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Evolution of Experimental Models of the Liver to Predict Human Drug Hepatotoxicity and Efficacy

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In this article, we review the past applications of in vitro models in identifying human hepatotoxins and then focus on the use of multiscale experimental models in drug development, including the use of zebrafish and human cell-based, 3-dimensional (3D), microfluidic systems of liver functions as key components in applying Quantitative Systems Pharmacology (QSP). We have implemented QSP as a platform to improve the rate of success in the process of drug discovery and development of therapeutics.^{1,2} Our working definition of QSP is “Determining the mechanism(s) of disease progression and mechanism(s) of action of drugs on multiscale systems through iterative and integrated computational and experimental methods to optimize the development of therapeutic strategies” (Fig. 1).

The stakeholders involved in drug development from academia, industry, and government agencies have long understood the need to improve drug candidate selection by optimizing efficacy while screening out potential toxins so as to concentrate efforts on candidates with favorable chances for market approval. A survey of the number of new drugs released between 2000 and 2009 demonstrated a 25-year low in drug approvals despite increases in research and development (R&D) investment.³ Lavery and colleagues⁴ reported that 66% of failed clinical trials were due to a lack of efficacy and 21% for unacceptable drug toxicity. However, Sacks and colleagues⁵ evaluated clinical drug trials between 2000 and 2012 using additional criteria to further refine the analysis and reported that lack of efficacy alone accounted for only 41% of failures, while the combination of poor efficacy and safety accounted for 35%, and safety alone accounted for 19% of drug failures. Together, cardiovascular and liver toxicity accounted for nearly 75% of all postmarket drug

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withdrawals in the United States between 1975 and 2007.⁶ Although cardiotoxicity has recently surpassed hepatotoxicity as the main organ toxicity ending clinical trials or causing postmarket drug withdrawal, hepatotoxicity has been the most frequent cause of drug product recalls between 1953 and 2014.⁷

CURRENT STATUS OF DRUG HEPATOTOXICITY PREDICTION USING MAMMALIAN IN VIVO MODELS

The liver is responsible for a wide range of functions, including xenobiotic detoxification, protein synthesis, synthesis and storage of glucose, production of the bile necessary for digestion, and regulation of blood cholesterol and triglycerides. The organ is positioned downstream of the gastrointestinal tract to enable “first-pass” clearance of orally ingested drugs and toxins. The structural organization of the liver sinusoidal space facilitates close contact between circulating compounds and the transporter-rich hepatocyte membrane proteins that allow for rapid and efficient transport of drugs from the portal blood. The high capacity for biotransformation in the hepatocyte also facilitates the generation of reactive metabolites that can cause liver damage.⁸

The key data for assessing hepatotoxicity derives from drug safety study protocols approved by regulatory agencies. Although evidence suggests that preclinical animal studies can predict up to approximately 70% of human toxicity, several problems are apparent from this approach.⁶ First, the traditional animal studies clearly fail to identify all possible adverse liver effects, because many compounds pass safely through animal testing only to be found hepatotoxic in the clinical trials or in the postmarketing. Second, the traditional drug safety protocols were designed only to ask broad questions with a simple yes or no answer; for example, is the compound hepatotoxic? Is the compound a reproductive toxin? Traditional preclinical drug safety assessments essentially ignored the why and the how of toxicity.⁹ Historically, few efforts were made to link the observational results from drug safety studies to molecular and cell level events, mechanisms of toxicity (MOTs), or to interactions between tissues and organs.

Adherence to the regulatory agencies’ drug safety protocols also failed to account for the poor concordance between animal and human organ toxicity (Table 1). The concordance can be as low as 40% for the liver to better than 90% for drugs with hematological liability.¹⁰ A good demonstration of how animal testing failed to identify clinical hepatotoxic drugs is exemplified by a series of structurally similar and marketed drug pairs for the same therapeutic indication (Table 2). In each case, one of the drugs in the pair exhibited no hepatotoxicity during preclinical and clinical trials or in postmarket surveillance, but the other was “silent” during the animal studies, yet induced hepatotoxicity in clinical trials or in the postmarket release. This discordance in the liver findings has been attributed to differences in the metabolism and metabolic clearance pathways between man and animal test species.¹¹

After expensive postmarket drug recalls for Troglitazone and Bromfenac and the restrictive labeling for Trovafloxacin and Tolcapone, the pharmaceutical industry initiated strategies to prescreen compounds for liver toxicity. In one example, the industry successfully

implemented in vitro and specialized in vivo pharmacokinetic (PK) screening early in the lead optimization process. Although in 1993 nearly 39% of compounds failed in clinical trials due to poor PK, this fell dramatically to 7% by 2003 following implementation of early drug discovery PK profiling.¹² However, during the same period of time, the rate of compounds failing in the clinic due to toxicity rose from 10% to 16%.¹² Based on the success in PK profiling, it was projected that a strategy similar to early PK screening also would be successful in identifying and eliminating potential hepatotoxins before proceeding into preclinical testing.

