. Although the number of active investigations in ''omics'' based profiling continues to increase, as yet no consensus has been reached on which platform technology or biomarkers should be universally applied.

Selection of validation compounds and testing concentrations

Liver toxicity has had a staggering impact on the pharmaceutical industry. Globally 5–10% of all adverse drug reactions result from liver toxicity, with over 1000 drugs reported to have potential liver toxic effects and a third of all post-market drug withdrawals were for unacceptable levels of liver toxicity.[30,31,115] Thus, a large selection of clinical compounds is available for testing and validating liver platforms. Table 3 provides an example list of 61 compounds that are chemically diverse and independent of therapeutic intent. The 30 ''liver toxic'' drugs on this example list were selected by virtue of having been withdrawn from clinical use due to hepatotoxicity or are in use but carry the ''black-box'' listing or warning labels for hepatotoxicity. If the goal of testing is to validate and train a computational tool for predictive modeling, then a large number of inactive compounds are also needed. Continuing in the example, the final compounds in Table 3 were selected by virtue that they have no effect on the liver, although some exhibit other organ toxicity, or have no clinically relevant toxicity. The list additionally includes matched pairs of compounds that are structurally related, have the same therapeutic intent, but upon entry into the broad marketplace, one of the paired compounds was found to be hepatotoxic. In addition, the screening concentration used for *in vitro* hepatotoxicity testing is most often selected to be 100 times the known or anticipated peak plasma level (C_{max}) or to 100 μM if the C_{max} is not known or cannot be estimated. That concentration limit was determined in 2008 when Xu et al. screened 300 compounds and

Table 3 Example of clinical compounds selected for validating liver platform toxicity responses

Non-Liver Toxic Drugs	Clinical Cmax (μg/mL)	Predominant Clearance route	Hepatotoxic drugs	Clinical Cmax (μg/mL)	Predominant Clearance route	Reactive metabolite
Amantadine HCl	0.65	Renal	Acitretin	0.61	–	No
Amiloride HCl	0.02	Renal, bile mixed	Alpidem**	0.065	–	Yes
Amitriptyline HCl	0.029	Renal	Benoxaprofen	0.775	–	Yes
Atenolol	1.33	Renal, bile mixed	Benzarone	2.3	–	Yes
Bupivacaine	0.067	Renal	Bosentan	0.082	Bile	–
Buspirone	0.00192	Renal	Bromfenac	9.2	–	Yes
Cimetidine	1.14	Renal	Chlormezanone	2.9	–	No
Clotrimazole	0.03	Bile	Cinchophen	4.5	–	No
Entacapone*	1.83	Bile	Dacarbazine	28.6	–	Yes
Famotidine	0.104	Renal	Dantrolene	1.24	Bile	Yes
Fluvastatin	0.273	Bile	Diclofenac	2.4	Renal	–
Gabapentin	2.474	Renal	Felbamate	0.0196	–	Yes
Gatifloxacin	4.35	Renal	Flutamide	0.1	–	Yes
Glimepiride	0.551	Renal, bile mixed	Gemtuzumab	2.86	–	Yes
Ibuprofen†	30.9	Renal	Glafenine	0.7	–	Yes
Levofloxacin	5.7	Renal	Ibufenac†	120∼	–	Yes
Lidocaine	8.5	Bile	Isonazid	10.5	–	Yes
Lovastatin	0.01	Bile	Ketoconazole	0.06	Renal, bile mixed	Yes
Montelukast	0.38	Bile	Methotrexate	0.351	Renal	–
Moxifloxacin‡	4.5	Renal, bile mixed	Naltrexone	0.02	–	No
Nadolol	0.13	Bile	Nefazodone§	0.4349	–	Yes
Pamidronate	2	Renal	Nevirapine	7.88	Renal	Yes
Paroxetine	0.02	Bile	Pemoline	4.5	–	No
Pilocarpine	0.0205	Renal	Pirprofen	2.8	–	Yes
Raloxifene	0.0005	Bile	Sulindac	11.4	Renal, bile mixed	–
Ranitidine	0.5	Renal	Tienilic acid	57	–	Yes
Rosiglitazone	0.373	Renal, bile mixed	Tolcapone*	6	Renal	Yes
Sertraline	0.0245	Bile	Troglitazone	2.82	–	Yes
Simvastatin	0.01	Bile	Trovafloxacin‡	2.09	–	No
Trazodone§	3.12	Renal	Valproic acid	7	Renal	Yes
Zolpidem**	0.12	Renal				

*Matched liver clean/liver toxic drug pair: entacapone/tolcapone.
†Matched liver clean/liver toxic drug pair: ibuprofen/ibufenac.
‡Matched liver clean/liver toxic drug pair: moxifloxacin/trovofloxacin.
§Matched liver clean/liver toxic drug pair: trazadone/nefazodone.
**Matched liver clean/liver toxic drug pair: zolpidem/alpidem.

published the $100 \times C_{max}$ as a ''reasonable'' level to separate a potential DILI compound from non-liver toxic compounds[84] and that value has since entered the *in vitro* toxicology lexicon.

