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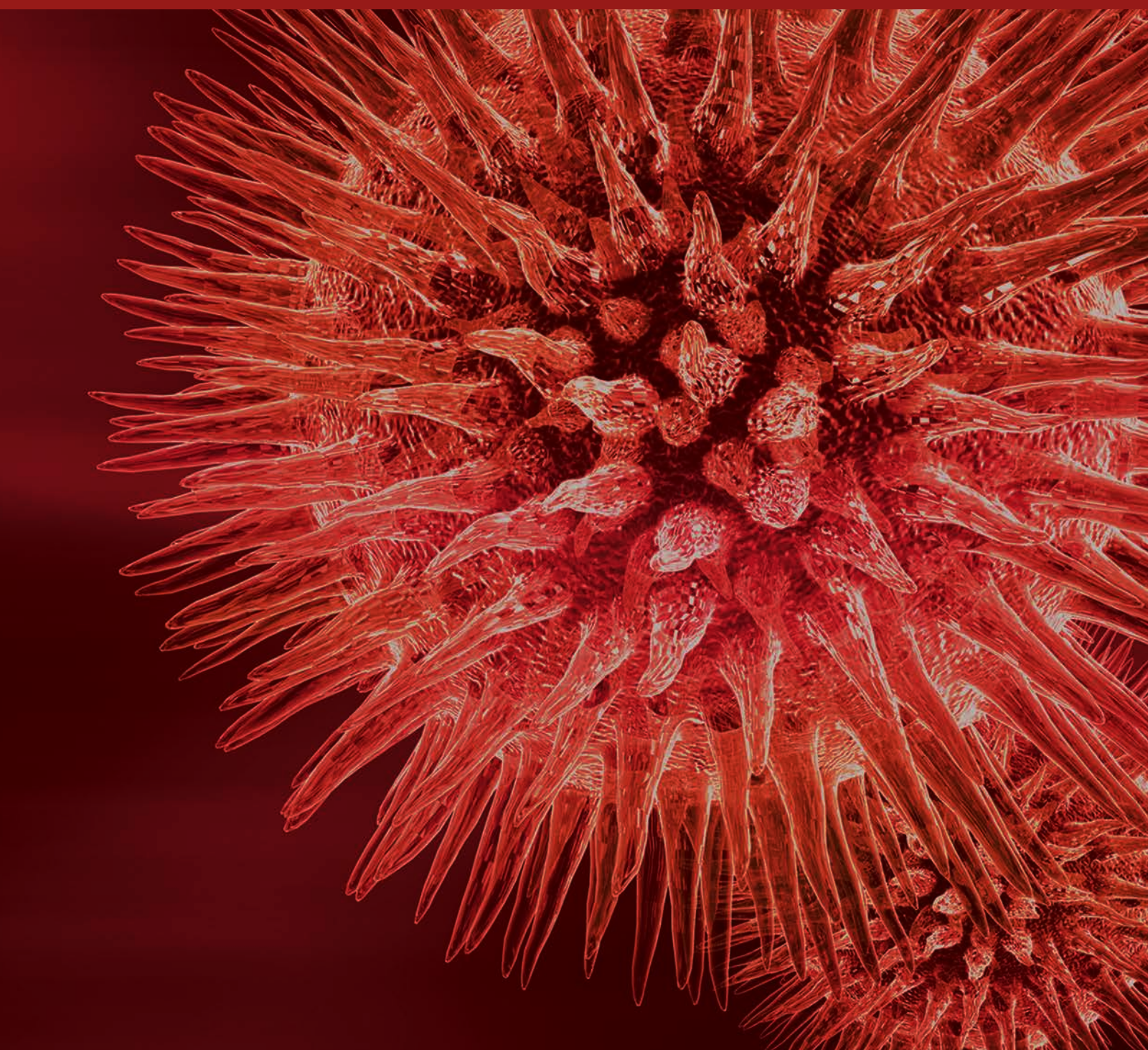
Drug-Induced Liver Injury

Guest Editors: Minjun Chen, Jürgen Borlak, Ayako Suzuki, and Eileen E. N. Almario



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Editorial
Drug-Induced Liver Injury

Eileen E. N. Almario,[1] Jürgen Borlak,[2] Ayako Suzuki,[3] and Minjun Chen[4]

[1]*Office of Computational Science, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD, USA*
[2]*Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany*
[3]*Department of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR, USA*
[4]*Division of Bioinformatics and Biostatistics, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA*

Correspondence should be addressed to Minjun Chen; minjun.chen@fda.hhs.gov

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Among acute hepatic injuries drug-induced liver injury (DILI) is rare; nonetheless it is one of the most common causes for withdrawal of drugs from the market. Progress has been made in the development of tools to identify the risk for DILI once it occurs in clinical trials. Recent efforts defined drug properties, for example, reactive metabolites, lipophilicity, and therapeutic dose which were found to correlate with the hepatotoxicity potential of chemical entities. More work is needed, however, to understand how to predict liver injury for individual patient.

This special issue addresses current needs for an improved understanding of DILI and highlights the importance for a systematic, collaborative linking of in vitro, preclinical, and clinical evidence data to enhance DILI prediction. A total of 17 submissions contributed to this special issue with 7 manuscripts published, including three reviews discussing the challenges of in vitro models, engineered liver models, and herbal hepatotoxicity, followed by two studies in zebrafish models and two mechanistic studies of hepatotoxicity induced by Cyclosporine A and valproic acid.

In a paper coauthored with colleagues from the regulated industry, F. A. Atienzar et al. review the critical challenges and opportunities associated with the use of in vitro models for predicting human DILI. Specifically, they emphasize lack of standardization as the major challenge and describe some practice issues (e.g., DILI classification, cut-off concentration for in vitro assay, and endpoint selection) that can further improve the development of such models. They highlight the need for an integrated approach in assessing toxicity, particularly for idiosyncratic DILI which is less reliably predicted by current tools, citing successful use of in vitro models for toxicity prediction in other domains (e.g., proarrhythmia risk).

C. Lin and S. R. Khetani systematically review recent technological progress in developing liver models, including micropatterned cocultures, spheroidal and bioprinted cultures, perfused biochips, precision cut liver slices, and humanized rodents together with high-content assays and in silico prediction. They highlight the challenges in developing a realistic model and the selection of measures and endpoints that correspond to phenotypic DILI in humans. They conclude that advances in engineered liver models will enable better prediction of toxicity and an understanding of idiosyncratic DILI to eventually reduce drug attrition, animal usage, and DILI risk in humans.

With the growing use of Chinese Herbal Medicine (CHM) worldwide, the implication of associated DILI needs to be considered. C. Liu et al. systematically summarize the historical literature and current scientific knowledge on herbal toxicity and reiterate safety regulation of herbal medicine in China. They emphasize the importance of accurate diagnosis and treatment of herb-induced liver injury and specifically discuss the contents and important implications of the recently released Chinese guideline for diagnosis and treatment for herb-induced liver injury.

Valproic acid (VPA) has been marketed for nearly 40 years although its mechanism of action is still not fully understood. Warnings and contraindications have been endorsed to reduce adverse drug reactions which may include serious or life threatening liver injury. R. Chang et al. examined the

effect of VPA on oleic acid-induced hepatocyte steatosis in the FL83B cell line and found VPA to enhance oleic acid-induced lipid droplet accumulations in a dose-dependent manner. They found that VPA triggered PPARγ nuclear translocation to endorse lipogenesis rather than lipolysis and to increase expression of the cell surface fatty acid transporter CD36, thus further augmenting VPA-induced hepatic steatosis.

A. Korolczuk et al. report a study with Cyclosporine A, that is, an immunosuppressive drug that revolutionized transplantation medicine some 30 years ago. After 28 days of drug treatment, they found that impaired liver function was associated with ultrastructural damage in mitochondria accompanied by significant changes in oxidative stress markers and lipid peroxidation products. Based on these observations, they suggested that oxidative stress and mitochondria damage might play a crucial role in the course of Cyclosporine A induced hepatotoxicity.

Zebrafish is a promising model in the assessment of DILI but its utility needs further exploration. D. Cheng et al. employ a biomolecular imaging approach that provides a full image set for ultrastructural mapping of the zebrafish larvae gastrointestinal system. They conclude that this imaging approach could be used for studying various digestive disorders and drug delivery pathways in the zebrafish.

In another study by H.-S. Nam et al., zebrafish exposed to escalating doses of tamoxifen were found to express miRNA-122 in the liver but not in other organs. Histological changes and tamoxifen blood concentration varied in a dose-dependent pattern similar to acetaminophen exposure. The authors conclude that miRNA-122 might be a potential marker for acute liver toxicity in zebrafish.

These selected articles portend well for the translation of basic science findings into tools that enhance DILI prediction, enable directed therapy to minimize harm, and preserve the availability of therapies in patients that are likely to benefit.