IN VITRO MODELS FOR PREDICTING DRUG-INDUCED HEPATOTOXICITY

Many commonly used in vitro hepatotoxicity assays rely on subcellular liver fractions, established hepatoma cell lines, primary animal and human hepatocytes, liver slices, and whole perfused livers. The use of in vitro data from microsomes, primary hepatocytes and S9 subcellular fractions to predict in vivo drug clearance is generally a well-accepted procedure.^{13,14} Whole perfused livers and liver slices on the other hand are low throughput, require continual usage of animals, are costly, and still suffer the lack of concordance with human toxic liabilities. These models have been reviewed elsewhere.¹¹

Rodent and human primary hepatocytes have become a mainstay of hepatotoxicity testing in the in vitro laboratory.¹¹ Large numbers of healthy hepatocytes can be isolated from a single rat or from a human liver resection or autopsy. This has allowed moderate to high-throughput screening to identify potential hepatotoxins, while at the same time reducing animal use, amount of test agent, cost per compound tested, and the time required to make a toxic liability decision. The primary hepatocyte has been a convenient model for investigating MOT, pharmacokinetics, identification of metabolites, and dose response toxicity. However, an important limitation in the use of isolated primary hepatocytes is a reduction in function and differentiation after 24 to 48 hours in culture.¹⁵ To avoid this issue, many in vitro hepatotoxicity tests have been conducted using established human hepatoma cell lines such as HepG2 and HepaRG or with immortalized primary hepatocytes such the Corning HepatoCell (Corning, NY).¹⁶⁻¹⁸ The limitation to these cell types, however, is low or absent biotransformation for many of the important cytochrome P450 enzymes and phase II conjugation reactions involved in drug clearance.^{11,19} The advantages and disadvantages of the more commonly used single-cell and cell fraction in vitro models are compared in Table 3.

The primary hepatocyte and immortalized hepatocytes have been used in 2D monolayers, 2D co-cultures, 3D single-cell type, and 3D co-cultures depending on the questions posed. Two-dimensional monolayer assays have been applied to collect simple cell death end points to triage large numbers of compounds.¹¹ In recent years, high-throughput screening (HTS) assays have been used to measure MOTs known to be relevant to mechanisms of clinical hepatotoxicity. The latter assay type, which is often referred to as “fit-for-purpose,” tests compounds in a model designed solely to identify a specific mechanism of toxicity. Examples of “fit-for-purpose” hepatocyte assays include mitochondrial dysfunction, oxidative stress, bile salt exporter protein inhibition, covalent binding, and pregnane X

receptor nuclear receptor modulation. These 5 mechanisms have a demonstrable association to increased risk for clinical hepatotoxicity.^{20–24}

Although preclinical animal testing remains critical for Investigational New Drug and New Drug Application approval, a significant shift to alternative approaches using quantitative structure-activity relationships (QSAR) computational models, simple in vitro cytotoxicity and “fit-for-purpose” HTS assays have been promoted by governmental agencies such as the Environmental Protection Agency, the National Center for the Advancement of Translational Sciences (NCATS), and the National Toxicology Program. These initiatives have resulted in the development of a number of databases (eg, Tox21, ToxCast) and models for prioritizing compounds based on HTS assay hepatotoxicity, as well as other organ toxicities.²⁵ In addition to the government initiatives, most R&D organizations in academia and the pharmaceutical industry have a slate of in vitro toxicity assays and computational approaches designed to eliminate unfavorable compounds early in the drug discovery process.²⁶

PAST EXPERIENCES WITH IN VITRO LIVER ABSORPTION, DISTRIBUTION, METABOLISM, EXCRETION, AND TOXICITY MODELS PREDICTING HUMAN CLINICAL TOXICITY

The concordance of in vitro toxicity testing with clinical hepatotoxicity varies from only 25% for the simple in vitro assays to nearly 80% for the more complex assays and analyses.^{17,27} Table 4 presents a subset of computational approaches: in vitro 2D human cell-based HTS assays; a human cell co-culture model; and one in vitro covalent binding assay selected from a joint Food and Drug Administration (FDA) National Center for Toxicology Research, California Institute of Technology, and Hannover Medical School report which cross-validated the concordance of these methods to human drug-induced liver injury (DILI).²⁷ Three interesting findings are noteworthy from the results: (1) multiparametric cell-based models performed better than the computational or cell-free covalent binding assays; (2) the predictive result for DILI-negative drugs (assay specificity) was always higher than the predictive result for DILI-positive drugs (assay sensitivity); and (3) an apparent upper limit to the predicted DILI nearing 75% to 80% was reached with the existing in vitro cell-based systems.²⁷ A likely explanation for the plateau of predictive concordance of 80% with existing in vitro cell models is the reductionist strategy used to simplify the organized, multicellular complexity of the liver microenvironment to single-cell or even 2-cell type testing assays.

ZEBRAFISH LARVAE AS A LOW-COST, MEDIUM-THROUGHPUT, WHOLE-ORGANISM PLATFORM TO PREDICT DRUG HEPATOTOXICITY

The use of animals for experimentation, especially warm-blooded species, presents ethical concerns, and governing bodies now strive to implement the reduction, refinement, and replacement (“3R”) of animals strategy in research.²⁸ The European Union Directive 2010/63/EU on the protection of animals used for scientific purposes requires the use of species with the lowest capacity to experience pain, suffering, and distress, and mandates

that the smallest number of animals be used to obtain scientifically valid results. Studies in rodents are further limited by the high costs for acquisition and maintenance.