Database and predictive modeling

The key to evaluating the performance of any model is the availability of a sufficiently large ''truth'' data-set to develop and validate the predictive signatures. In the case of liver toxicity, this would ideally include human clinical and detailed mechanistic toxicology for a set of compounds at least as large as the list in Table 3. Currently, such toxicity data are widely dispersed and often not sufficiently annotated or fully accessible for computational use. To address this need, the US Food and Drug Administration (FDA) is compiling the Liver Toxicity Knowledge Base (LTKB).[116] The project involves the collection of diverse data (e.g. DILI mechanisms, drug metabolism, histopathology, therapeutic use, targets, side effects, etc.) associated with individual drugs and the use of systems biology analysis to integrate these data for DILI assessment and prediction. In a similar effort, the National Library of Medicine (NLM) and the National Institute of Diabetes and Digestive and Kidney Diseases have established the LiverTox website (Livertox.nih.gov, 2013). LiverTox provides up-to-date, comprehensive and unbiased information about DILI caused by prescription and nonprescription drugs, herbals, and dietary supplements as a mixture of text and data with extensive references. PharmaPendium (Elsevier, New York, NY, USA) is a commercial source of excerpted preclinical, clinical, and post-release safety data in a single longitudinal database with searchable pages of FDA approval packages. Although both LiverTox and Pharmapendium provide extensive and valuable information on drug safety, the data are organized for human interpretation and generally requires some reorganization for computational modeling. In addition to these drug-focused resources, there are safety databases focused on other classes of compounds such as the Environmental Protection Agency's ACToR, the Aggregated Computational Toxicology Resource,[117] and the Toxin and Toxin-Target Database (T3DB).[118]

The *in vitro* human liver models, and in particular the microfluidic liver models provide a significant new opportunity to understand drug toxicity at the cellular and molecular levels. The databases cited earlier, while providing a wealth of information on human adverse effects, have limited information on the cellular functions and molecular markers leading to those reactions. To optimally mine the rich data generated from these models will require new databases to capture the detailed functional and molecular data and computational tools to associate that data with the preclinical, clinical, and post-release safety data. Promising results have already been demonstrated. Improved DILI prediction over animal models was demonstrated using a simple *in vitro* hepatocyte assay and classifier,[84] and an *in silico* SAR model that relates chemical structures to the liver side effect data was demonstrated in the LTKB with a high degree of accuracy.[119] New databases of cellular and molecular safety data from sophisticated organ models, combined with computational toxicology tools that integrate that data with in silico models of chemical-target interactions, are expected to greatly enhance the ability to predict *in vivo* human drug effects.[120,121]

Conclusion

In the past two decades, there has been significant progress in the development of *in vitro* liver models including commercially available models. While macro-scale approaches have significant impact on developing models with parenchymal and non-parenchymal cells, the liver-on-a-chip provides a scale-down strategy for recreating tissue microenvironment. Building a predictive liver platform will require competent liver cell types in physiologically relevant organization within a microfluidic platform to mimic sinusoid-like media flow to deliver nutrients, oxygen, drugs, and drain metabolic waste processes and drug metabolites. Further, several analysis techniques need to be incorporated into these platforms to not only monitor the function of these devices, but also provide information for *in vitro–in vivo* correlation, which is essential for successful identification of DILI and its mechanisms. An essential feature of such valuable screening tool is that all parts of the system need validation to the robustness and reproducibility for low dose exposure over extended lengths of time. The platform should be able to identify many of the ''silent'' hepatotoxins that manifest after clearing pre-clinical and even some phases of clinical trials. The test system should be designed to capture real-time data that can provide a critical understanding of the MOT's that trigger hepatotoxicity and the integrated cellular system response that dampens or amplifies the effect so that the former leads to an adaptive response and the latter to a pathological injury. Finally, the use of computer modeling to link the experimental MOT and cellular effect data to pre-clinical and clinical experience of known drugs will provide a mechanistic tool for predictive assessment of unknown test articles.

