Published in final edited form as:

Exp Biol Med (Maywood). 2014 September ; 239(9): 1180–1191. doi:10.1177/1535370214531872.

In Vitro Platforms for Evaluating Liver Toxicity

Shyam Sundhar Bale^{#1}, Lawrence Verneti^{#2,3}, Nina Senutovitch^{2,3}, Rohit Jindal¹, Manjunath Hegde¹, Albert Gough^{2,3}, William J McCarty¹, Ahmet Bakan³, Abhinav Bhushan¹, Tong Ying Shun², Inna Golberg¹, Richard DeBiasio², Berk Osman Usta¹, D. Lansing Taylor^{2,3}, and Martin L. Yarmush^{1,#}

¹Center for Engineering in Medicine (CEM) at Massachusetts General Hospital, Harvard Medical School, Shriners Hospital for Children, Boston MA 02114

²University of Pittsburgh Drug Discovery Institute, Pittsburgh PA 15260

³University of Pittsburgh Department of Computational and Systems Biology, Pittsburgh PA 15260

[#] These authors contributed equally to this work.

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

[#]corresponding author: Dr. Martin L. Yarmush, Center for Engineering in Medicine (CEM), Massachusetts General Hospital, Harvard Medical School, Shriners Hospital for Children, 51 Blossom Street, Boston, MA 02114, ireis@sbi.org.

Contribution of Authors

The entire team had input into conceiving and outlining the manuscript. SSB and LV contributed equally to the writing of the manuscript. The entire team proof-read and edited the manuscript.

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

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, 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 drug induced liver injury (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 through 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 cell 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 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 co-culturing 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 are the embryonic stem cells (ESCs) or the adult induced pluripotent stem cells (iPSCs) that can be matured into a 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 7 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 macro-scale 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) 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 3 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-1 β 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 micro-tissue spheroid culture using a gravity-enforced cellular assembly, enabling formation of cellular contacts (51). Briefly, hepatocytes and non-parenchymal cells in a specifically designed multi well plate are introduced into a hanging drop which forms a micro-tissue spheroid in 3 days (Figure 1B). After formation, the spheroids can be transferred into a spheroid-specific 96 well plate and cultured for up to 5 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 TC₅₀ values when compared with 2D cultures.

Another approach is the co-culture of discrete micro-patterned hepatocyte islands surrounded and stabilized by stromal cells (3T3-J2 fibroblasts) and this approach is commercially available as Hepatopac (Hepregen, Medford, MA) (52-56). The system uses a standard 24 well plate format with reusable elastomeric molds to pattern 500 μ m diameter hepatocyte islands surrounded by the stromal cells (Figure 1C). The co-culture system maintained its function for up to 6 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, and that 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 micro systems 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) 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 7 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) (64) 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 μ M to 50 μ M 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) (65) adapts a microfluidic microscale cell culture analog (μ CCA) 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 hours. 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 8 days (66).

The above described commercial liver-on-a-chip platforms 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 (MOT)

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 (67, 68). 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 (69). 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 (70).

In recent years, led by the case for BSEP inhibition produced liver injury gaining significant clinical concordance (71), 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 (68, 72). 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 lead to liver injury has been noted with compounds such as acetaminophen, chloramphenicol, danazol, diclofenac, flutamide, ibuprofen, imipramine, indomethacin, isoniazid, hydralazine, nitrofurantoin, piroxicam, procainamide, sulpha-methoxazole, tacrine and tamoxifen (73). 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 (74, 75).

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, 76).

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 (77, 78). Fluorescent molecules (probes) and protein-based fluorescent biosensors are powerful tools

for the reporting of spatiotemporal dynamics (79). Fluorescence-based probes that are targeted to particular substrates and subcellular compartments are widely used for live cell studies (79). 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, 79-82). 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, 83). 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 well as protein modifications such as conformational change, translocation, ligand binding, analyte changes and post-translational modifications (83-85). 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, 86). The abundant research efforts to understand and further improve fluorescent proteins led to the extension of their applications into fluorescence-based protein biosensors (86, 87). Biosensors have been built from a wide range of proteins with inherent fluorescent chromophores (XFPs) and extrinsic chromophores like small molecule sensors such as aptamers (88) and fluorophore-binding proteins (89, 90). 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 (91), translocation (83), protein modification (92, 93) and the presence of intracellular ions (94). 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 (95, 96). 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 (97, 98). 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) (99). 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 (100, 101). (100, 101). 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.