In recent years, there has been an increased recognition that in vitro phenotypic experimental cell models, as well as small multicellular organisms, can be used in the multiscale approach described as part of QSP.¹ Zebrafish in particular have attracted attention not only as a model for drug discovery, but also as a preclinical model for toxicity assessments.^{29–32} Their prospective position in drug discovery and toxicity assessment is envisioned to be a bridge between simple cell-based and the still mandated mammalian testing.³³

Zebrafish are uniquely positioned for large-scale experimentation. Zebrafish are vertebrate animals with high similarity to mammals, both organotypically and physiologically. They have a tractable, diploid genome that is 70% to 80% similar to humans and that is amenable to both forward and reverse genetics. Because of their small size, zebrafish, at the larval stage, are compatible with multiwell plate formats used in HTS, requiring only small amounts of compounds/drugs. Their high fecundity makes it possible to obtain large numbers of specimens for experimentation, dramatically reducing cost compared with rodent models. The zebrafish embryo therefore provides a cost-effective opportunity to discover potential drug liabilities using functional assays in a living animal as a complement to the emerging human tissue models.

The zebrafish embryonic liver is completely developed and functional by 72 hours post fertilization (hpf), as judged by organ appearance and functional markers, such as phase 1 and phase 2 biotransformation capabilities, serum protein secretion, glycogen storage, and lipogenesis.^{34–36} Importantly, transgenic zebrafish larvae expressing human Cyp3A4 have been developed and will find use in PK and toxicity testing.³⁷

Assays for zebrafish hepatotoxicity have thus far mainly been observational. In zebrafish larvae, necrotic cells can be visually identified by a change in appearance from translucency to opaque black.³¹ A major shortcoming of macroscopic cell death assays is that they are not sensitive enough to detect early toxicity.³⁸ Nonetheless, a variant of this methodology using liver degeneration, changes in size, and yolk sac retention as endpoints has recently been published and shown to predict 8 of 8 known hepatotoxicants.³⁹ Changes in liver appearance, for example, cellular organization, interactions, and shape, also can be detected by histopathology from tissue slices, although this method is time-consuming, requires a trained pathologist, and therefore is usually reserved to validate observations by other measurements. Last, hepatotoxicity can be assessed in the adult zebrafish using canonical liver enzyme assays (eg, alanine aminotransferase), although the use of adults eliminates the convenience, ethical impact, and high-throughput compatibility that embryos offer.⁴⁰ Our own data suggest that even gross organism toxicity, assessed by visual inspection of morphologic changes in 72 hpf larvae (ie, bent tails, distended peritoneum and edema, pericardial congestion) can distinguish hepatotoxic from nontoxic agents (Table 5).

Zebrafish toxicity research is now shifting from observational to mechanism-based toxicity assays. Mesens and colleagues⁴¹ explored a molecular endpoint that captures effects on lipid metabolism because liver injury is frequently associated with perturbations in lipid

metabolism. Hence, the group looked at expression of liver-specific fatty acid binding protein 10a (L-FABP 10a) as a molecular biomarker for hepatotoxicity and found that changes in expression were predictive of specific mechanisms. A corollary example was recently published by Verstraelen and colleagues,⁴² in which they evaluated the expression of 5 liver-specific genes (including 2 apoptosis, and 2 metabolism-related) following exposure with 5 known toxicants. Their results confirmed those of Mesens and colleagues⁴¹ with L-FABP 10a and further documented that biomarker responses are compound-dependent, mechanism-dependent, and concentration-dependent. At the present time, the utility of biomarkers for prediction of toxicity appears to have potential in “fit-for-purpose” studies. If highly predictive biomarkers can be found, the zebrafish offers the opportunity to generate transgenic reporter lines that would greatly increase throughput.

Our own work has embraced adaptation of in vitro, human mechanism-based toxicity models and screening in zebrafish. In addition to morphologic observations (see Table 5), this suite of assays includes measurements of reactive oxygen species (ROS) and mitochondrial membrane perturbations because they are very good predictors of clinical toxicity.²⁴ Fig. 2 illustrates the utility of ROS measurements in zebrafish larvae using menadione. Menadione is a naphthoquinone that generates ROS in cells through redox cycling. Menadione caused time-dependent and dose-dependent generation of ROS in zebrafish that correlated with embryonal toxicity and ROS induction and death in cultured hepatocytes, although there were quantitative differences between these types of models, likely due to differences in drug uptake, glutathione levels, and possibly metabolism. Additional development of these methods is required.