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REFERENCES

1. Schuster D, Laggner C, Langer T. Why drugs fail – a study on side effects in new chemical entities. *Curr Pharm Des* 2005;**11**:3545–59
2. Maddrey WC. Drug-induced hepatotoxicity: 2005. *J Clin Gastroenterol* 2005;**39**:S83–9
3. Wilke RA, Lin DW, Roden DM, Watkins PB, Flockhart D, Giacomini KM, Krauss RM. Identifying genetic risk factors for serious adverse drug reactions: current progress and challenges. *Nat Rev Drug Discov* 2007;**6**:904–16
4. Blomme EaG, Yang Y, Waring JF. Use of toxicogenomics to understand mechanisms of drug-induced hepatotoxicity during drug discovery and development. *Toxicol Lett* 2009;**186**:22–31

5. Kaplowitz N. Acetaminophen hepatoxicity: what do we know, what don't we know, and what do we do next? *Hepatology* 2004;**40**:23–6
6. Mitchell JR, Jollow DJ, Potter WZ, Davis DC, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J Pharmacol Exp Ther* 1973;**187**:185–94
7. Fourches D, Barnes JC, Day NC, Bradley P, Reed JZ, Tropsha A. Cheminformatics analysis of assertions mined from literature that describe drug-induced liver injury in different species. *Chem Res Toxicol* 2010;**23**:171–83
8. Greaves P, Williams A, Eve M. First dose of potential new medicines to humans: how animals help. *Nat Rev Drug Discov* 2004;**3**:226–36
9. Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, Lilly P, Sanders J, Sipes G, Bracken W, Dorato M, Van Deun K, Smith P, Berger B, Heller A. Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regul Toxicol Pharmacol* 2000;**32**:56–67
10. Kaplowitz N. Idiosyncratic drug hepatotoxicity. *Nat Rev Drug Discov* 2005;**4**:489–99
11. Roth RA, Ganey PE. Intrinsic versus Idiosyncratic drug-induced hepatotoxicity — two villains or one? 2010;**332**:692-7
12. Di L. The role of drug metabolizing enzymes in clearance. *Expert Opin Drug Metab Toxicol* 2014;**10**:379–93
13. 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* 2013;**8**:e59432.
14. LeCluyse EL, Witek RP, Andersen ME, Powers MJ. Organotypic liver culture models: meeting current challenges in toxicity testing. *Crit Rev Toxicol* 2012;**42**:501–48
15. Yi F, Liu GH, Izpisua Belmonte JC. Human induced pluripotent stem cells derived hepatocytes: rising promise for disease modeling, drug development and cell therapy. *Protein Cell* 2012;**3**:246–50
16. Subba Rao M, Sasikala M, Nageshwar Reddy D. Thinking outside the liver: induced pluripotent stem cells for hepatic applications. *World J Gastroenterol* 2013;**19**:3385–96
17. Shan J, Schwartz RE, Ross NT, Logan DJ, Thomas D, Duncan SA, North TE, Goessling W, Carpenter AE, Bhatia SN. Identification of small molecules for human hepatocyte expansion and iPS differentiation. *Nat Chem Biol* 2013;**9**:514–20
18. Zguris JC, Itle LJ, Hayes D, Pishko MV. Microreactor microfluidic systems with human microsomes and hepatocytes for use in metabolite studies. *Biomed Microdevice* 2005;**7**:117–25
19. Emoto C, Murase S, Iwasaki K. Approach to the prediction of the contribution of major cytochrome P450 enzymes to drug metabolism in the early drug-discovery stage. *Xenobiotica* 2006;**36**:671–83
20. Bovenkamp MVD, Groothuis GMM, Meijer DKF, Olinga P. Precision-cut fibrotic rat liver slices as a new model to test the effects of anti-fibrotic drugs in vitro. *System* 2006;**45**:696–703
21. Olinga P, Schuppan D. Precision-cut liver slices: a tool to model the liver ex vivo. *J Hepatol* 2013;**58**:1–8.
22. van de Bovenkamp M, Groothuis GMM, Draaisma AL, Merema MT, Bezuijen JI, van Gils MJ, Meijer DKF, Friedman SL, Olinga P. Precision-cut liver slices as a new model to study toxicity-induced hepatic stellate cell activation in a physiologic milieu. *Toxicol Sci: Official J Soc Toxicol* 2005;**85**:632–8
23. Vickers AEM, Saulnier M, Cruz E, Merema MT, Rose K, Bentley P, Olinga P. Organ slice viability extended for pathway characterization: an in vitro model to investigate fibrosis. *Toxicol Sci: Official J Soc Toxicol* 2004;**82**:534–44
24. Stieger B. In vitro techniques: isolated organ perfusion, slices, cells and subcellular elements. In: Rodes J (ed.) *Textbook of hepatology*. MA, USA: Blackwell Publishing, 2007