Acknowledgments

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Eileen E. N. Almario
Jürgen Borlak
Ayako Suzuki
Minjun Chen

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Review Article

Research Advances on Hepatotoxicity of Herbal Medicines in China

Changxiao Liu,[1] Huirong Fan,[2] Yazhuo Li,[1] and Xiaohe Xiao[3]

[1]*Research Center for New Drug Evaluation, Tianjin Institute of Pharmaceutical Research, Tianjin 300193, China*
[2]*Institute of Radiation Medicine, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin 300192, China*
[3]*China Military Institute of Chinese Medicine, Integrative Medical Center, 302 Military Hospital of China, Beijing 10003, China*

Correspondence should be addressed to Changxiao Liu; liuchangxiao@163.com

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In general, herbal medicines have been considered as safe by the general public, since they are naturally occurring and have been applied in treatment for over thousands of years. As the use of herbal medicine is rapidly increasing globally, the potential toxicity of herbal drugs, in particular drug-induced liver injury (DILI), has now become a serious medical issue. According to the literature, the authors analyzed and discussed the hepatotoxicity problem of Chinese herbal medicines (CHM), including global overview on herbal-induced liver injury (HILI), current research progress on toxic CHM, diagnosis and treatment of HILI, and modern approaches and technologies of study of hepatotoxicity. As to promote the recognition of HILI and tackle the issue, a guideline for the diagnosis and treatment of HILI has recently been drafted by Chinese scientists. As suggested by the guideline, the hepatotoxicity issue of CHM, as a matter of fact, is overestimated. Up to date, the investigation of hepatotoxicity of CHM is now booming with worldwide application of CHM. This review therefore provides useful information for investigating hepatotoxicity of herbal medicine and characterizing DILI caused by CHM. In addition, authors describe in which way further efforts should be made to study the rationale of CHM and liver injury.

1. Introduction

In general, herbal medicines have been considered as safe by the general public, since they are naturally occurring and have been applied in treatment for over thousands of years. As the use of herbal medicine is rapidly increasing globally, the potential toxicity of herbal drugs, in particular drug-induced liver injury (DILI), has now become a serious medical issue. Many herbal medicines exhibit pharmacological activities and toxic effects by chemical substances as secondary metabolites. Some herbal medicines have been associated with hepatotoxicity, which is reversible upon discontinuation of the treatment by patients [1].

A large number of studies have identified chemical substances with a hepatotoxic effect, which has been linked to liver injury; therefore, the hepatotoxic effect is defined as drug-induced liver injury (DILI). Oh et al. analyzed herbal-induced liver injury (HILI) by performing literature review from eight databases, including PubMed, Medline, the Cochrane Library, EMBASE, and four Korean electronic databases. Based on the comprehensive data, the analysis indicated the incidence of hepatotoxicity in patients using herbal drugs and the possibility of increased risk of HILI by coadministration of herbal and conventional medicines [2]. In China, herbal hepatotoxicity and diagnosis of HILI have been reported widely from preclinical studies to clinical observation. However, HILI study remains still rather difficult.

In this paper, the authors provide an analysis and discussion of the hepatotoxicity of CHM. They also include a global overview of herbal-induced liver injury (HILI), current research progress on toxic CHM, diagnosis and treatment of HILI, and modern approaches and technologies used to study hepatotoxicity. The investigation of CHM hepatotoxicity is now booming with worldwide application of CHM; however, the hepatotoxicity of CHM is overestimated. A guideline for the diagnosis and treatment of HILI has now been drafted. This review therefore provides useful information for

Table 1: Selected herbal medicines listed in LiverTox.

Herbal medicines	Annotation in Chinese
Polygonum multiflorum Thunb.	*Heshouwu*
Tripterygium wilfordii Hook. f.	*Leigongteng*
Bupleurum chinense DC.	*Chaihu*
Epimedium brevicornum Maxim.	*Yinyanghuo*
Silybum marianum (L.) Gaertn.	*Shuifeiji*
Cassia acutifolia Delile	*Fanxieye*
Rhamnus purshiana DC. (cascara)	*Shuli*

investigating hepatotoxicity of herbal medicine and characterizing DILI caused by CHM and outlines the direction of further efforts in the study of CHM and liver injury.

2. Research Overview on DILI/HILI

Over 30 herbal medicines were reported to cause DILI by LiverTox, a database maintained by the US National Library of Medicine (Table 1). Meanwhile, herb-related products were ranked as the second among the most common causes to cause DILI in the US based on the recent guideline published by the American College of Gastroenterology (ACG) [3].

However, in clinical practice, the definitive DILI diagnosis is extremely difficult because there are not a test and criteria existing for accurate diagnosis. RUCAM (Roussel Uclaf Causality Assessment Method) [4], along with the Maria and Victorino scale [5], and the structured methodologies based on expert opinion proposed by the US DILIN (Drug-Induced Liver Injury Network) recently, are the most popularly used methods for assessing the causality between liver injury and the implicated medications [6]. Unfortunately, test-retest reliability and interrater reliability were reported as 0.54 and 0.45, respectively, when using the RUCAM scales reported by a study from the DILIN, suggesting that the reliabilities of applying such standardized causality assessment methods are rather low [7].

Although identifying all medications is a vital element in determining DILI causality, it is extremely difficult to achieve for an accurate DILI assessment if solely relying on patient recall [8] because of increased polypharmacy from multiple independent providers. As for herbal DILI, this scenario is particularly complicated due to polypharmacy and the combined use of chemical pharmaceuticals.

As reported by WHO (World Health Organization) [9], herbs in the marketplace are frequently contaminated with the hazardous materials (e.g., heavy metals, mycotoxins, and pesticides), alternative plant species, and fillers that are not listed on their labels, which could cause misidentification of causality and lead to confounding of the scientific diagnosis of herbal DILI, overdiagnosis, and overreporting [10]. For example, the hepatotoxicity of black cohosh [11, 12] and *Pelargonium sidoides* [13] have been suspected with controversial arguments from the confounding variables. As commented by Dr. Teschke et al. [14], quality is far more important than the quantity for the causality assessment. So far, the universal causality assessment methods (e.g., the RUCAM scale) still heavily rely on the exclusion of other causes of liver injury, and consequently the identification of the real risk associated with herbal DILI has been minimally addressed.

3. Long-Term Application of Toxic Drug in China and Current Status of Toxic CHM Research

3.1. Documented Toxic CHM in China. "*Zhou day official*" (*Zhouli Tiangong* in Chinese), an ancient medical book, recorded that physicians are in charge of the government and apply poly poisons for medical use, which reflects the concept of coexistence of therapeutic effects and adverse reaction of drugs. *People's Republic of China Pharmacopoeia* (2005 Edition) contains 72 toxic drugs, including 10 toxic medicines, 38 moderate toxic medicines, and 24 mild toxic medicines. Table 2 lists the typical toxic Chinese herbal medicines originated from including medicinal herbal plants, minerals, and animals.

3.2. Chinese Medicines with HILI in Animal Experiments. Chinese medicines with HILI that have been identified in animal experiments are listed in Table 3.

In addition, some topical medications, such as fish guts, rotenone, and realgar, have been identified with liver toxicity in animal studies which may lead to liver damage with varying degrees if taken orally. Since the toxic dose is far more over the amount used in the treatment, the possibility of occurrence of toxicity remains at very low level. Apart from this, the hepatotoxicity of certain herbal medicines cannot be confirmed, due to lack of reliable clinical evidence.

3.3. Chinese Medicines with HILI in Clinics. Chinese medicines associated with HILI in clinical use have been reported. Chinese medicines associated with HILI in clinical use have been reported as shown in Table 4. There are also several known CHM compound formulations associated with liver injury in clinical observation. CHM formulations with DILI are listed in Table 5.

For example, *Polygonum multiflorum* Thunb. (*Heshouwu* in Chinese), which is officially listed in the Chinese Pharmacopoeia, is one of the most popular perennial Chinese traditional medicines but is also associated with HILI. Currently, pharmacological studies have unveiled its key benefits in the treatment of various diseases, providing information relevant to pharmacokinetics-pharmacodynamics analysis, neurodegenerative diseases, dyslipidemia treatment, and sleep disorders [18].

3.4. Categories of Chemical Substances with HILI. Herbal drug-induced liver injury (HILI) is now a hot spot in the field of CHM safety study. Rapid screening and evaluation of herbal drug-induced liver injury have become one of the key techniques of herbal research. In this section, the research status of HILI from active, toxic ingredient, mechanism of toxicity, and toxicity reduction of Chinese herbal drugs is discussed. According to the structural information of the

Table 2: Typical toxic Chinese herbal medicines.