CASE STUDY: USING “FIT-FOR-PURPOSE” ASSAY EVALUATIONS TO RANK-ORDER COMPOUNDS

Given the limits to predicting human hepatotoxicity from current in vitro and in vivo methods, additional improvements to the test systems and analytical methods are needed to select better compounds for preclinical testing. A case study is presented to illustrate one strategy used at the University of Pittsburgh Drug Discovery Institute to rank-order compounds for hepatotoxicity risk. Compounds are screened through zebrafish embryos and a set of in vitro “fit-for-purpose” cytotoxicity and mechanism of toxicity assays are then applied (Fig. 3). Rank ordering does not rely on any one single assay, but as a profile of risk factors calculated from the safety margin (the ratio of toxic level to therapeutic level) categorically binned into high, moderate, or low risk. The development of this approach was an extension of the time-dependent and concentration-dependent multiplexed MOT endpoint assays for mitochondrial function, oxidative stress, and cytotoxicity analyses developed and validated in the CellCiphr HepG2 and primary hepatocyte toxicity panels. Our use of the safety margin to classify risk, taken together with a Pfizer study that reported an increase in concordance between in vitro assays and clinical hepatotoxicity with drugs that induced 2 or more MOTs, suggested that some improvements are possible.^{17,23,43} In the case study described here, the chance of clinical hepatotoxicity increases in compounds with more high and moderate risk factors.

EMERGENCE OF HUMAN TISSUE AND ORGAN MODELS

For the reasons presented previously, new strategies are required to better identify hepatotoxic compounds, especially chronic toxicity, as well as to develop better human efficacy and disease models. The development of biomimetic, multicellular, 3D, microfluidic microphysiology models of the human liver and other organs are in development.^{44,45}

Cellular responses to drugs in the intact human organ are more accurately represented by 3D human cell cultures than the traditional static 2D cell cultures, with additional advantages provided by the inclusion of media perfusion to provide nutrients, oxygen, chemicals, and remove waste products.⁴⁶⁻⁴⁸ Researchers are now capitalizing on the increased availability of human primary, immortalized, or induced pluripotent stem cell (iPSC)-derived hepatocytes, new bioengineering materials, microfabrication techniques, and microfluidic devices to construct reasonable representations of the adult human liver acinus in 3D multicellular microphysiological systems (MPS).^{48,49} These MPS can be maintained for a month or longer, allowing chronic, as well as acute, responses to drug challenges. In our recent study, we demonstrated acute and chronic drug effects, including the induction of fibrosis by methotrexate and the induction of immune-mediated hepatotoxicity.⁴⁸ A comparison of some current static and perfused 3D, multicell models are presented in Table 6.

Of particular interest to those who study hepatotoxicity is the creation of a “liver on a chip” with iPSC-derived adult hepatocytes from patients who have susceptibility to DILI events or other defined genetic and disease backgrounds. This would place liver MPS platforms at the center of personalized medicine and in the continuum of the QSP approach to drug discovery and evaluation of disease progression.^{1,2} The potential ramifications and promises of this new paradigm for drug discovery, disease progression, and toxicity assessments are discussed in more detail in the Prospectus.

PROSPECTUS: MOVING TO THE FUTURE: INTEGRATING THE HUMAN LIVER ON A CHIP, COMPUTATIONAL MODELS, AND QUANTITATIVE SYSTEMS PHARMACOLOGY

Collectively, the limited concordance of laboratory animal drug safety testing with human safety, the apparent 80% limit of success of human-based 2D in vitro models and the 65% to 75% rate of success with computational models to predict drug-induced clinical hepatotoxicity has shifted the focus to the creation of human, 3D, microfluidic systems, referred to as MPS. One such platform has been developed at the University of Pittsburgh Drug Discovery Institute and integrates an MPS liver model using 4 human liver cell types organized into a microfluidic, 3D, sinusoidal complex, with the capacity for live cell monitoring of MOT using fluorescence-based biosensors over a period of several weeks. Secreted proteins, cytokines, and metabolites collected from the efflux are analyzed by biochemical assays and mass spectrometry along with the results from imaging biosensors for parameters such as apoptosis, ROS production, and free calcium levels. All of the data are linked in a database designed to collect, manage, and model the data (Fig. 4). Integrated

human MPS platforms that are biomimetics of normal organ structure and function have the potential to improve on the current predictive limit (approximately 80%) and diminish the odds of “silent” human hepatotoxins from being introduced into clinical trials or the market.

Continued improvements to the MPS liver models will include the application of renewable cells (eg, human iPSC-derived adult hepatocytes, as well as the nonparenchymal cells) to permit the investigation of the heterogeneous human genetic backgrounds, as well as specific diseases (eg, nonalcoholic fatty liver disease, hepatocarcinoma, and rare childhood liver diseases). Further advances also will include liver metabolic zonation and higher throughput arrays of MPS. The microphysiology database also will continue to evolve as a tool to manage, mine, and model the experimental data, as well as public sources of preclinical and clinical findings, expert-based drug knowledge, physical properties, pharmacology targets, adverse event reporting, and large datasets from “omics,” including toxicogenomics, metabolomics, proteomics, reactive metabolite proteomics, and transcriptomics.⁵⁰ Furthermore, QSP is expected to increase our understanding of the integrated and interacting cellular, tissue, and organ networks; genes; proteins; and metabolic processes that give rise to liver disease progression, therapeutic efficacy, and drug-induced hepatotoxicity.^{1,50}

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KEY POINTS

- Quantitative Systems Pharmacology is a multiscale, iterative, and integrated computational and experimental approach for optimizing the development of therapeutic strategies.
- Limited efficacy and drug safety methods continue to limit the efficiency of the discovery and development of therapeutics.
- Mammalian in vivo models have low concordance to clinical hepatotoxicity.
- The predictive concordance between the existing in vitro experimental models coupled with computational modeling and clinical hepatotoxicity has reached a limit.
- Three-dimensional, multicellular, microfluidic, human tissue and organ experimental models are a promising alternative to whole animal and cultured cell methods for efficacy and safety testing.