25. Gebhardt R, Hengstler JG, Muller D, Glockner R, Buenning P, Laube B, Schmelzer E, Ullrich M, Utesch D, Hewitt N, Ringel M, Hilz BR, Bader A, Langsch A, Koose T, Burger HJ, Maas J, Oesch F. New hepatocyte in vitro systems for drug metabolism: metabolic capacity and recommendations for application in basic research and drug development, standard operation procedures. *Drug Metab Rev* 2003;**35**:145–213
26. Guillouzo A. Liver cell models in in vitro toxicology. *Environ Health Perspect* 1998;**106**:511–32
27. LeCluyse EL. Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. *Eur J Pharm Sci* 2001;**13**:343–68
28. Sivaraman A, Leach JK, Townsend S, Iida T, Hogan BJ, Stolz DB, Fry R, Samson LD, Tannenbaum SR, Griffith LG. A microscale in vitro physiological model of the liver: predictive screens for drug metabolism and enzyme induction. *Curr Drug Metab* 2005;**6**:569–91.
29. Hewitt NJ, Lechon MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM, Hengstler JG. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* 2007;**39**:159–234
30. Dunn JC, Tompkins RG, Yarmush ML. Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. *Biotechnol Prog* 1991;**7**:237–45
31. Bjornsson E. Drug-induced liver injury: Hy's rule revisited. *Clin Pharmacol Ther* 2006;**79**:521–8
32. Chiba M, Ishii Y, Sugiyama Y. Prediction of hepatic clearance in human from in vitro data for successful drug development. *AAPS J* 2009;**11**:262–76
33. Dunn JC, Yarmush ML, Koebe HG, Tompkins RG. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. *Faseb J* 1989;**3**:174–7
34. Dunn JC, Tompkins RG, Yarmush ML. Hepatocytes in collagen sandwich: evidence for transcriptional and translational regulation. *J Cell Biol* 1992;**116**:1043–53
35. Dunn JC, Tompkins RG, Yarmush ML. Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. *Biotechnol Prog* 1991;**7**:237–45
36. Dunn JC, Tompkins RG, Yarmush ML. Hepatocytes in collagen sandwich: evidence for transcriptional and translational regulation. *J Cell Biol* 1992;**116**:1043–53
37. Semler EJ, Ranucci CS, Moghe PV. Mechanochemical manipulation of hepatocyte aggregation can selectively induce or repress liver-specific function. *Biotechnol Bioeng* 2000;**69**:359–69
38. Chen AA, Khetani SR, Lee S, Bhatia SN, Van Vliet KJ. Modulation of hepatocyte phenotype in vitro via chemomechanical tuning of polyelectrolyte multilayers. *Biomaterials* 2009;**30**:1113–20
39. Rajagopalan P, Shen CJ, Berthiaume F, Tilles AW, Toner M, Yarmush ML. Polyelectrolyte nano-scaffolds for the design of layered cellular architectures. *Tissue Eng* 2006;**12**:1553–63
40. Bjornsson TD, Callaghan JT, Einolf HJ, Fischer V, Gan L, Grimm S, Kao J, King SP, Miwa G, Ni L, Kumar G, McLeod J, Obach RS, Roberts S, Roe A, Shah A, Snikeris F, Sullivan JT, Tweedie D, Vega JM, Walsh J, Wrighton SA. The conduct of in vitro and in vivo drug-drug interaction studies: a Pharmaceutical Research and Manufacturers of America (PhRMA) perspective. *Drug Metab Dispos* 2003;**31**:815–32
41. Bjornsson TD, Callaghan JT, Einolf HJ, Fischer V, Gan L, Grimm S, Kao J, King SP, Miwa G, Ni L, Kumar G, McLeod J, Obach SR, Roberts S, Roe A, Shah A, Snikeris F, Sullivan JT, Tweedie D, Vega JM, Walsh J, Wrighton SA. The conduct of in vitro and in vivo drug-drug interaction studies: a PhRMA perspective. *J Clin Pharmacol* 2003;**43**:443–69
42. Grecco N, Cohen N, Warner AW, Lopez-Correa C, Truter SL, Snapir A, Piccoli SP, Wang D, Westelinck A, Hinman L, Franc MA. PhRMA survey of pharmacogenomic and pharmacodynamic evaluations: what next? *Clin Pharmacol Therapeut* 2012;**91**:1035–43
43. Chu V, Einolf HJ, Evers R, Kumar G, Moore D, Ripp S, Silva J, Sinha V, Sinz M, Skerjanec A. In vitro and in vivo induction of cytochrome p450: a survey of the current practices and recommendations: a pharmaceutical research and manufacturers of america perspective. *Drug Metab Dispos* 2009;**37**:1339–54
44. Karara AH, Edeki T, McLeod J, Tonelli AP, Wagner JA. PhRMA survey on the conduct of first-in-human clinical trials under exploratory investigational new drug applications. *J Clin Pharmacol* 2010;**50**:380–91
45. Poulin P, Jones HM, Jones RD, Yates JW, Gibson CR, Chien JY, Ring BJ, Adkison KK, He H, Vuppugalla R, Marathe P, Fischer V, Dutta S, Sinha VK, Bjornsson T, Lave T, Ku MS. PhRMA CPCDC initiative on