High Content Screening (HCS) Approach to Measuring Hepatotoxicity

HCS permitted the moderate throughput of cell-based assays in drug discovery and development (83, 102). Evaluation of DILI using multi-parameter cell feature analysis and measured by fluorescence imaging was introduced by Haskins et al (103) and was implemented by O'Brien et al (104) and later extended by Xu et al (78) 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 (105). 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% (77). Collectively, these efforts established the combination of HCS multiplexed data measurements coupled to computer driven analysis can translate to useful predictive models.

'Omics - Measurements'

Ever since the introduction of microarray genomics technology over two decades ago, 'omics' platforms have expanded to include 3 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 (106) 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 (107). 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 2-week rat studies (108). 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, 109). Thus, a large selection of clinical compounds is available for testing and validating liver platforms. Table 3 provides an

example list of 105 compounds that are chemically diverse and independent of therapeutic intent. The 56 ‘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 is 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 published the $100 \times C_{\max}$ as a ‘reasonable’ level to separate a potential DILI compound from non-liver toxic compounds (78) 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) (110). 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 drug induced liver injury 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) 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 is 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 (111) and the Toxin and Toxin-Target Database (T3DB) (112).

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 above, 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 (78), 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 (113). 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 (114, 115).

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.

Acknowledgments

This work was supported by NIH # 1UH2TR00503-01.

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(27):3545–59. [PubMed: 16248807]
2. Maddrey WC. Drug-induced hepatotoxicity: 2005. *Journal of clinical gastroenterology.* 2005; 39:S83–9. [PubMed: 15758665]
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(11):904–16. [PubMed: 17971785]

4. Blomme, EaG; Yang, Y.; Waring, JF. Use of toxicogenomics to understand mechanisms of drug-induced hepatotoxicity during drug discovery and development. *Toxicology letters*. 2009; 186:22–31. [PubMed: 18996174]
5. Kaplowitz N. Acetaminophen hepatotoxicity: what do we know, what don't we know, and what do we do next? *Hepatology*. Jul; 2004 40(1):23–6. [PubMed: 15239082]
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*. Oct; 1973 187(1):185–94. [PubMed: 4746326]
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*. Jan; 2010 23(1):171–83. [PubMed: 20014752]
8. Greaves P, Williams A, Eve M. First dose of potential new medicines to humans: how animals help. *Nat Rev Drug Discov*. Mar; 2004 3(3):226–36. [PubMed: 15031736]
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*. Aug; 2000 32(1):56–67. [PubMed: 11029269]
10. Kaplowitz N. Idiosyncratic drug hepatotoxicity. *Nat Rev Drug Discov*. Jun; 2005 4(6):489–99. [PubMed: 15931258]
11. Roth RA, Ganey PE. Intrinsic versus Idiosyncratic Drug-Induced Hepatotoxicity — Two Villains or One?. 2010; 332:692–7. [PubMed: 20019161]
12. Di L. The role of drug metabolizing enzymes in clearance. *Expert Opin Drug Metab Toxicol*. Jan 7.2014
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(3):e59432. [PubMed: 23516635]
14. LeCluyse EL, Witek RP, Andersen ME, Powers MJ. Organotypic liver culture models: meeting current challenges in toxicity testing. *Crit Rev Toxicol*. Jul; 2012 42(6):501–48. [PubMed: 22582993]
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*. Apr; 2012 3(4):246–50. [PubMed: 22441839]
16. Subba Rao M, Sasikala M, Nageshwar Reddy D. Thinking outside the liver: induced pluripotent stem cells for hepatic applications. *World journal of gastroenterology: WJG*. Jun 14; 2013 19(22): 3385–96. [PubMed: 23801830]
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*. Aug; 2013 9(8):514–20. [PubMed: 23728495]
18. Zguris JC, Itle LJ, Hayes D, Pishko MV. Microreactor microfluidic systems with human microsomes and hepatocytes for use in metabolite studies. *Biomedical microdevices*. 2005; 7:117–25. [PubMed: 15940424]
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*. Aug; 2006 36(8):671–83. [PubMed: 16891248]
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. *Journal of Hepatology*. 2013:1–8.
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. *Toxicological sciences: an official journal of the Society of Toxicology*. 2005; 85:632–8. [PubMed: 15728706]
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.