Source types	Chinese herbal medicines	Medicinal source
Plants	Radix Aconiti Kusnezoffii	*Aconitum kusnezoffii* Reichb.
	Radix *Aconiti penduli*	*Aconitum pendulum* N. Busch
	Rhizoma Arisaematis	*Arisaema erubescens* (Wall.) Schott
	Flos Daturae	Datura *metel* L.
	Rhizoma Typhonium	*Typhonium giganteum* Engl.
	Radix Aconiti Lateralis	*Aconitum carmichaelii* Debx.
	Rhizoma Pinellia	*Pinellia ternate* (Thunb.) Breit.
	Semen Nut-vomitive	*Strychnos nux-vomica* Linn.
	Herba Euphorbia	*Euphorbia kansui* L.
	Radix euphorbiae lantu	*Stellera chamaejasme* Linn.
	Garcinia	*Garcinia pedunculata* Roxb.
	Leptochloa	*Leptochloa chinensis* (L.) Ness
	Radix Rhododendroni molli	*Rhododendron molle* (Blume) G. Don
	Semen Hyoscyami	*Hyoscyamus niger* L.
	Croton	*Croton tiglium* L.
Animals	Venenum Bufonis	Asiatic toad
	Cantharidin	*Lytta caragana* Pallas
	Huechys sanguinea	*Huechys sanguinea* (De Geer)
Minerals	Arsenic	Arsenic trioxide
	Arsenolite	Arsenolite
	Arsenic stone	Arsenic stone
	Rabiagar	Arsenic disulfide
	Calomelas	Mercurous chloride
	Hydrargyri oxydum Rubrum	Hydrargyri oxydum Rubrum
	Mercury	Mercury

Table 3: Chinese medicines with HILI on animals.

Chinese herbal medicines	Annotation in Chinese	Medicinal plant
Anisi Stellati Fructus	*Bajiao*	*Illicium verum* Hook. f.
Radix Sanguisorbae	*Diyu*	*Sanguisorba officinalis* L.
Fructus *Gardeniae*	*Zhizi*	*Gardenia jasminoides* Ellis
Galla Chinensis	*Wubeizi*	*Rhus chinensis* Mill
Cortex Granati	*Shiliupi*	*Punica granatum* L.
Chebulae Fructus	*Hezi*	*Terminalia chebula* Retz.
Rhizoma Acori tatarinowii	*Shichangpu*	*Acorus tatarinowii* Schott
Fructus Foeniculi	*Xiaohuixiang*	*Foeniculum vulgare* Mill.
Cortex Cinnamomi	*Guipi*	*Cinnamomum cassia* Presl
Fructus Aristolochiae	*Madouling*	*Aristolochia debilis* Sieb. et Zucc.
Caulis Akebiae	*Mutong*	*Akebia quinata* (Thunb.) Decne

chemical composition, toxic substances can be divided into the seven categories (in Table 6) [19].

3.5. Current Status of HILI in China. *Heshouwu* (dried root of *P. multiflorum*) has been traditionally used in China as a tonic for liver and kidney conditioning for thousands of years without significant adverse effects found. Currently, *Heshouwu* has been widely used to prevent hair loss and graying, prevent aging, and extend lifespan, which also has potential for treatment of Parkinson's disease, liver injury, hyperlipidemia, and Alzheimer's disease. However, a typical example of DILI causality determination of *Heshouwu* was presented by Wang et al. [15]. The major active ingredient might be 2,3,5,4′-tetrahydroxy trans-stilbene-2-O-β-glucoside (TSG), and it was found to expand the lifespan of model organisms by activating Sirtuin 1 (Sirt1) [20, 21] and cause autophagy stimulation [22]. In Hong Kong, *Heshouwu*-related DILI cases were identified in 1996 and consequently led to the announcement by the Medicines and Healthcare Products Regulatory Agency in 2006. Therefore, the reports related to *Heshouwu* DILI have caused increased concerns regarding the safety of herbs usage.

Table 4: Herbal medicines with HILI used in treatment.

Chinese herbal medicines	Annotation in Chinese	Medicinal plant
Cortex Albiziae	*Hehuanpi*	*Albizia julibrissin* Durazz
Semen Strychni	*Maqianzi*	*Brucea javanica* (L.) Merr.
Rhizoma Alismatis	Zexie	*Alisma orientale* (Sam.) Juzep.
Folium Sennae	*Fanxieye*	*Cassia angustifolia* Vahl.
Semen *Cassiae*	*Juemingzi*	*Cassia tora* Linn.
Herba Chenopodii	*Tujinjie*	*Chenopodium ambrosioides* L.
Folium *Clerodendri*	*Chouwutong*	Clerodendrum trichotomum Thunb.
Radix Dioscoreae bulbifera	*Huangdu*	*Dioscorea bulbifera* Linn
Omoto Nipponlily	*Wannianqing*	*Rohdea japonica* (Thunb.) Roth
Herba Ephedrae	*Mahuang*	*Ephedra sinica* Stapf
Herba Gynurae	*Jusanqi*	Gynura japonica (Thunb.) Juel.
Semen Hydnocarpi Hainanensis	*Dafengzi*	Hydnocarpi Hainanensis (Merr.) Sleum.
Folium Ilexi pubescensi	*Maodongqing*	*Ilex pubescens* Hook. et Arn.
Fructus Trichosanthis	*Gualou*	*Trichosanthes kirilowii* Maxim.
Fructus Meliaceae	*Kulianzi*	*Melia azedarach* L.
Semen *Myristicae*	*Roudoukou*	*Myristica fragrans* Houtt.
Herba Papaveri somniferi	*Yingsu*	*Papaver somniferum* L.
Radix Phytolaccae	*Shanglu*	*Phytolacca acinosa* Roxb.
Radix Polygoni cuspidati	*Huzhang*	*Polygonum cuspidatum* Sieb. et Zucc.
Radix Polygoni multiflori	*Heshouwu*	*Polygonum multiflorum* Thunb.
Rhubarb	*Dahuang*	*Rheum officinale* Baill.
Semen Ricini	*Bimazi*	*Ricinus communis* L.
Radix et Rhizoma Salviae miltiorrhizae	*Danshen*	*Salvia miltiorrhiza* Bunge
Radix Scutellariae	*Huangqin*	*Scutellaria baicalensis* Georgi
Herba Senecioe scandensi	*Qianniguang*	*Senecio scandens* Buch.-Ham. ex D. Don
Herba Scutellariae Barbatae	*Banzhilian*	*Scutellariae Barbatae* D. Don
Radix Stephaniae tetrandrae	*Hanfangji*	*Stephania tetrandra* S. Moore
Flos Syzygii aromatici	*Dingxiang*	*Syzygium aromaticum* (L.) Merr. et Perry
Herba Taxilli	*Sangjisheng*	*Taxillus sutchuenensis* (Lecomte) Danser
Radix et Rhizoma Tripterygii	*Leigongteng*	*Tripterygium wilfordii* Hook. f.
Herba Typhae	*Xiangpu*	*Typha angustifolia* L.
Radix Valerian	*Xiecao*	*Valeriana officinalis* L.
Fructus Xanthii	*Cangerzi*	*Xanthium sibiricum* Patrin ex Widder

Table 5: CHM formulations with DILI.

Formulations	CHM preparations
Pill	*Zhuangguguanjie* pill; *Baishi* pill; *Liushen* pill; *Shiduqing* pill; *Xuedu* pill; *Zhifeng-tougu* pill; *Xiaokechuan* pill; *Tianma* pill
Decoction	*Xiaochaihu* decoction; *Dachaihu* decoction; *Gegen* decoction; *Dahuangmudanpi* decoction
Capsule	Complex *Qingdai* capsule; *Baidianfeng* capsule; *Zhuangushenjin* capsule; *Diaoxinxuekang* capsule
Tablet	*Xiaoying* tablets; *Xiaohe* tablets; *Guxian* tablets; *Zengshengping* tablets; *Niuhuang Jiedu* tablets; *Kunming-Shanhaitang* tablets
Granule	*Baifukang* granule
Injection	Complex *Danshen* injection
Powder	*Ganji* powder; *Fangfeng-Tongsheng* powder

Table 6: Categories of chemical substance with HILI.

Categories	Substance with HILI
Alkaloids	Aconitine; febrifugine; ajmaline; vincristine
Glycosides	Cardiac glycosides; cyanogenic glycosides; saponins; *bulbifera*
Toxic proteins	toxin fruit; croton, castor; abrinjatropha; trichosanthes; centipede; snake venom; viper
Terpene and lactones	Toosendan; spurge; *Coriaria* leaf; wormwood leaves
Tannins	Gall; peel; holly leaf
Heavy metals	Cinnabar; realgar; light powder; litharge; vitriol; red lead
Other toxic components	*Garcinia*; red fennel root; *Hydnocarpus*, *Pinellia ternata*; daphne; melon pedicle acids; *Asarum*; rue; mint; *Asarum forbesii*; musk grass

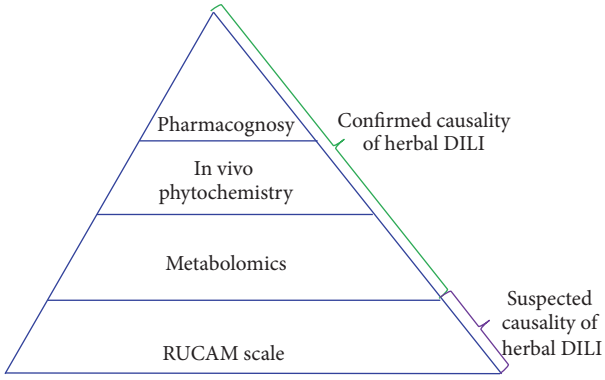

Figure 1: Proposed RUCAM-based stepwise strategy to causality assessment of herbal DILI [15].