predictive models of human pharmacokinetics, part 1: Goals, properties of the PhRMA dataset, and comparison with literature datasets. *J Pharm Sci* 2011;**100**:4050–73
46. Jones RD, Jones HM, Rowland M, Gibson CR, Yates JW, Chien JY, Ring BJ, Adkison KK, Ku MS, He H, Vuppugalla R, Marathe P, Fischer V, Dutta S, Sinha VK, Bjornsson T, Lave T, Poulin P. PhRMA CPCDC initiative on predictive models of human pharmacokinetics, part 2: Comparative assessment of prediction methods of human volume of distribution. *J Pharm Sci* 2011;**100**:4074–89
47. Ring BJ, Chien JY, Adkison KK, Jones HM, Rowland M, Jones RD, Yates JW, Ku MS, Gibson CR, He H, Vuppugalla R, Marathe P, Fischer V, Dutta S, Sinha VK, Bjornsson T, Lave T, Poulin P. PhRMA CPCDC initiative on predictive models of human pharmacokinetics, part 3: Comparative assessement of prediction methods of human clearance. *J Pharm Sci* 2011;**100**:4090–110
48. Vuppugalla R, Marathe P, He H, Jones RD, Yates JW, Jones HM, Gibson CR, Chien JY, Ring BJ, Adkison KK, Ku MS, Fischer V, Dutta S, Sinha VK, Bjornsson T, Lave T, Poulin P. PhRMA CPCDC initiative on predictive models of human pharmacokinetics, part 4: Prediction of plasma concentration-time profiles in human from in vivo preclinical data by using the Wajima approach. *J Pharm Sci* 2011;**100**:4111–26
49. Poulin P, Jones RD, Jones HM, Gibson CR, Rowland M, Chien JY, Ring BJ, Adkison KK, Ku MS, He H, Vuppugalla R, Marathe P, Fischer V, Dutta S, Sinha VK, Bjornsson T, Lave T, Yates JW. PHRMA CPCDC initiative on predictive models of human pharmacokinetics, part 5: Prediction of plasma concentration-time profiles in human by using the physiologically-based pharmacokinetic modeling approach. *J Pharm Sci* 2011;**100**:4127–57
50. Kostadinova R, Boess F, Applegate D, Suter L, Weiser T, Singer T, Naughton B, Roth A. A long-term three dimensional liver co-culture system for improved prediction of clinically relevant drug-induced hepatotoxicity. *Toxicol Appl Pharmacol* 2013;**268**:1–16
51. Messner S, Agarkova I, Moritz W, Kelm JM. Multi-cell type human liver microtissues for hepatotoxicity testing. *Arch Toxicol* 2013;**87**:209–13
52. Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. *Nat Biotechnol* 2008;**26**:120–6
53. March S, Hui EE, Underhill GH, Khetani S, Bhatia SN. Microenvironmental regulation of the sinusoidal endothelial cell phenotype in vitro. *Hepatology* 2009;**50**:920–8
54. Khetani SR, Kanchagar C, Ukairo O, Krzyzewski S, Moore A, Shi J, Aoyama S, Aleo M, Will Y. Use of micropatterned cocultures to detect compounds that cause drug-induced liver injury in humans. *Toxicol Sci* 2013;**132**:107–17
55. Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. *Nat Biotechnol* 2008;**26**:120–6
56. Wang WW, Khetani SR, Krzyzewski S, Duignan DB, Obach RS. Assessment of a micropatterned hepatocyte coculture system to generate major human excretory and circulating drug metabolites. *Drug Metab Dispos* 2010;**38**:1900–5
57. Andersson H, van den Berg A. Microfabrication and microfluidics for tissue engineering: state of the art and future opportunities. *Lab Chip* 2004;**4**:98–103
58. Powers MJ, Domansky K, Kaazempur-mofrad MR, Kalezi A, Capitano A, Upadhyaya A, Kurzawski P, Wack KE, Stolz DB, Kamm R, Grif LG. A microfabricated array bioreactor for perfused 3D liver culture. *Biotechnol Bioeng* 2002;**78**:257–69
59. Powers MJ, Janigian DM, Wack KE, Baker CS, Beer Stolz D, Griffith LG. Functional behavior of primary rat liver cells in a three-dimensional perfused microarray bioreactor. *Tissue Eng* 2002;**8**:499–513
60. Boyer TD, Wright TL, Manns MP, Zakim D. *Zakim and Boyer's hepatology: a textbook of liver disease*, 5th ed. Philadelphia, PA: Elsevier Saunders, 2006
61. Arias IM (ed.). *The liver*. West Sussex: John Wiley & Sons Ltd, 2009
62. Lee PJ, Hung PJ and Lee LP. An artificial liver sinusoid with a microfluidic endothelial-like barrier for primary hepatocyte culture *Biotechnol Bioeng* 2007;**97**:1340-6
63. Zhang MY, Lee PJ, Hung PJ, Johnson T, Lee LP, Mofrad MRK. Microfluidic environment for high density hepatocyte culture. *Biomed Microdevice* 2008;**10**:117–21