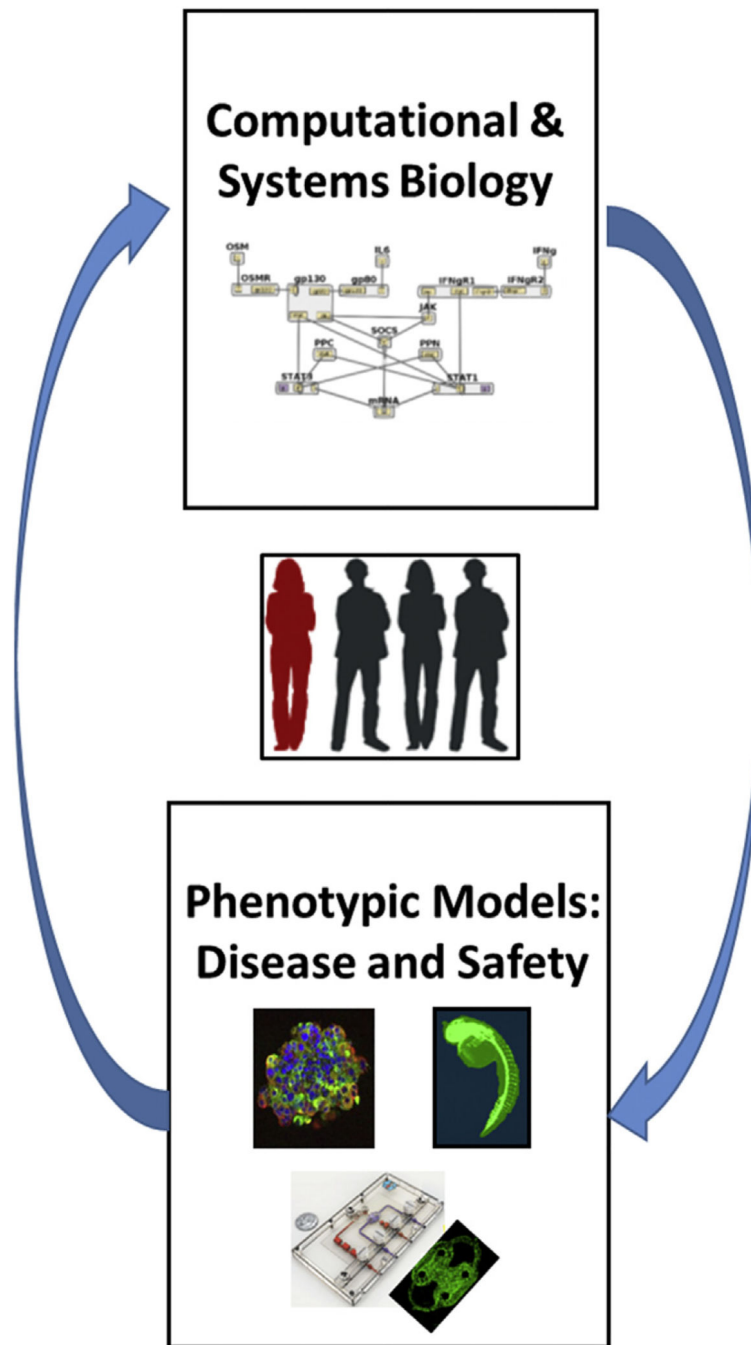
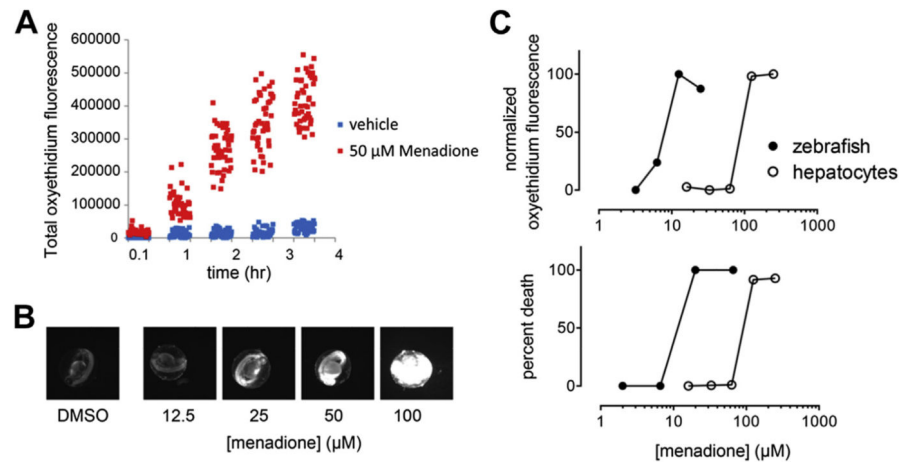


Fig. 1. QSP is an approach to drug discovery and development that applies iterative and integrated computational and experimental methods to determine the mechanism(s) of disease progression and mechanism(s) of action of drugs on multiscale systems. QSP starts with patients and patient samples, applies computational and experimental models, and ends with fundamental knowledge that optimizes therapeutic treatments for patients. This article focuses on the use of multiscale experimental models for liver toxicity and efficacy testing, especially phenotypic models.