Currently, *Heshouwu* remained to be controversial in terms of its safety and effects, and functions and role of *Heshouwu* are not entirely conclusive, due to absence of excluding confounding DILI causality factors (such as heavy metals, pesticides, and fungal toxin contamination) and lack of pharmacognostic identification of patients' digested materials.

Based on the general RUCAM assessment, Wang et al. established a model for assessing HILI causality that combines translational laboratory tests with pharmacognosy, phytochemistry, and metabolomics [15, 20–23] (Figure 1). This model might improve our capability for the diagnosis and causality assessment of HILI.

3.6. Safety Regulation of Chinese Herbal Products. For safe application of Chinese herbal products, apart from conventional investigation of mechanisms of safety, efficacy, and toxicity, regulation of Chinese medicine products is needed to enhance the recognition of safety in the following aspects: (1) understanding the role of Chinese herbal medicine treatment and adverse drug reactions (ADR) coexisting; (2) the proper use of CHMs; and (3) avoiding overdosing of herbs and unnecessary prolonged treatment, so as to reduce the incidence of ADR. Most importantly, active academic exchanges and cooperation with foreign regulatory agency, with respect to safety issues of herbal medicines, are urgently needed to promote communal understanding and communication. Safe and effective herbal medicines can then be recognized globally.

According to Chinese Drugs Act and ADR monitoring and reporting system, the appropriate research and monitoring medicine safety management system is established. The main work was done in the following six areas: (1) adverse drug reactions monitoring by strengthening communications to promote understanding of drug manufacturers, medical practitioners, and public; (2) introducing investigation methods of Chinese herbal medicine to improve the clinical application; (3) encouraging scientific groups to actively participate in society of herbal medicines, in terms of safety issues; (4) encouraging pharmaceutical companies to carry out postmarketing product safety reevaluation to enhance the effectiveness and safety of Chinese medicine; (5) performing hospital-based medicine safety monitoring; and (6) establishing the national and local research institutions to perform systematical studies on the clinical manifestations, mechanisms, and prevention measures of ADR of CHMs.

4. Diagnosis and Treatment of HILI in China

4.1. Understanding of Herbal-Induced Liver Injury. In recent years, with the rising worldwide use of herbs and the improvement of adverse drug reaction monitoring system, HILI reports rapid increase [3]. Factors affecting HILI are complicated and diverse. In addition, there are other factors, for instance, individual variation and irrational drug use [24]. Due to the lack of standards reflecting the complexity of HILI diagnosis, the current clinical diagnosis of HILI is often inaccurate. In addition, an integrated drug classification system has not been established for comparison of liver damage, resulting in a higher level of HILI [25]. Therefore, the establishment of guidelines for diagnosis of HILI and treatment with the characteristics of Chinese herbal medicine becomes very important for patients with hepatic impairment. Enhanced recognition of scientific objectivity and judgment of HILI may improve diagnosis and treatment, as well as reduction of liver damage, in particular for development of pharmaceutical industry.

4.2. Proportion of HILI, in Terms of DILI, Varies in Different Countries and Regions. These documents are associated with more than a single-center retrospective investigation and diagnosis center, which is related to different levels of HILI. In addition, the drug and level of liver damage caused are determined by the statistical methods adopted [26–33]. Most herbs are used as a whole or a certain type of pharmaceutical (such as anti-TB drugs) and are even compared to certain pharmaceuticals (such as acetaminophen), in terms of efficacy and safety. However, other ingredients of the herbs are not taken into account, leading to higher proportions of one-sided conclusions [34].

4.3. HILI Factors Affecting the Complexity and Diversity. Herbal factors affecting HILI complexity and diversity include improper clinical use, individual variation, and combination of pharmaceuticals.

(1) Herbal Factors. These factors include the following: (a) some herbs may produce direct damage on the liver, such as *Gynura japonica* L. (*Zusanqi* in Chinese) [33] and *Tripterygium wilfordii* Hook. f. (*Leigongteng* in Chinese) [35]; (b) variety mix: some herbs homonym, pseudo product mix, such as clinical application of *Gynura japonica* L. as *Panax notoginseng* can cause liver injury [36]; and (c) improper processing: unreasonable concocted herbal medicine may increase the risk of liver damage, such as nonstandard cooked or raw *Radix Polygonum*. Risk of liver injury is higher than that of the standard stipulated *Polygonum* [37]. Exogenous harmful substances: herbal drugs may be contaminated during growth, processing, storage, transportation, and other courses, resulting in seriously high level of herbal pesticide residues, heavy metals, and microbial toxins, which causes liver damage [38, 39]. Meanwhile, inadequate processing could augment the toxic risk of the clinical usage of *Radix Polygonum* [15].

(2) The Irrational Clinical Use. Chinese herbal medicine should be applied in accordance with the theory of CHM, according to the diagnosis and judgment made. Cross-checks of medication, dose selection, and appropriate compatibility of toxic drugs are necessary for treatment of diseases. Overdosed drug, unconventional dosing or treatment, and improper drug compatibility may increase risk of liver damage [40]. For example, not as other well-known hepatotoxic herbs, the potential risk of *Heshouwu* was not fully clarified in the clinical community and therefore the hepatotoxic risk among herbs should be paid attention to in clinical practice and by regulatory administration [15]. Patients with different physical conditions, for instance, underlying chronic diseases, genetic differences, and other factors, may also have increased risk of liver damage [41].

(3) Combination with Pharmaceuticals. Combination of herbal medicines and Western medicines may result in higher risk of liver damage. In treatment, it can lead to liver damage while taking chemical drugs such as cholesterol-lowering statin drugs. Some of the herbal drug preparation is actually in the compound formulation, which contain a hepatotoxic pharmaceutical ingredient, for example acetaminophen. Consequently, the liver injury caused by the combination of Chinese and chemical medicines cannot be simply attributed to herbal and related agents.

4.4. Clinical Features of DILI/HILI. DILI has been reported to be widely caused by a variety of pharmaceuticals, herbs, and other toxic substances. In Western countries, 1.2%–6.6% of acute liver disease cases reported at tertiary referral centers were related to DILI, and it also accounts for 13% of acute liver failure [42, 43]. According to the R-value between ALT (serum alanine aminotransferase) and ALP (alkaline phosphatase), clinical DILI cases were classified into hepatocellular, cholestatic, and mixed types [44]. In Western countries the common causative agents and their clinical chemistry type of DILI have been documented [45, 46].

Hepatocellular injury was mostly frequently cited as the liver injury caused by herbs, dietary supplements, and folk remedies. The etiology of DILI in Western countries usually differs from those in Asian countries, particularly in China and Korea. In Western countries, DILI were mainly caused by the prescription of chemical drugs (e.g., analgesics, antibiotics, and CNS agents) [47, 48]. The ingredients of prescription drugs are generally known, while the exact causative ingredients among the herbs and folk remedies used in traditional medicine remain unclear [49]. Similar with prescription medications, the liver injury type observed among herbal and dietary supplements is predominantly hepatocellular too [50]. Thus, the R-values based methodologies for DILI classification might not be an appropriate approach for Asian patients as compared with the patients in Western countries.

In a case study of DILI reported by 302 military hospitals from 2009 to 2014, of 96857 inpatients, 1985 DILI cases with liver dysfunction were retrospectively collected. The portions of patients prescribed CHM, pharmaceuticals, and combination of both were 28.4, 43.8, and 27.8%, respectively. By comparison with pharmaceuticals, the DILI caused by CHM presented a higher mortality, but there was no significant difference, in terms of rates of chronic DILI and acute liver failure (12.9 versus 12.4%, $P = 0.807$; 7.6 versus 7.6%, $P = 0.971$). Additionally, 75.6% of cases caused by CHM were considered as probable. The highly probable cases were only 16.6%, based on Roussel Uclaf Causality Assessment Method. The diagnostic criteria used have been illustrated in Figure 2 [25].

4.5. Risk Factors and Future Challenges on HILI Research. A large portion of DILI reported in Western countries is HILI. Several risk factors for HILI, for instance, components of herb, their side effects, and pesticides used on the crop and other environmental factors should therefore be given special consideration. In pathogenic aspects of HILI, there are several theories about the pathophysiology of liver injury, the majority of which are based on DILI. The "damage hypothesis" suggests that HILI may be caused by the following aspects: (1) formation of parent drug-protein complexes, (2) irreversible generation of reactive metabolites, (3) inhibition of the bile salt export pump, and (4) intracellular damage indirectly

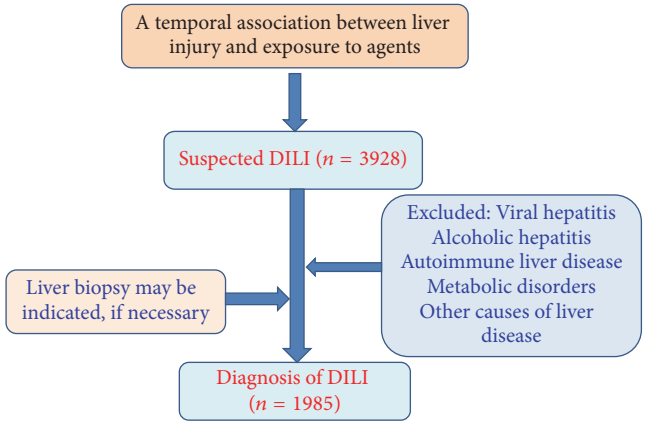

Figure 2: The flowchart illustrating drug-induced liver injury diagnosis.