64. Chan T, Yu H, Moore A, Khetani S, Tweedie DJ. Meeting the challenge of predicting hepatic clearance of compounds slowly metabolized by cytochrome P450 using a novel hepatocyte model, HepatoPacTM. *Drug Metab Dispos: Biol Fate Chem* 2013;**41**:2024–32
65. van Midwoud PM, Groothuis GM, Merema MT, Verpoorte E. Microfluidic biochip for the perifusion of precision-cut rat liver slices for metabolism and toxicology studies. *Biotechnol Bioeng* 2010;**105**:184–94
66. Chao P, Maguire T, Novik E, Cheng KC, Yarmush ML. Evaluation of a microfluidic based cell culture platform with primary human hepatocytes for the prediction of hepatic clearance in human. *Biochem Pharmacol* 2009;**78**:625–32
67. Kidambi S, Yarmush RS, Novik E, Chao P, Yarmush ML, Nahmias Y. Oxygen-mediated enhancement of primary hepatocyte metabolism, functional polarization, gene expression, and drug clearance. *Proc Natl Acad Sci U S A* 2009;**106**:15714–9
68. Messner S, Agarkova I, Moritz W, Kelm JM. Multi-cell type human liver microtissues for hepatotoxicity testing. *Arch Toxicol* 2013;**87**:209–13
69. Zhang MY, Lee PJ, Hung PJ, Johnson T, Lee LP, Mofrad MR. Microfluidic environment for high density hepatocyte culture. *Biomed Microdevice* 2008;**10**:117–21
70. Domansky K, Inman W, Serdy J, Dash A, Lim MHM, Griffith LG. Perfused multiwell plate for 3D liver tissue engineering. *Lab Chip* 2010;**10**:51–8
71. Chao P, Maguire T, Novik E, Cheng K-C, Yarmush ML. Evaluation of a microfluidic based cell culture platform with primary human hepatocytes for the prediction of hepatic clearance in human. *Biochem Pharmacol* 2009;**78**:625–32
72. Novik E, Maguire TJ, Chao P, Cheng KC, Yarmush ML. A microfluidic hepatic coculture platform for cell-based drug metabolism studies. *Biochem Pharmacol* 2010;**79**:1036–44
73. Leigh DA, Lusby PJ, McBurney RT, Morelli A, Slawin AM, Thomson AR, Walker DB. Getting harder: cobalt(III)-template synthesis of catenanes and rotaxanes. *J Am Chem Soc* 2009;**131**:3762–71
74. Saab L, Peluso J, Muller CD, Ubeaud-Sequier G. Implication of hepatic transporters (MDR1 and MRP2) in inflammation-associated idiosyncratic drug-induced hepatotoxicity investigated by microvolume cytometry. *Cytometry Part A: J Int Soc Analytic Cytol* 2013;**83**:403–8
75. Kaplowitz N. Drug-induced liver injury: introduction and overview. In: Kaplowitz N, DeLeve LD (eds) *Drug induced liver disease*. 3rd ed. CA, USA: Elsevier, 2013
76. Taub R. Liver regeneration: from myth to mechanism. *Nat Rev Mol Cell Biol* 2004;**5**:836–47
77. Morgan RE, van Staden CJ, Chen Y, Kalyanaraman N, Kalanzi J, Dunn RT, 2nd, Afshari CA, Hamadeh HK. A multifactorial approach to hepatobiliary transporter assessment enables improved therapeutic compound development. *Toxicol Sci: Official J Soc Toxicol* 2013;**136**:216–41
78. Keppler D. The roles of MRP2, MRP3, and OATP1B1 and OATP1B3 in conjugated hyperbilirubinemia. *Drug Metab Dispos* 2014;**42**:561–5
79. Kalgutkar AS, Soglia JR. Minimising the potential for metabolic activation in drug discovery. *Expert Opin Drug Metab Toxicol* 2005;**1**:91–142
80. Guengerich FP, MacDonald JS. Applying mechanisms of chemical toxicity to predict drug safety. *Chem Res Toxicol* 2007;**20**:344–69
81. Tujios S, Fontana RJ. Mechanisms of drug-induced liver injury: from bedside to bench. *Nat Rev Gastroenterol Hepatol* 2011;**8**:202–11
82. Benet LZ, Broccatelli F, Oprea TI. BDDCS applied to over 900 drugs. *AAPS J* 2011;**13**:519–47
83. Giuliano KA, Gough AH, Taylor DL, Vernetti LA, Johnston PA. Early safety assessment using cellular systems biology yields insights into mechanisms of action. *J Biomol Screen* 2010;**15**:783–97
84. Xu JJ, Henstock PV, Dunn MC, Smith AR, Chabot JR, de Graaf D. Cellular imaging predictions of clinical drug-induced liver injury. *Toxicol Sci* 2008;**105**:97–105
85. Liu X, LeCluyse EL, Brouwer KR, Lightfoot RM, Lee JI, Brouwer KL. Use of Ca2+ modulation to evaluate biliary excretion in sandwich-cultured rat hepatocytes. *J Pharmacol Exp Ther* 1999;**289**:1592–9