**Fig. 2.**

ROS generation correlates with death in zebrafish larvae and rat hepatocytes. (A) Zebrafish larvae at 72 hpf were arrayed in 96-well microplates, loaded with dihydroethidium (DHE) for 30 minutes, and treated with menadione (50 μ M). At the indicated time points, plates were scanned and analyzed for red oxyethidium fluorescence on an ArrayScan VTi high-content reader (ThermoFisher, Waltham, MA, USA). (B) Fluorescence micrographs of dose-dependent menadione-induced oxyethidium generation at 48 hpf. (C) Zebrafish larvae at 72 hpf (*closed circles*) or cultured rat hepatocytes (*open circles*) were treated in 96-well plates with various concentrations of menadione and oxyethidium fluorescence quantified. Toxicity correlated with production of ROS in both zebrafish embryos and hepatocytes.

SAR Compound Series Rank Ordered by Hepatotoxicity Risk

Compound	ZebraFish Embryo	HepG2		Primary Hepatocytes			Physical Characteristics	
	μ MALD ^a	1 h mito function μ MIC50	72 h cytotoxicity μ MIC50	1 h mito function μ MIC50	4 h ROS μ MIC50	5-d Cytotoxicity μ MIC50	cLogP	pKa
Cmpd 1							4.16	2.41
Cmpd 2							2.75	2.22
Cmpd 3		34					2.54	2.21
Cmpd 4		73					1.59	3.38
Cmpd 5		48	169	284			2.37	3.39
Cmpd 6			49			155	4.03	2.21
Cmpd 7		169	28			280	4.03	2.21
Cmpd 8		70	202				4.61	3.77
Cmpd 9			16				2.92	3.4
Cmpd 10	86		183		197	210	3.74	5.28
Cmpd 11		11	160				3.48	2.41
Cmpd 12	21	35	75				4.24	2.22
Cmpd 13	21	20	24			140	4.61	4.61
Cmpd 14	5	8	18	80	143	147	4.16	2.42
Cmpd 15	6.2	25	15				5.05	4.91
Cmpd 16	<5 ^b	4	6	2	180	230	5.86	2.42
Cmpd 17	0.3	2	31	10	6	62	4.46	2.42
Cmpd 18	5	8	7	33	17	63	4.82	2.43
Cmpd 19	14	6	11	13	15	45	4.92	2.42

Less Risk



More Risk

Color Coded Safety Margin

<10 X Cmax	10-30 X Cmax	>30 X Cmax
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Fig. 3.

Case use of multiple “fit-for-purpose” assays to determine hepatotoxicity risk. The concentration of inhibitor in which the response is reduced by half (IC_{50}) results from 6 different assays in zebrafish, HepG2 and primary hepatocytes are used to calculate the safety margin, defined as the toxic IC_{50} response/ C_{max} blood concentration. The lower the safety window the higher the risk for hepatotoxicity. To rank-order compounds, the safety margin is categorized into high (*red*), moderate (*yellow*), or low (*green*) risk to generate the heat map. The overall rank order is determined by the number of high and moderate risks. ^a Acute lethal dose at which one-half of zebrafish embryos are dead by 24-hour exposure. ^b Drug quantity not sufficient (QNS) to repeat study to calculate safety margin.