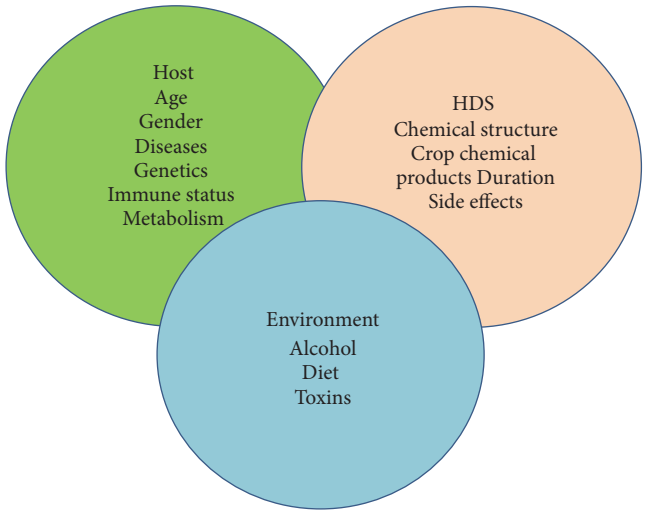

Figure 3: A variety of theories related to the pathogenesis of HILI [16].

mediated by oxidative endoplasmic reticulum stress and mitochondrial damage. The "hapten hypothesis" suggests that (1) metabolite-protein and (2) the drug-protein adducts lead to inadvertent activation of the adaptive immune system. Figure 3 depicts a variety of theories related to the pathogenesis of HILI. The overlap is mostly with those for DILI in host, herb-drug supplements (HDS), and environment factors [16, 51].

Up to date, a large portion of medicines used in developing countries are herb drugs. It is unlikely to evaluate safety of herbal drugs. Herbal medicines are considered to be safe though liver injury may still be a potential risk. It is difficult to determine the exact incidence of HILI, which might have been seriously underestimated. As to determine the toxicity and adverse effects, multiple components are needed to be carefully separated, combined with ethnopharmacological and toxicological investigations. Therefore, the recognition of indicative symptoms of liver dysfunction is important because a potential differential diagnosis of the liver injury could improve the patient prognosis.

4.6. Chinese Guideline for Diagnosis and Treatment of HILI. At present, liver histopathology does not establish the diagnosis of DILI with the required certainty. To fulfill the requirement of diagnoses of DILI and HILI, clinical and structured causality assessments are the better approaches than liver histopathology results obtained from liver biopsy, an invasive procedure with a low complication rate. Scientists and physicians now face difficulties and challenge for HILI diagnosis. HILI is a hot issue in the field of CHM study. We thought that this guideline has a great significance since it could make clear the correlation of liver injury and CHM, improve the diagnosis and treatment level of HILI, guide the rational application of CHM, reduce occurrence of HILI, and promote healthy and sustainable development of CHM. For the past 10 years, the Chinese medicine experts have been dedicated to this research, making a significant contribution in the following two aspects.

(1) Rapid Screening and Evaluation of HILI Have Become One of the Key Techniques of Herbal Research. For example, Li et al. analyzed the toxic ingredient, mechanism of toxicity, and reduction of toxicity of CHM [19]. The studies performed highlighted how to investigate toxicity of CHM with a scientific approach and rational. First, the toxic effects of CHM with HILI diminished or disappeared by reducing the level of toxic ingredients or by modifying the chemical structure. Second, through the research on the integrated evidence chain-based identification strategy for HILI and the CHM characteristic-oriented toxicity attenuating and rational use strategy, it would be helpful to provide scientific support for HILI diagnosis and CHM prescription and to promote integrated solutions for individualization therapy of clinical precision medicine using CHMs [52].

(2) The Released "Guideline for Diagnosis and Treatment of Herbal-Induced Liver Injury." Since there are still many issues, in terms of HILI diagnosis and treatment, the China Association of Chinese Medicine (CACM) organized experts and developed "Guideline for diagnosis and treatment of herbal-induced liver injury," which requires constant revision and improvement under the support of evidence-based medical science. This guideline describes HILI terms and definitions, epidemiological studies, affecting factors, clinical characteristics and type of injury, histopathologic features, the degree of damage classification, differential diagnosis, diagnosis strategies and methods, diagnostic criteria, and others. Currently, the guideline has been approved and published by CACM (T/CACM 005-2016) in April 2016 [53].

In this guideline, the Integrated Evidence Chain-Based Causality Identification Algorithm (IECCIA), a new perspective and workflow, was proposed and recommended in HILI diagnosis for Chinese medical practitioners, which are composed of five nodal segments: (1) thorough exclusion of confusing liver diseases; (2) thorough history review of implicated drugs, especially excluding the combination of Western medicines and CHM; (3) pharmacognostic

identification and quality assessment for the implicated herbs, including plant origin, exogenous toxin contamination, and synthetic drugs adulteration; (4) detection of characteristic in vivo metabolites of implicated herbs; and (5) detection of specific HILI biomarkers of implicated herbs. Based on this workflow, the guideline proposed the three-level diagnosis system for the first time, suspected, clinical, and confirmed diagnosis for HILI. Some misunderstandings of HILI and the rational usages of CHM were also explained elaborately in the guideline. Notably, the proposed HILI diagnosis workflow could also be used in diagnosing DILI to obtain integrated evidence chain.

The proposed HILI guidelines with the characteristics of Chinese medicines for liver damage and clear relationship between medicine and scientific judgment of HILI are objective in terms of improving the diagnosis and treatment, clinical therapy, and modern scientific research. CACM experts suggest a scientific and rational classification of comparison of drugs with liver injury: a classification would divide drugs with induced liver injury into Chinese herbal medicine, small molecule pharmaceuticals, and biologics; a secondary classification could classify Chinese herbal medicine and pharmaceuticals, according to their comparative efficacy; a third tier would compare a particular species of herb to its pharmaceutical [53].

5. Modern Methods and Technologies of Hepatotoxicity Study

Accurately predicting the potential hepatotoxic characteristics of pharmaceutical products remains a challenge. Therefore, improved models, methods, and technologies are needed to study liver metabolism and identify drug hepatotoxicity in humans.

5.1. Drug Metabolism Enzymes and Liver Enzymes. Herbal medicines are a complex system. Due to the diversity and complexity of their chemical composition, the same chemical substances in various pharmaceutical formulations exhibited differences in drug metabolism and dynamic behavior [54–59]. Such differences are a reflection of drug-drug interactions (DDIs) or herb-drug interactions (HDIs). The complex results of DDIs or HDIs are related to transporters and metabolizing enzymes.

Cryopreserved human hepatocytes or AREHCs (Assay-Ready Expanded Hepatocytes) are widely applied in researches for hepatic metabolism, CYP induction/inhibition, compound uptake, genotoxicity, hepatotoxicity, and 3D coculture [60]. AREHCs have functional CYP activity. Therefore, AREHCs are qualified for study of CYP induction, metabolism [57]. AREHCs are comparable to HepaRG cells, in terms of basal phase I enzyme activities. AST, ALT, GLDH, and LDH are measured for the indication of liver injury, and these enzymes are released upon membrane leakage when hepatocytes are damaged [61, 62].

Rhein, as the mainly absorbable anthraquinone derivative, is taken into systemic circulation after oral administration. Wang et al. carried out the toxicokinetic analysis of water extract of *Rheum palmatum* L. [17]. Their study suggested that rhein was majorly used to evaluate the toxicokinetics of rhubarb. Figure 4 depicts the profiles of average concentration of rhein versus time. When given to the same dosage, the AUC, $C_{\max k}$, $t_{1/2}$ *Ka,* and $t_{1/2}$ of chronic renal failure (CRF) groups were generally lower than those of normal groups. As dose given increased, the AUC and $C_{\max}$ values did not increase proportionally in both chronic renal failure (CRF) rat model and normal groups. This might be on account of a nonlinear pharmacokinetic course, or because the indigestion led to severe diarrhea induced by the rhubarb itself at a high dosage. Therefore, the study suggests that the possibility of renal lesion using rhubarb in treatment of CRF would be limited when the dose was properly controlled.

5.2. Hepatic Transporter Study. AREHCs express hepatic transporter genes that are expressed by primary hepatocytes [60]. The mRNA expression level of the hepatic transporter genes is significantly higher compared to HepG2 cells. The expanded hepatocytes can be used for compound uptake and metabolism studies. The resazurin assay can be repetitively used to bioreactor cultures with pHH or HepG2 [63]. However, a toxic effect of resazurin upon long-term exposure was observed in other studies that could impair the diclofenac exposure effect.