- Toxicological sciences: an official journal of the Society of Toxicology. 2004; 82:534–44. [PubMed: 15456927]
24. Stieger, B. In vitro techniques: isolated organ perfusion, slices, cells and subcellular elements. In: Rodes, J., editor. Textbook of Hepatology. 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, Koese 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.* May-Aug;2003 35(2-3):145–213. [PubMed: 12959414]
 26. Guillouzo A. Liver cell models in in vitro toxicology. *Environ Health Perspect.* Apr; 1998 106(Suppl 2):511–32. [PubMed: 9599700]
 27. LeCluyse EL. Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. *Eur J Pharm Sci.* Jul; 2001 13(4):343–68. [PubMed: 11408150]
 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.* Dec; 2005 6(6):569–91. [PubMed: 16379670]
 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(1):159–234. [PubMed: 17364884]
 30. Dunn JC, Tompkins RG, Yarmush ML. Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. *Biotechnol Prog.* May-Jun;1991 7(3):237–45. [PubMed: 1367596]
 31. Bjornsson E. Drug-induced liver injury: Hy's rule revisited. *Clin Pharmacol Ther.* Jun; 2006 79(6): 521–8. [PubMed: 16765139]
 32. Chiba M, Ishii Y, Sugiyama Y. Prediction of hepatic clearance in human from in vitro data for successful drug development. *AAPS J.* Jun; 2009 11(2):262–76. [PubMed: 19408130]
 33. Dunn JC, Yarmush ML, Koebe HG, Tompkins RG. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. *Faseb J.* Feb; 1989 3(2):174–7. [PubMed: 2914628]
 34. Dunn JC, Tompkins RG, Yarmush ML. Hepatocytes in collagen sandwich: evidence for transcriptional and translational regulation. *The Journal of cell biology.* Feb; 1992 116(4):1043–53. [PubMed: 1734019]
 35. Dunn JC, Tompkins RG, Yarmush ML. Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. *Biotechnol Prog.* May-Jun;1991 7(3):237–45. [PubMed: 1367596]
 36. Dunn JC, Tompkins RG, Yarmush ML. Hepatocytes in collagen sandwich: evidence for transcriptional and translational regulation. *The Journal of cell biology.* 1992; 116:1043–53. [PubMed: 1734019]
 37. Semler EJ, Ranucci CS, Moghe PV. Mechanochemical manipulation of hepatocyte aggregation can selectively induce or repress liver-specific function. *Biotechnology and bioengineering.* Aug 20; 2000 69(4):359–69. [PubMed: 10862674]
 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.* Feb; 2009 30(6): 1113–20. [PubMed: 19046762]
 39. Rajagopalan P, Shen CJ, Berthiaume F, Tilles AW, Toner M, Yarmush ML. Polyelectrolyte nano-scaffolds for the design of layered cellular architectures. *Tissue Engineering.* 2006; 12:1553–63. [PubMed: 16846351]
 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.* Jul; 2003 31(7):815–32. [PubMed: 12814957]
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. *Journal of clinical pharmacology.* May; 2003 43(5):443–69. [PubMed: 12751267]
 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? *Clinical pharmacology and therapeutics.* Jun; 2012 91(6):1035–43. [PubMed: 22534869]
 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.* Jul; 2009 37(7):1339–54. [PubMed: 19389860]
 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. *Journal of clinical pharmacology.* Apr; 2010 50(4):380–91. [PubMed: 20097935]
 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.* Apr 26.2011
 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.* Mar 30.2011
 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 assessment of prediction methods of human clearance. *J Pharm Sci.* May 3.2011
 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.* Apr 7.2011
 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.* May 3.2011
 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.* Apr 1; 2013 268(1):1–16. [PubMed: 23352505]
 51. Messner S, Agarkova I, Moritz W, Kelm JM. Multi-cell type human liver microtissues for hepatotoxicity testing. *Arch Toxicol.* Jan; 2013 87(1):209–13. [PubMed: 23143619]
 52. Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. *Nat Biotechnol.* Jan; 2008 26(1):120–6. [PubMed: 18026090]
 53. March S, Hui EE, Underhill GH, Khetani S, Bhatia SN. Microenvironmental regulation of the sinusoidal endothelial cell phenotype in vitro. *Hepatology.* Sep; 2009 50(3):920–8. [PubMed: 19585615]
 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.* Mar; 2013 132(1):107–17. [PubMed: 23152190]

55. Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. *Nat Biotechnol.* Jan; 2008 26(1):120–6. [PubMed: 18026090]
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.* Oct; 2010 38(10):1900–5. [PubMed: 20595376]
57. Andersson H, van den Berg A. Microfabrication and microfluidics for tissue engineering: state of the art and future opportunities. *Lab Chip.* Apr; 2004 4(2):98–103. [PubMed: 15052347]
58. Powers MJ, Domansky K, Kaazempur-mofrad MR, Kalezi A, Capitano A, Upadhyaya A, Kurzwski P, Wack KE, Stolz DB, Kamm R, Grif LG. A Microfabricated Array Bioreactor for Perfused 3D Liver Culture. 2002
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.* Jul; 2002 8(3):499–513. [PubMed: 12167234]
60. Boyer, TD.; Wright, TL.; Manns, MP.; Zakim, D. Zakim and Boyer's hepatology: a textbook of liver disease. 5th ed.. Edinburgh]: Elsevier Saunders; Philadelphia, Pa: 2006.
61. Arias, IM. The Liver. Arias, IM., editor. John Wiley & Sons Ltd; West Sussex: 2009.
62. Lee PJ, Hung PJ, Lee LP. An Artificial Liver Sinusoid With a Microfluidic Endothelial-Like Barrier for Primary Hepatocyte Culture. 2007; 97:1340–6.
63. Zhang MY, Lee PJ, Hung PJ, Johnson T, Lee LP, Mofrad MRK. Microfluidic environment for high density hepatocyte culture. *Biomedical microdevices.* 2008; 10:117–21. [PubMed: 17682945]
64. Domansky K, Inman W, Serdy J, Dash A, Lim MHM, Griffith LG. Perfused multiwell plate for 3D liver tissue engineering. *Lab on a Chip.* 2010; 10:51–8. [PubMed: 20024050]
65. 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. [PubMed: 19463793]
66. 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. [PubMed: 19925779]
67. 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.* Mar 18; 2009 131(10):3762–71. [PubMed: 19275264]
68. 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: the journal of the International Society for Analytical Cytology.* Apr; 2013 83(4):403–8. [PubMed: 23401306]
69. Kaplowitz, N. Drug-Induced Liver Injury: Introduction and Overview. In: Kaplowitz, N.; DeLeve, LD., editors. *Drug Induced Liver Disease. Third Edition.* Elsevier; 2013.
70. Taub R. Liver regeneration: from myth to mechanism. *Nat Rev Mol Cell Biol.* 2004; 5(10):836–47. [PubMed: 15459664]
71. 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. *Toxicological sciences: an official journal of the Society of Toxicology.* Nov; 2013 136(1):216–41. [PubMed: 23956101]
72. Keppler D. The Roles of MRP2, MRP3, and OATP1B1 and OATP1B3 in Conjugated Hyperbilirubinemia. *Drug Metab Dispos.* Jan 23.2014
73. Kalgutkar AS, Soglia JR. Minimising the potential for metabolic activation in drug discovery. Expert opinion on drug metabolism & toxicology. Jun; 2005 1(1):91–142. [PubMed: 16922655]
74. Guengerich FP, MacDonald JS. Applying mechanisms of chemical toxicity to predict drug safety. *Chemical research in toxicology.* Mar; 2007 20(3):344–69. [PubMed: 17302443]
75. Tujios S, Fontana RJ. Mechanisms of drug-induced liver injury: from bedside to bench. *Nat Rev Gastroenterol Hepatol.* Apr; 2011 8(4):202–11. [PubMed: 21386809]
76. Benet LZ, Broccatelli F, Oprea TI. BDDCS applied to over 900 drugs. *The AAPS journal.* Dec; 2011 13(4):519–47. [PubMed: 21818695]