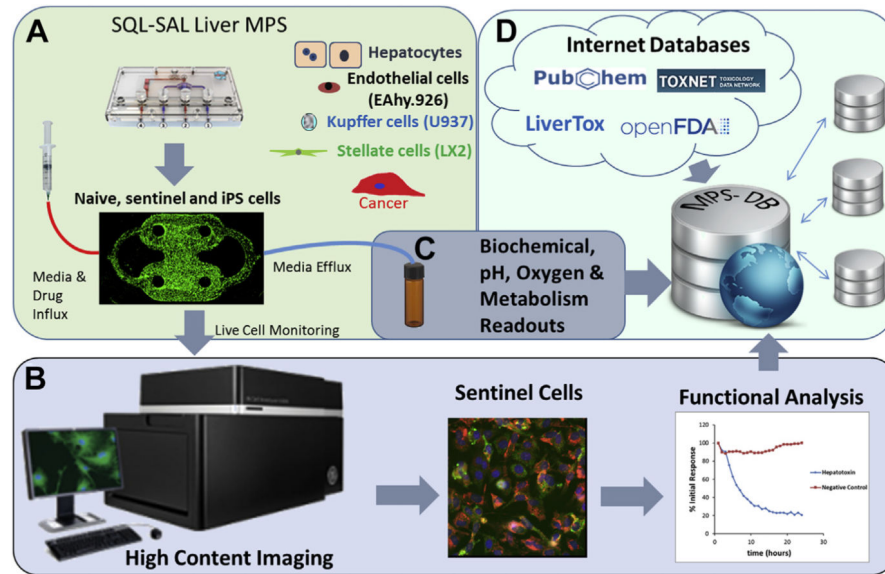


Fig. 4. Overview of the Human Liver Microphysiology Platform for studying human liver physiology, disease models and drug safety testing. The platform is composed of the following: (A) the Sequentially Layered, Self-Assembly Liver model (SQL-SAL) constructed from a microfluidic device and 4 human cell types, a fraction of which are “sentinel” cells expressing fluorescence-based biosensors, and that can include disease-specific cells, such as cancer cells. Data are collected from the model via (B) high-content imaging readouts of transmitted light contrast and fluorescence; and (C) biochemical and mass spectrometry readouts.^{48,51} (D) The multiplexed data are uploaded into the *Microphysiology Systems Database* (MPS-DB) to manage data, associate external data sources, and build predictive models of human efficacy and toxicity.⁵⁰

Table 1

Concordance of animal and human organ toxicity

Target Organ	Concordance, %
Liver	40–54 ^{10,52}
Cutaneous/Ophthalmic	36
Endocrine	60
Urinary tract	64
Neurologic	70
Cardiovascular	80
Gastrointestinal	85
Hematologic	91

Data from Olson H, Betton G, Robinson D, et al. Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regul Toxicol Pharmacol* 2000;32(1):56–67.

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Table 2

Clinical liver effects of structurally similar drugs found safe in animals

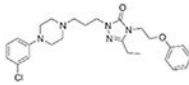
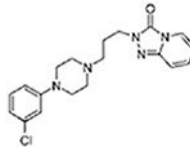
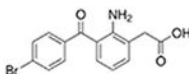
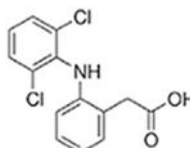
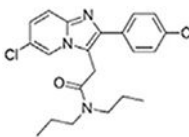
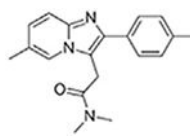
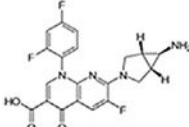
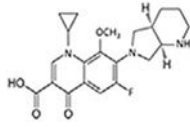
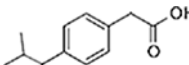
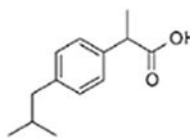
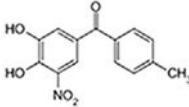
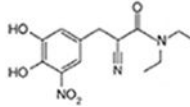
Human Liver Toxic Drug	Structure	Human Liver Safe Drug	Structure
Nefazodone		Trazodone	
Bromfenac		Diclofenac	
Alpidem		Zolpidem	
Trovafloxacin		Moxifloxacin	
Ibufenac		Ibuprofen	
Tolcapone		Entacapone	

Table 3

Commonly used cell and cellular fraction in vitro models for absorption, distribution, metabolism, excretion, and toxicity (ADMET)

Model	Application	Advantage	Disadvantage	Reference
Hepatocyte cell fractions microsomes	Liver clearance, metabolite ID	Fast, inexpensive, individual variation can be studied	Overestimates in vivo metabolism; only CYP and UGT enzymes	14,53
S9	Liver clearance, metabolite ID	Phase I and phase II activity	Lower enzyme activity in the S9 fraction, may miss low level metabolites	14
1° hepatocyte suspensions	Liver clearance, metabolite ID	In vivo levels of drug metabolism and transport proteins, cryopreservation	4-h time limit	19
Transgenic cell lines for PK	Metabolite ID	Single enzyme reactions generate high levels of metabolites for structural ID	Overestimate involvement of one enzyme species	14
Established liver cells lines HepG2	Toxicity testing, MOT, induction	Established cell line, inexpensive	Absence or low expression of most phase I and phase II enzymes	14,19
HepaRG	Toxicity testing, MOT, induction, metabolite ID	CYP1A2 and 3A4 inducible, established cell line	High CYP3A4, Cyp 7A1 expression, but low in all other CYP levels compared with primary hepatocytes	19,54
Primary Heps	PK, toxicity testing, metabolite ID	Well characterized, intact metabolism, intact transporters, cryopreservation	Decline in differentiated functions; no immune or fibrosis cells; single donor variation	14
Spheroids: established cell lines HepG2, HepaRG	PK, toxicity testing, metabolite ID	Extend differentiated cell functions from days to weeks, 3D, improved metabolism	Cell functions lower than primary hepatocytes, low urea, albumin production	55–57

Abbreviations: CYP, cytochrome p450; Heps, hepatocytes; MOT, mechanisms of toxicity; PK, pharmacokinetic; UGT, udp-glucuronosyltransferase