86. Liu X, Chism JP, LeCluyse EL, Brouwer KR, Brouwer KL. Correlation of biliary excretion in sandwich-cultured rat hepatocytes and in vivo in rats. *Drug Metab Dispos* 1999;**27**:637–44
87. Post PL, DeBiasio RL, Taylor DL. A fluorescent protein biosensor of myosin II regulatory light chain phosphorylation reports a gradient of phosphorylated myosin II in migrating cells. *Mol Biol Cell* 1995;**6**:1755–68
88. Le Vee M, Gripon P, Stieger B, Fardel O. Down-regulation of organic anion transporter expression in human hepatocytes exposed to the proinflammatory cytokine interleukin 1beta. *Drug Metab Dispos* 2008;**36**:217–22
89. Vickers AE, Fisher R, Olinga P, Dial S. Repair pathways evident in human liver organ slices. *Toxicol In Vitro* 2011;**25**:1485–92
90. Morris MC. Fluorescent biosensors of intracellular targets from genetically encoded reporters to modular polypeptide probes. *Cell Biochem Biophys* 2010;**56**:19–37
91. Donato MT, Jimenez N, Castell JV, Gomez-Lechon MJ. Fluorescence-based assays for screening nine cytochrome P450 (P450) activities in intact cells expressing individual human P450 enzymes. *Drug Metab Dispos* 2004;**32**:699–706
92. Frommer WB, Davidson MW, Campbell RE. Genetically encoded biosensors based on engineered fluorescent proteins. *Chem Soc Rev* 2009;**38**:2833–41
93. Goodchild S, Love T, Hopkins N, Mayers C. Engineering antibodies for biosensor technologies. *Adv Appl Microbiol* 2005;**58C**:185–226
94. Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind specific ligands. *Nature* 1990;**346**:818–22
95. Szent-Gyorgyi C, Schmidt BF, Creeger Y, Fisher GW, Zakel KL, Adler S, Fitzpatrick JA, Woolford CA, Yan Q, Vasilev KV, Berget PB, Bruchez MP, Jarvik JW, Waggoner A. Fluorogen-activating single-chain antibodies for imaging cell surface proteins. *Nat Biotechnol* 2008;**26**:235–40
96. Adams SR, Campbell RE, Gross LA, Martin BR, Walkup GK, Yao Y, Llopis J, Tsien RY. New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications. *J Am Chem Soc* 2002;**124**:6063–76
97. Shekhawat SS, Ghosh I. Split-protein systems: beyond binary protein-protein interactions. *Curr Opin Chem Biol* 2011;**15**:789–97
98. Zhang J, Ma Y, Taylor SS, Tsien RY. Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. *Proc Natl Acad Sci U S A* 2001;**98**:14997–5002
99. Ting AY, Kain KH, Klemke RL, Tsien RY. Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. *Proc Natl Acad Sci U S A* 2001;**98**:15003–8
100. Dooley CT, Dore TM, Hanson GT, Jackson WC, Remington SJ, Tsien RY. Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators. *J Biol Chem* 2004;**279**:22284–93
101. Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, Tsien RY. Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. *Nature* 1997;**388**:882–7
102. Persechini A, Lynch JA, Romoser VA. Novel fluorescent indicator proteins for monitoring free intracellular Ca2+. *Cell Calcium* 1997;**22**:209–16
103. Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY. Understanding, improving and using green fluorescent proteins. *Trends Biochem Sci* 1995;**20**:448–55
104. Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nat Biotechnol* 2004;**22**:1567–72