77. Giuliano KA, Gough AH, Taylor DL, Verneti LA, Johnston PA. Early safety assessment using cellular systems biology yields insights into mechanisms of action. *J Biomol Screen*. Aug; 2010 15(7):783–97. [PubMed: 20639501]
78. Xu JJ, Henstock PV, Dunn MC, Smith AR, Chabot JR, de Graaf D. Cellular imaging predictions of clinical drug-induced liver injury. *Toxicol Sci*. Sep; 2008 105(1):97–105. [PubMed: 18524759]
79. Liu X, LeCluyse EL, Brouwer KR, Lightfoot RM, Lee JJ, Brouwer KL. Use of Ca²⁺ modulation to evaluate biliary excretion in sandwich-cultured rat hepatocytes. *J Pharmacol Exp Ther*. Jun; 1999 289(3):1592–9. [PubMed: 10336557]
80. 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*. Jun; 1999 27(6):637–44. [PubMed: 10348791]
81. 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*. Dec; 1995 6(12):1755–68. [PubMed: 8590803]
82. 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 Metabolism and Disposition*. Feb; 2008 36(2):217–22. [PubMed: 17991769]
83. Vickers AE, Fisher R, Olinga P, Dial S. Repair pathways evident in human liver organ slices. *Toxicol In Vitro*. Oct; 2011 25(7):1485–92. [PubMed: 21575709]
84. Morris MC. Fluorescent biosensors of intracellular targets from genetically encoded reporters to modular polypeptide probes. *Cell biochemistry and biophysics*. 2010; 56(1):19–37. [PubMed: 19921468]
85. 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*. Jul; 2004 32(7):699–706. [PubMed: 15205384]
86. Frommer WB, Davidson MW, Campbell RE. Genetically encoded biosensors based on engineered fluorescent proteins. *Chemical Society reviews*. Oct; 2009 38(10):2833–41. [PubMed: 19771330]
87. Goodchild S, Love T, Hopkins N, Mayers C. Engineering Antibodies for Biosensor Technologies. *Advances in applied microbiology*. 2005; 58C:185–226. [PubMed: 16543034]
88. Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind specific ligands. *Nature*. Aug 30; 1990 346(6287):818–22. [PubMed: 1697402]
89. 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*. Feb; 2008 26(2):235–40. [PubMed: 18157118]
90. 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. *Journal of the American Chemical Society*. May 29; 2002 124(21):6063–76. [PubMed: 12022841]
91. Shekhawat SS, Ghosh I. Split-protein systems: beyond binary protein-protein interactions. *Current opinion in chemical biology*. Dec; 2011 15(6):789–97. [PubMed: 22070901]
92. Zhang J, Ma Y, Taylor SS, Tsien RY. Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. *Proceedings of the National Academy of Sciences of the United States of America*. Dec 18; 2001 98(26):14997–5002. [PubMed: 11752448]
93. Ting AY, Kain KH, Klemke RL, Tsien RY. Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. *Proceedings of the National Academy of Sciences of the United States of America*. Dec 18; 2001 98(26):15003–8. [PubMed: 11752449]
94. Dooley CT, Dore TM, Hanson GT, Jackson WC, Remington SJ, Tsien RY. Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators. *The Journal of biological chemistry*. May 21; 2004 279(21):22284–93. [PubMed: 14985369]
95. 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*. Aug 28; 1997 388(6645):882–7. [PubMed: 9278050]