Table 4

Concordance of large-scale in vitro screening to human hepatotoxicity^a

Model	Name/Type	# Compounds	Overall ^b Concordance, %	True Positives, %	True Negatives, %	Reference
In silico models						
Structural alert	DEREK ^c	623	56	46	73	58
QSAR/Molecular descriptors	4 commercial programs ^d	~1600	63	39	87	59
2D molecular descriptor	2D molecular descriptor	382	76	76	75	60
	PaDel molecular descriptor ^e	1087	69	67	70	27,61
	ECF 6 molecular descriptors ^f	295	59	53	65	62
2D in vitro cell models						
HepG2	6 endpoint ^g	102	70	40	100	63
HepG2	11 endpoint ^h	136	76	N/A ⁱ	N/A	17
1° Human hepatocytes	4 endpoint ^j	344	75–80	50–60	95–100	64
Co-cultured 1° human hepatocytes, stromal cells	Micropattern surface 4 endpoint ^k	45	78	66	90	65
In vitro cell-free assays	Covalent binding assay-glutathione adduct formation	223	68	45	90	66

Abbreviations: QSAR, quantitative structure-activity relationship; 2D, 2-dimensional.

^aCross-validated results.

^bConcordance = True positives (sensitivity) + true negatives (specificity)/2.

^cDEREK - toxicity prediction software (Lhasa, Leeds, UK)

^dMC4PC, MDL-QSAR, BioEpisteme, Predictive Data Miner.

^ePaDel open source software for calculating molecular descriptors.

^fECF (extended connectivity functional fingerprints).

^gCell counts, nuclear area, plasma membrane integrity, lysosomal activity, mitochondrial membrane potential and mitochondrial area.

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^h Cell count, DNA degradation, nuclear size, cytoskeletal disruption, DNA damage response, oxidative stress, mitosis, stress kinase, mitochondrial membrane potential and area, cell cycle arrest. Results not cross validated.

ⁱ Not available.

^j Cell count, mitochondrial damage, oxidative stress, and intracellular glutathione.

^k Glutathione levels, ATP levels, albumin, and urea secretion.

Data from Chen M, Bisgin H, Tong L, et al. Toward predictive models for drug-induced liver injury in humans: are we there yet? *Biomark Med* 2014;8(2):201–13.

Table 5

Gross morphologic observations in zebrafish larvae identify known toxicants including hepatotoxic amiodarone

Compound/Dose Range	Gross Morphology	Concentration, μ M						
		200	66	20	6.6	2	0.66	DMSO
Dose range		200	66	20	6.6	2	0.66	DMSO
Menadione	Live/dead ^a	0/4	0/4	0/4	4/0	4/0	4/0	4/0
	Visual toxicity ^b				1/4	2/4	0/4	0/4
Amiodarone	Live/dead	0/4	0/4	4/0	4/0	4/0	4/0	4/0
	Visual toxicity			2/4	3/4	0/4	1/4	0/4
Dose range		1000	300	100	30	10	3	DMSO
Caffeine	Live/dead	4/0	4/0	4/0	4/0	4/0	4/0	4/0
	Visual toxicity	3/4	4/4	0/4	0/4	0/4	0/4	0/4
Dose range		20	6.6	2	0.66	0.2	0.07	DMSO
CCCP	Live/dead	1/4	4/0	4/0	4/0	4/0	4/0	4/0
	Visual toxicity	0/1	2/4	3/4	3/4	4/4	2/4	0/4
Rotenone	Live/dead	1/4	3/4	4/0	4/0	4/0	4/0	4/0
	Visual toxicity	1/1	0/3	1/4	2/4	3/4	2/4	0/4

Abbreviations: CCCP, Carbonyl cyanide m-chlorophenyl hydrazone; DMSO, dimethyl sulfoxide.

^a Translucent (live), Opaque (dead).

^b Bent tail, distended peritoneum, edema, pericardial congestion.

Table 6

Examples of multicellular static and microfluidic liver models for absorption, distribution, metabolism, excretion, and toxicity (ADMET) testing

Model	Application	Advantage	Disadvantage	References
Static models				
Co-culture spheroids	Stellate cell activation	Long term 3D culturing with improved drug metabolism and output of albumin, urea	Specialized plates to from spheroids, specialized culturing techniques	67
Co-culture micropatterned primary hepatocytes with fibroblasts	Hepatotoxicity Metabolite ID Disease models	Hepatocytes maintain differentiated function 2–3 wk	2D cultures, specialized plates	65,68
Four-cell spheroids primary hepatocytes, primary liver NPC	Hepatotoxicity Metabolite ID	Hepatocytes maintain differentiated function >3 wk, 3D, immune-mediated toxicity	Specialized culturing techniques	69
3D microfluidic, multicellular liver models				
Primary hepatocytes, endothelial cells	Hepatotoxicity	Hepatocytes maintain differentiated function >3 wk, microfluidic improves function	Specialized culturing techniques, perfusion system	70
Hepatocytes, endothelial cells, stellate cells, Kupffer-like immune cells	PK, toxicity, therapeutic intervention, liver disease model	Hepatocytes maintain differentiated function >3 wk, immune-mediated toxicity, fibrosis activation microfluidic improves function	Specialized culturing techniques, perfusion system	48,51,71

Abbreviations: 2D, 2 dimensional; 3D, 3 dimensional; NPC, non parenchymal cells; PK, pharmacokinetic.