5.3. Hepatotoxicity Technology. Primary hepatocytes and liver cell lines are important for in vitro toxicogenomic studies and RT-qPCR technology. Gene expression profiles following exposure to potential hepatotoxicants can be analyzed. Identification of reference gene with stable expression during in vitro toxicology studies is critical. A study performed by Fox et al. was aimed to analyze stabilities of reference genes in HepG2 and primary rat hepatocytes with two different culture systems [64]. The genes E2F7 and IL-1IRA were then identified as potential toxicity biomarkers for acetaminophen treatment. Are hepatocytes can be used for hepatotoxicity studies and predictive screens of novel compounds? The expanded primary hepatocytes have high tolerance ($IC_{50} > 200\,\mu M$) to nontoxic compounds (such as phenytoin and ciprofloxacin). AREHCs are responsive to moderate hepatic toxins (such as methotrexate) and are highly sensitive ($IC_{50} < 30\,\mu M$) to hepatotoxins (such as ketoconazole and tacrolimus) [60].

5.4. Genotoxicity Technology. The validated hepatocytes can be used for micronucleus assays for genotoxicity application, when grown in AREHC Genotoxicity Assay Medium [60]. This medium permits hepatocyte cell division allowing the observation of micronucleus formation when genotoxic compounds were used to treat hepatocytes. Genotoxicity Assay Validated AREHCs can be used for micronucleus assays to determine potential genotoxic effects of novel compounds that alter cell division and DNA replication to be observed. Genotoxicity Assay Validated AREHCs are division-competent hepatocytes unlike uncultured primary hepatocytes and therefore have wider applicability and can be used for assays that require cell division such as micronucleus assays, for instance, the effect of cyclophosphamide on AREHC viability and micronucleus formation [60].

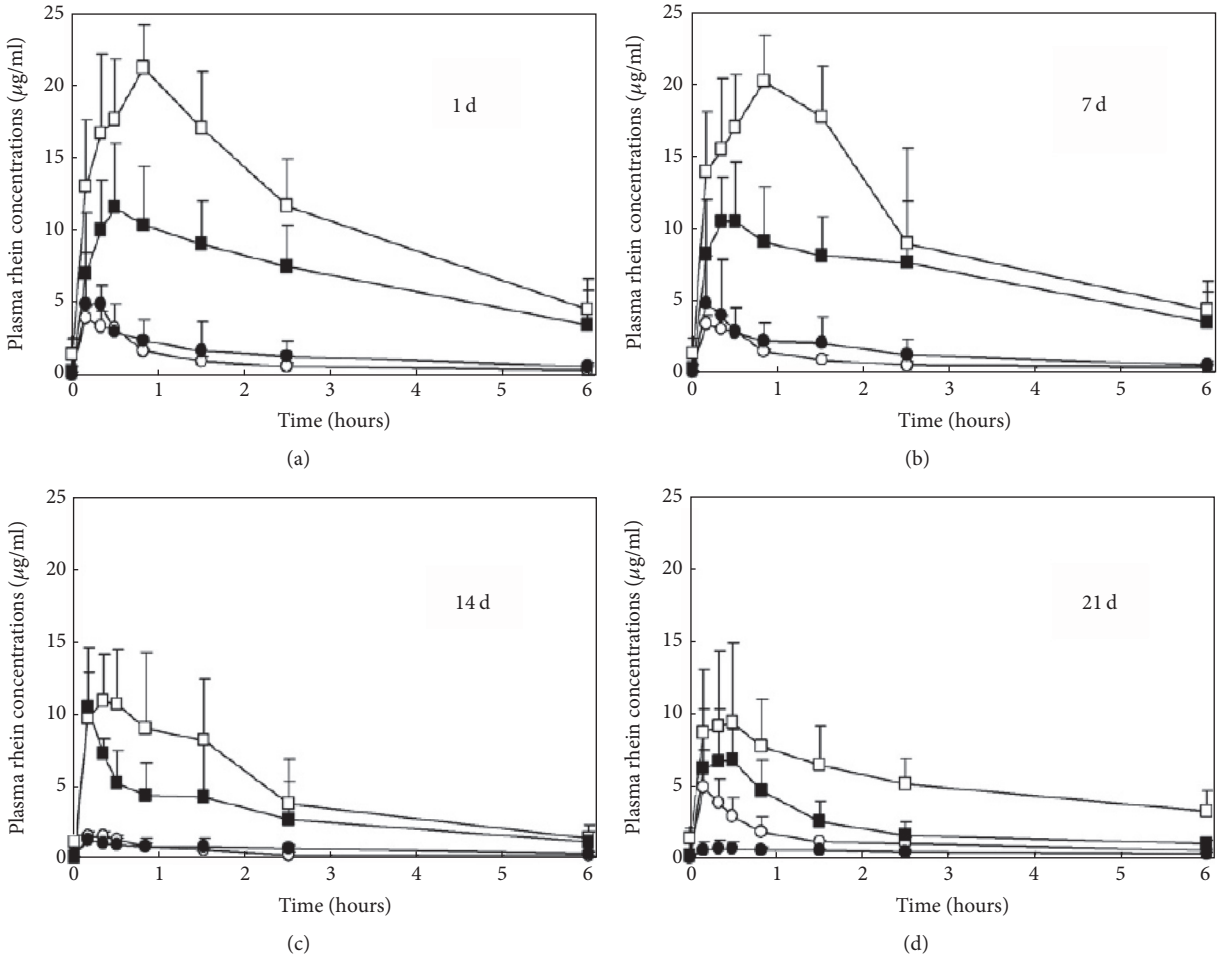

Figure 4: Profiles of average plasma concentration of rhein versus time after orally administrating *Rheum palmatum* extract. Normal rats and observed data of CRF are represented by opened and filled symbols. Low and high dosage groups are represented with circle and square, respectively [17].

Cyclophosphamide is a DNA alkylating agent that interferes with DNA replication and is used for cancer chemotherapy. In a study by using a combination of metabolomic and genomic analyses, senecionine hepatotoxicity was determined. Significantly elevated conjugated bile acids after senecionine exposure were observed via serum profiling of bile acids. The hepatic mRNA levels of several key bile acid metabolism related genes were found to be changed significantly. Cholesterol 7-α hydroxylase, bile acid CoA-amino acid N-acetyltransferase, organic anion-transporting polypeptides, multidrug-resistance-associated protein, and sodium taurocholate cotransporting polypeptide were involved in the process. The cross-omics approach provides a comprehensive view for the study of the toxicity induced by senecionine, a hepatotoxic pyrrolizidine alkaloid. Moreover, the change in the respective transporters and bile acid metabolism could provide another toxic mechanism for pyrrolizidine alkaloids [65].

5.5. Culture Technology. A dynamic 3D bioreactor system cultured with primary human liver cells could be useful to investigate hepatic drug effects. It is suggested that a stable performance of the hepatocytes subsequent to three days of an adaption phase based on the data from untreated bioreactors (control BR), which is in the line with other studies that indicated hepatocyte functionality preserved in the 3D bioreactor, was longer than those in 2D cultures [66]. Therefore, it is a main advantage for the perfused 3D bioreactor system over conventional 2D cultivation to perform long-term in vitro toxicity studies [67]. A marked and rapid decrease of CYP gene expression has been observed when compared with the freshly isolated human hepatocytes [68]. In addition, Liver Sinusoidal Endothelial Cells are specialized scavenger cells that have a high capacity for uptake of soluble molecules up to 0.2 μm in size. The liver endothelial cells can be cocultured with the expanded hepatocytes to determine paracrine signaling between these two cell types. Coculture can also be used to determine the effect of compounds or molecules on hepatocytes that are taken up exclusively by liver endothelial cells such as bile acids and phalloidin. Hepatic organoids can be grown by coculturing hepatocytes, Liver Sinusoidal Endothelial Cells, and Mesenchymal Stem Cells.

5.6. Bioinformatics in DILI/HILI Research. Currently, bioinformatics, including network pharmacology and network toxicology, is an important tool to study the effectiveness and safety of drugs. Network pharmacology has been developed from the principles of systems biology and network theory. Systems biology aimed to integrate all levels of biological organization including cell, organ, organism, or population to explain biological complexity. The concept of network pharmacology is established on the belief that, rather than individual molecules, targeting multiple nodes in interconnected molecular systems could result in enhanced efficacy and low occurrence of adverse effects [69, 70]. Combining systems biology with network biology may enable a new network pharmacology approach to be applied in drug research. The network of drug action is built by drug-target networks and biological networks. Thus, network pharmacology could be used to investigate the complex dynamics of interconnected molecular and organic systems in drug discovery, development, and drug safety [71, 72]. The recent progress in applying the network pharmacology in Chinese herbal medicine research is shown in the following seven aspects, such as predicting new drug targets, action mechanism, new drug discovery, drug evaluation for PD/PK, safety and toxicology, quality control, and bioinformatics.