96. Persechini A, Lynch JA, Romoser VA. Novel fluorescent indicator proteins for monitoring free intracellular Ca²⁺ Cell calcium. Sep; 1997 22(3):209–16. [PubMed: 9330791]
97. Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY. Understanding, improving and using green fluorescent proteins. Trends in biochemical sciences. Nov; 1995 20(11):448–55. [PubMed: 8578587]
98. 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. Nature biotechnology. Dec; 2004 22(12):1567–72.
99. Nagai T, Sawano A, Park ES, Miyawaki A. Circularly permuted green fluorescent proteins engineered to sense Ca²⁺ Proceedings of the National Academy of Sciences of the United States of America. Mar 13; 2001 98(6):3197–202. [PubMed: 11248055]
100. 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. Bioorganic & medicinal chemistry. Oct 15; 2009 17(20):7381–6. [PubMed: 19762247]
101. 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. Journal of biomolecular screening. Aug 16.2013
102. Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. Drug Metabolism and Disposition. Aug; 2003 31(8):1035–42. [PubMed: 12867492]
103. 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. Oct 21; 2010 10(20):2778–86. [PubMed: 20835427]
104. 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. Apr 6.2006
105. Verneti, L.; Irwin, W.; Giuliano, KA.; Gough, A.; Johnston, K.; Taylor, DL. Cellular Systems Biology Applied to Preclinical Safety Testing: A Case Study of CellCiphr™ Profiling. In: Xu, SEaJJ., editor. Drug Efficacy, Safety, and Biologics Discovery: Emerging Technologies and Tools Hoboken. John Wiley & Sons, Inc; NJ: 2008. p. 53-73.
106. Yang Y, Blomme EA, Waring JF. Toxicogenomics in drug discovery: from preclinical studies to clinical trials. Chemico-biological interactions. Nov 1; 2004 150(1):71–85. [PubMed: 15522262]
107. Paules R. Phenotypic anchoring: linking cause and effect. Environ Health Perspect. May; 2003 111(6):A338–9. [PubMed: 12760838]
108. 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. Apr 15; 2011 252(2): 73–84. [PubMed: 20955723]
109. 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. Mar; 2009 90(3):388–94. [PubMed: 19254601]
110. 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. Aug; 2011 16(15-16):697–703. [PubMed: 21624500]
111. 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. Nov 15; 2008 233(1):7–13. [PubMed: 18671997]
112. 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 acids research. Jan; 2010 38(Database issue):D781–6. [PubMed: 19897546]

113. 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 computational biology. Dec.2011 7(12):e1002310. [PubMed: 22194678]
114. Merlot C. Computational toxicology--a tool for early safety evaluation. Drug Discov Today. Jan; 2010 15(1-2):16–22. [PubMed: 19835978]
115. Sun H, Xia M, Austin CP, Huang R. Paradigm shift in toxicity testing and modeling. The AAPS journal. Sep; 2012 14(3):473–80. [PubMed: 22528508]
116. 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, HepatoPac™. Drug metabolism and disposition: the biological fate of chemicals. Aug 19.2013
117. van Midwoud PM, Groothuis GM, Merema MT, Verpoorte E. Microfluidic biochip for the perfusion of precision-cut rat liver slices for metabolism and toxicology studies. Biotechnol Bioeng. Jan 1; 2010 105(1):184–94. [PubMed: 19718695]
118. 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. Sep 15; 2009 78(6):625–32. [PubMed: 19463793]
119. 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. Sep 15; 2009 106(37):15714–9. [PubMed: 19720996]
120. Messner S, Agarkova I, Moritz W, Kelm JM. Multi-cell type human liver microtissues for hepatotoxicity testing. Archives of toxicology. Jan; 2013 87(1):209–13. [PubMed: 23143619]
121. Zhang MY, Lee PJ, Hung PJ, Johnson T, Lee LP, Mofrad MR. Microfluidic environment for high density hepatocyte culture. Biomedical microdevices. Feb; 2008 10(1):117–21. [PubMed: 17682945]

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 (50)). B) Hanging drop strategy by Insphero. Hepatocytes and non-parenchymal cells are introduced into the drop and allowed to form micro-tissue that is transferred into a 96 well plate and cultured. C) Hepregen micro-pattern strategy used elastomeric molds to pattern hepatocytes followed by addition of 3T3-J2 cells. D) CellAsic device structure uses a micro-structure pattern to shield the hepatocytes from flow, mimicking an endothelial-like layer (adapted from (62, 63)). E) The Zyoxel platform consists of two reservoirs, with cells in one and media for re-circulation in the other (adapted from (64)). F) The Hurel Platform incorporates a Cell Culture Analog (CCA) with cell seeding area with multiple devices in parallel.