105. Nagai T, Sawano A, Park ES, Miyawaki A. Circularly permuted green fluorescent proteins engineered to sense Ca2+. *Proc Natl Acad Sci U S A* 2001;**98**:3197–202
106. Sakaguchi R, Endoh T, Yamamoto S, Tainaka K, Sugimoto K, Fujieda N, Kiyonaka S, Mori Y, Morii T. A single circularly permuted GFP sensor for inositol-1,3,4,5-tetrakisphosphate based on a split PH domain. *Bioorg Med Chem* 2009;**17**:7381–6
107. Albrecht SC, Sobotta MC, Bausewein D, Aller I, Hell R, Dick TP, Meyer AJ. Redesign of genetically encoded biosensors for monitoring mitochondrial redox status in a broad range of model eukaryotes. *J Biomol Screen* 2013;**19**:379–86
108. Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. *Drug Metab Dispos* 2003;**31**:1035–42
109. van Midwoud PM, Merema MT, Verpoorte E, Groothuis GM. A microfluidic approach for in vitro assessment of interorgan interactions in drug metabolism using intestinal and liver slices. *Lab Chip* 2010;**10**:2778–86
110. O'Brien PJ, Irwin W, Diaz D, Howard-Cofield E, Krejsa CM, Slaughter MR, Gao B, Kaludercic N, Angeline A, Bernardi P, Brain P, Hougham C. High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. *Arch Toxicol* 2006;**80**:580–604
111. Vernetti L, Irwin W, Giuliano KA, Gough A, Johnston K, Taylor DL. Cellular systems biology applied to preclinical safety testing: a case study of CellCiphrTM profiling. In: SEaJJ Xu (ed.). *Drug efficacy, safety, and biologics discovery: emerging technologies and tools*. Hoboken, NJ: John Wiley & Sons, Inc, 2008:53–73
112. Yang Y, Blomme EA, Waring JF. Toxicogenomics in drug discovery: from preclinical studies to clinical trials. *Chem-Biol Interact* 2004;**150**:71–85
113. Paules R. Phenotypic anchoring: linking cause and effect. *Environ Health Perspect* 2003;**111**:A338–9
114. Suter L, Schroeder S, Meyer K, Gautier JC, Amberg A, Wendt M, Gmuender H, Mally A, Boitier E, Ellinger-Ziegelbauer H, Matheis K, Pfannkuch F. EU framework 6 project: predictive toxicology (PredTox)-overview and outcome. *Toxicol Appl Pharmacol* 2011;**252**:73–84
115. Gill SD, McBurney H, Schulz DL. Land-based versus pool-based exercise for people awaiting joint replacement surgery of the hip or knee: results of a randomized controlled trial. *Arch Phys Med Rehabil* 2009;**90**:388–94
116. Chen M, Vijay V, Shi Q, Liu Z, Fang H, Tong W. FDA-approved drug labeling for the study of drug-induced liver injury. *Drug Discov Today* 2011;**16**:697–703
117. Judson R, Richard A, Dix D, Houck K, Elloumi F, Martin M, Cathey T, Transue TR, Spencer R, Wolf M. ACToR–aggregated computational toxicology resource. *Toxicol Appl Pharmacol* 2008;**233**:7–13
118. Lim E, Pon A, Djoumbou Y, Knox C, Shrivastava S, Guo AC, Neveu V, Wishart DS. T3DB: a comprehensively annotated database of common toxins and their targets. *Nucleic Acid Res* 2010;**38**:D781–6
119. Liu Z, Shi Q, Ding D, Kelly R, Fang H, Tong W. Translating clinical findings into knowledge in drug safety evaluation – drug induced liver injury prediction system (DILIps). *PLoS Computat Biol* 2011;**7**:e1002310
120. Merlot C. Computational toxicology – a tool for early safety evaluation. *Drug Discov Today* 2010;**15**:16–22
121. Sun H, Xia M, Austin CP, Huang R. Paradigm shift in toxicity testing and modeling. *AAPS J* 2012;**14**:473–80