Since Chinese herbal medicine is a very complicated system, the network toxicology, as an important branch of network pharmacology, in terms of safety prediction of CHM, faces three great challenges. It refers to study on the toxicological features and their interaction and regulation in biological systems and investigates the mechanism of toxicity. Network toxicology now develops rapidly in safety prediction of CHM. The application of network toxicology to safety and toxicology study on CHM is extremely beneficial to identify the toxic ingredients and potential incompatibility of CHM, including integration studies of bioinformatics, innovation of methods, and risk assessment in future development of the network toxicology in CHM research.

Network toxicology is another intriguing field for CHM research [73]. In network toxicology, adverse outcomes in human and toxicological mechanisms of medications can be investigated through network modeling of the complex relationships among adverse reactions, targets, and chemical entities [74]. Specifically, the knowledge about the chemical entities, genes, proteins, toxicological endpoints, and adverse reactions needed to be collected from literature, public database, and experiments. A network can be constructed based on comprehensive relationships among the nodes (such as genes, proteins, toxicological endpoints, and adverse reactions), and network analysis will help to infer the unknown relationships among the interesting nodes (such as active ingredients and targets). For the CHM studies, the network modeling can be utilized to identify the active toxic ingredients in herbs, to understand the toxicological mechanisms, and to predict the potential adverse outcomes in human and contradiction of herb combinations. Overall, network toxicology can be a promising tool that can provide the scientific evidences to support the safety evaluation of CHM.

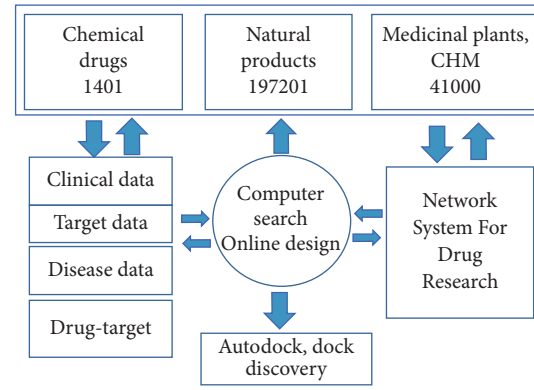

Figure 5: CHM Network pharmacology database.

Network toxicology of CHM aims at describing the network toxicology, which refers to the study on the toxicological properties of the model by building a network, using the established network model to analyze toxic substances interaction and regulation in biological systems, understanding the toxic effects of drugs or drug-drug on the body, and investigating the mechanisms of toxicity. CHM network pharmacology database is established by Guangdong Hospital of Traditional Chinese Medicine and Peking University for online use [75]. The basic framework of CHM Database is shown in Figure 5.

1401 chemical drugs, 197201 natural products, and 4100 herbal medicines or medicinal plants are collected. Tremendous amount of information obtained supports the following investigations: (1) CHM network is applied to predict the interaction of CHM molecules-pathway and formula contained; (2) interaction of target and target protein is used to study the mechanism of CHM; (3) the pharmacological responses of the active molecules from a population of molecules and combined drugs at the levels of multitargets, indications, side effects, and molecular-drug-targets-disease relationships are evaluated and summarized; (4) systems biology and translational medicine from basic research to clinical outcome are combined; and (5) the possible active molecules and active targets in CHM prescriptions are predicted [72]. The successful application of the network toxicology to safety and toxicology study of CHM is extremely beneficial to identify the toxic ingredients and potential incompatibility of CMMs. In the future, the network toxicology will develop rapidly in safety prediction of CHM by utilizing public and/or open database. The network toxicology may act as a new approach for screening the potential toxic ingredients in herbs.

6. Discussion and Conclusions

In China, more than 5000 species of recorded medicinal herbs are used in treatment of diseases. Over the past 15 years, a series of adverse events have taken place, in terms of safety, such as aristolochic acid in 2000, *Longdanxiegan* pill in 2003, and *Houttuynia* injection in 2006. Special consideration

should be given to the safety issue of CHM, but effectiveness and efficacy of CHM cannot be less weighted.

Apart from China, Mexico owns the second largest number of herbs recorded with approximately 4500 species [76]. The use of medicinal plants by modern populations has been explored by many publications in Latin America and Mexico. In addition, most herb products are used for the purpose of health care in Japan and South Korea.

In Mexico, a total of 5% state flora is used for medicinal plants with 235 different herbs recorded for medicinal use. During the 1990s, ethnomedicinal study, combining ethnopharmacy and ethnobotany, was developed in Mexico and Latin America [77]. Based on the records in the library of traditional Mexican medicine, seven herbal products daily used in Mexico have been warned about due to hepatotoxicity: *Citrus aurantium* L. (citrus orange), *Tilia mexicana* Schlechtendal (tilia), *Scoparia dulcis* L. (maidenhair), *Prunus persica* L. (peach), *Morus alba* L. (white mulberry), *Equisetum hyemale* L. (horse tail), and *Rosmarinus officinalis* L. (rosemary). A prospective study between 2004 and 2013 to characterize hepatotoxicity and results, comparing herbal and dietary supplements (HDS) versus medications, was described by Navarro et al. [27]. Of the 839 subjects enrolled, 130 patients were sampled, the incidence of liver injury caused by bodybuilding and nonbodybuilding HDS was from 7% to 20%.

The clinical manifestations of HILI varied from asymptomatic or abnormal hepatic biochemical tests to acute liver failure [78]. 28 patients with DILI in the US DILI Network database were described by Chalasani et al. Hepatocellular injury was founded as the most common side effect in 63% of the patients sampled, and cholestatic injury was approximately 17%; of all DILI cases, 88% were mild to moderate and the rest were severe or fatal. Patients who required a liver transplant were limited and 9% of patients eventually developed chronic DILI [46].

A prospective nationwide study of DILI/HILI in South Korea performed by Suk et al. [79] reported 371 cases. The causes of DILI/HILI included herbal medications in 27.5% of cases, prescription or nonprescription medications in 27.3%, health foods or dietary supplements in 13.7%, medicinal herbs or plants in 9.4%, folk remedies in 8.6%, and herbal preparations in 3.2%.

According to the WHO Collaborating Centre for International Drug Monitoring reports, adverse reaction reports of herbs were 4960 cases prior to 1994. By the end of 1999, it has increased to 8986 cases; great concern has been given to safety issue of CHM. Since then, measures have been taken; for instance, the US Food and Drug Administration terminated use of the preparation almonds made; Singapore banned the import and sale of preparations containing berberine; Japan reevaluated the efficacy and safety of *Bupleurum* preparations; the use of aristolochic acid-containing medicines was prohibited, and sale of the Senecio plant medicines is banned in the UK.

As a matter of fact, it is hard to draw a conclusion that the safety issues of CHM become increasingly serious. The chemical composition of CHM is complex and diverse, but the content of toxic substances is minimal. It requires comprehensive investigation of adverse reactions and drug safety issues of CHM. Due to the complex of CHM, in terms of planting, processing, and other factors, it is not proper to make judgment on CHM by simply applying the standard and criteria to assess overall effect of CHM.

Based on the information available and literature, the authors analyzed and discussed the hepatotoxicity problem of CHM, including global overview on herbal-induced liver injury (HILI), current research progress on toxic CHM, diagnosis and treatment of HILI, and modern approaches and technologies of study of hepatotoxicity. A guideline for the diagnosis and treatment of HILI has now been drafted. The hepatotoxicity issue of Chinese herbal medicine, as a matter of fact, is overestimated. The investigation of hepatotoxicity of CHM is now booming with worldwide application of CHM. This review, therefore, provides useful information for investigating hepatotoxicity of herbal medicine and characterizing DILI caused by CHM. In addition, in which way further efforts should be made to study the rationale of CHM and liver injury has been discussed.

Competing Interests

The authors declare that there is no conflict of interests related to this paper.

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Review Article

Advances in Engineered Liver Models for Investigating Drug-Induced Liver Injury

Christine Lin[1,2] and Salman R. Khetani[2]

[1]*School of Biomedical Engineering, Colorado State University, Fort Collins, CO 80523, USA*
[2]*Department of Bioengineering, University of Illinois at Chicago, Chicago, IL 60607, USA*

Correspondence should be addressed to Salman R. Khetani; skhetani@uic.edu

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Drug-induced liver injury (DILI) is a major cause of drug attrition. Testing drugs on human liver models is essential to mitigate the risk of clinical DILI since animal studies do not always suffice due to species-specific differences in liver pathways. While primary human hepatocytes (PHHs) can be cultured on extracellular matrix proteins, a rapid decline in functions leads to low sensitivity (<50%) in DILI prediction. Semiconductor-driven engineering tools now allow precise control over the hepatocyte microenvironment to enhance and stabilize phenotypic functions. The latest platforms coculture PHHs with stromal cells to achieve hepatic stability and enable crosstalk between the various liver cell types towards capturing complex cellular mechanisms in DILI. The recent introduction of induced pluripotent stem cell-derived human hepatocyte-like cells can potentially allow a better understanding of interindividual differences in idiosyncratic DILI. Liver models are also being coupled to other tissue models via microfluidic perfusion to study the intertissue crosstalk upon drug exposure as in a live organism. Here, we review the major advances being made in the engineering of liver models and readouts as they pertain to DILI investigations. We anticipate that engineered human liver models will reduce drug attrition, animal usage, and cases of DILI in humans.