Table 1

Classifications of Drug-Induced Human Liver Injury

Classification	Pattern of Liver Injury	Example Drug
<i>Simple Classification</i>		
Intrinsic	Dose dependent, predictable, 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
<i>Clinical Laboratory Classification</i>		
Autoimmune	Circulating antibodies	tienilic acid
Hepatocellular	↑AST, ↑ALT, ↑Bilirubin	acetaminophen
Cholestatic	↑ALP, ↑Bilirubin	chlorthalidone
Infiltrative	↑ALP	tamoxifen
<i>Histopathologic Classification</i>		
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, troglitazone, 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 carbamazepine, 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 Phosphatase

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 ¹	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 (116)			Glutathione (50, 117)
Macromolecular interactions (reactive metabolites, covalent binding)		Clearance(116) Metabolites (55, 56)		Clearance, Metabolism (118, 119)	Clearance, cyp induction/inhibition(117)
Mitochondrial function, respiration, Calcium Flux		ATP(54) ² , MTT(54) ¹			ATP(117) ²
BSEP – canalicular flow		IHC (MRP2, Zo1)(54) CMFDA(55)		CMFDA(118)	
Immune stress	IL-6 release after LPS stimulation (120)				LPS stimulation, cytokine profile(117)
Protein synthesis inhibition (multiple levels)	Albumin (120)	Albumin, urea secretion(55)	Albumin(121)		Albumin, urea, fibrinogen, transferrin(117)
Drugs evaluated	Diclofenac, Acetaminophen, Trovafloxacin	Alprazolam, Atazanavir, Atomoxetine, Diazepam, Diclofenac, Flecainide, Glimepiride, Lidocaine, Meloxicam, Midazolam, Prednisolone, Riluzole, Risperidone, Theophylline, Tolbutamide, Atomoxetine, Trypan Blue, Voriconazole, others [3,4,8]	Diclofenac	Caffeine, Buspirone, Imipramine, Tadalafil, Sildenafil, Metoprolol, Carbamazepine, Antipyrine	Fenofibrate, Troglitazone, Trovafloxacin, Trovafloxacin, Pioglitazone, Acetaminophen

¹ NPC – non-parenchymal liver cell.

² ATP used as marker of cell viability.

Table 3
Example of Clinical Compounds Selected for Validating Liver Platform Toxicity Responses

Non-Liver Toxic Drugs	Clinical C _{max} (ug/ml)	Predominant Clearance route	Hepatotoxic drugs	Clinical C _{max} (ug/ml)	Predominant Clearance route	Reactive metabolite
Amantadine HCl	0.65	renal	Acitretin	0.61	-	No
Amiloride HCl	0.02	renal, bile mixed	Alpidem ⁵	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	-
Bupirone	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	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 ²	30.9	renal	Glaferine	0.7	-	Yes
Levofloxacin	5.7	renal	Ibuprofen ²	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 ³	4.5	renal, bile mixed	Naltrexone	0.02	-	No
Nadolol	0.13	bile	Nefazodone ⁴	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

Non-Liver Toxic Drugs	Clinical C _{max} (ug/ml)	Predominant Clearance route	Hepatotoxic drugs	Clinical C _{max} (ug/ml)	Predominant Clearance route	Reactive metabolite
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 ¹	6	renal	Yes
Sertraline	0.0245	bile	Troglitazone	2.82	-	Yes
Simvastatin	0.01	bile	Trovafloracin ³	2.09	-	No
Trazodone ⁴	3.12	renal	Valproic acid	7	renal	Yes
Zolpidem ⁵	0.12	renal				

¹ Matched liver clean/liver toxic drug pair: Entacapone/Tolcapone

² Matched liver clean/liver toxic drug pair: Ibuprofen/Ibuprofen

³ Matched liver clean/liver toxic drug pair: Moxifloxacin/Trovafloracin

⁴ Matched liver clean/liver toxic drug pair: Trazadone/Nefazodone

⁵ Matched liver clean/liver toxic drug pair: Zolpidem/Alpidem