1. Introduction

Drug-induced liver injury (DILI) is a leading cause of pharmaceutical attrition and acute liver failures in the US [1]. In particular, DILI has been linked to almost 1000 marketed drugs [2]. DILI can mimic many forms of acute or chronic liver diseases, such as necrosis (cellular death), hepatitis (inflammation), cholestasis (reduction or stoppage of bile flow), fibrosis (scarring), or a mixture of different injury types. While some drugs cause DILI that is predictable and dependent on the dose of the drug (i.e., acetaminophen), many cases of DILI are termed "idiosyncratic" since overt liver injury occurs unpredictably in a small number of patients potentially due to other factors such as environmental stimuli, coadministered drugs, and host risk factors (i.e., age, sex, preexisting disease, and genetics). Idiosyncratic DILI can be mediated by the innate and adaptive immune systems that are triggered by injury to hepatocytes or other cell types of the liver. Ultimately, the interplay between hazardous and adaptive cellular responses can determine whether the liver of a particular patient adapts following a mild injury or proceeds to severe injury due to a drug. In order to mitigate the risk of DILI (and toxicities to other types of tissues), regulatory agencies require testing on live animals before a drug candidate can proceed to human clinical trials. However, animal testing is only 50% predictive of human DILI, likely due to the significant differences in drug metabolism pathways between the livers of animals and humans [3]. In addition, use of young animals with limited genetic diversity under well-defined nutritional conditions for drug safety assessment does not capture the aforementioned host risk factors present in humans. Therefore, given the challenges with screening drugs in animals, regulatory agencies and the pharmaceutical industry are under increased pressures to develop and adopt human-relevant methods to evaluate drug safety prior to exposing live patients to drugs.

In the case of the liver, several different model systems have been developed to provide human-specific data

on drug behavior [4]. These include microsomes, cancerous/immortalized cell lines, isolated primary human liver cells, liver slices, and humanized rodents. While these models have already been used in some instances to mitigate the risk of DILI during drug development, there remains a need for model systems that are better predictive of clinical outcomes, with respect to the type and severity of DILI, and can be used to elucidate interindividual variability in drug outcomes. Furthermore, how metabolism in the liver affects toxicity in other tissue types needs to be investigated further using newer culture platforms that link tissue types together through the exchange of culture medium [5].

A spectrum of human liver model systems is being developed to address the above mentioned challenges using engineering tools (i.e., micropatterning, microfluidics, and biomaterials) that enable greater control over the cellular microenvironment to influence cell functions *in vitro*. Here, we will describe the most recent advances in engineered human liver models that have utility for early DILI prediction and to obtain a better understanding of the diverse mechanisms underlying different forms of DILI. We begin with a brief description of conventional/traditional culture models and then discuss engineered liver models starting with static micropatterned cocultures (2D), followed by static spheroids and bioprinted liver models (3D). We then discuss perfused culture platforms that are more technologically complex than static plate-based systems but can be adapted to both 2D and 3D cultures. Precision cut liver slices and humanized liver rodent models are discussed as the most complex and *in vivo*-like liver models currently available. High content readouts and *in silico* predictions are briefly discussed as they pertain to DILI detection. Finally, Section 10 summarizes the key trends as well as the pending issues/questions in the field. We highlight key published studies that demonstrate the different types of model systems and data sets generated for detection of DILI, while referring the reader to other review articles that provide more comprehensive information on specific technologies and/or methodologies.

2. Conventional Cultures

Culture of hepatic cell lines and primary hepatocytes on adsorbed or gelled extracellular matrix (ECM) has been carried out for several decades and other reviews cover the genesis and development of this field [6]. Here, we briefly summarize the key conventional/traditional model systems that are useful for assessing the liver toxicity potential of pharmaceuticals. For instance, cancerous hepatic cell lines (i.e., HepG2, HepaRG) in 2D monolayers are widely used for evaluating the toxicities of candidate compounds, especially in early stages of drug development [7]. In some cases, such cell line cultures also enable the study of drug-induced lipid accumulation (i.e., steatosis) [8] and alterations in bile canaliculi dynamics [9], which constitute alterations in hepatic functions that can ultimately lead to liver injury. While cancerous/immortalized hepatic cell lines provide for nearly inexhaustible sources of liver cells for early drug screening, they have some limitations for accurately modeling complex physiological outcomes. In particular, cell lines are ultimately limited to single donors (whereas DILI can vary across multiple individuals), known to display abnormal morphology and levels of liver functions [10], and not always highly sensitive for detection of DILI [11], at least in 2D monolayers.

In contrast to hepatic cell lines, primary hepatocytes, especially from humans, can vary in phenotype across donors and are not always readily available due to sourcing limitations; however, they are the closest representation of human liver physiology if cultured appropriately *in vitro* [6]. Primary hepatocytes in suspension can only be incubated with drugs for 4–6 hours, thereby requiring very high doses of drug to cause any cellular toxicity. Confluent monolayers of hepatocytes adhered to adsorbed collagen can be incubated with drugs for 4–72 hours, but drug metabolism capacities in such cultures are known to show severe downregulation, which negatively impacts correlation with clinical DILI outcomes [12]. Over the years, investigators have devised non-engineering-based techniques to slow down such downregulation. For instance, overlaying confluent hepatocyte monolayers with an ECM gel, such as Matrigel or collagen, can slow down the functional decline of hepatocytes [13]; however, within the first few days, hepatocyte functions in such ECM-overlay culture models still decline to levels that are <10% of those measured in freshly isolated hepatocytes [14]. Culturing hepatocytes in spheroids with tight cell-cell interactions is another technique to mitigate the rapid loss of hepatic functions; however, creating spheroids in a random configuration (such as via nonadhesive plates) leads to nonhomogenous diameters and necrosis in the interior of large spheroids (>200–300 μm). Coculturing with both liver- and non-liver-derived stromal cells has been long known to keep hepatocytes functional for prolonged times as compared to pure monolayers on adsorbed collagen [15]. However, randomly distributed cocultures of hepatocytes and stromal cells display inherent instability in functions over time due to areas of the monolayer that contain suboptimal homotypic and heterotypic cell-cell interactions [14]. Thus, while the aforementioned techniques constitute advances in hepatocyte culture, they are not sufficient to fully stabilize the hepatic phenotype to allow for significant improvements in the prediction of clinical DILI. As we show in subsequent sections, engineering-based tools can augment the techniques discussed above such that the microenvironment around cells is controlled to an extent where functions are better stabilized in a reproducible manner across many experiments and donors so as to be useful for screening drugs with improved prediction of clinical DILI.

3. Micropatterned Cocultures

Microfabrication tools adapted from the semiconductor industry allow for the creation of heterogeneous surfaces with precise features that can range in sizes from a few nanometers to micrometers depending on the type of tools utilized [16]. In the case of the liver, Khetani and Bhatia pioneered micropatterned cocultures (MPCCs) of primary human hepatocytes (PHHs) and stromal cells such that controlled cell-cell interactions (i.e., architecture) led to

high and stable liver functions for 4-6 weeks *in vitro* as compared to unstable randomly distributed cocultures of the same two cell types (Figure 1) [14]. Soft-lithography that utilizes elastomeric polymers such as polydimethylsiloxane (PDMS) was used to miniaturize MPCCs in 24- and 96-well plate-based screening formats. Interestingly, a nonliver murine embryonic 3T3 fibroblast subclone (3T3-J2) induces higher functions in PHHs than even liver-derived stromal cells (manuscript in preparation), potentially due to the embryonic fibroblasts invoking developmental pathways that are complementary across the two species [15]. Nonetheless, MPCCs have been designed to be modular in that the stromal population can be modified without significantly affecting the hepatocyte homotypic interactions on the micropatterned domains, which are important for maintaining cell polarity. For instance, preestablished MPCCs were augmented with primary human Kupffer macrophages once the hepatic phenotype was stable after ~1 week in culture [17]. Stimulating the macrophages with bacterial-derived endotoxin, lipopolysaccharide (LPS), led to cytokine-mediated downregulation of specific cytochrome P450 (CYP450) enzymes in hepatocytes, which can affect DILI outcomes. We are now augmenting MPCCs with liver sinusoidal endothelial cells and hepatic stellate cells to enable the crosstalk between liver cell types in modeling different types of DILI.

MPCCs created with either primary human or rat hepatocytes were incubated for 5 to 9 days with a set of 35 drugs with known DILI liabilities in the clinic, while 10 drugs served as non-liver-toxic controls [18]. The doses tested ranged from $1 * C_{\max}$ to $100 * C_{\max}$ for each drug, where $C_{\max}$ is the reported maximal drug concentration in human plasma. Xu et al. justified the use of doses up to $100 * C_{\max}$ due to interindividual differences in drug concentrations within the liver [12]. The DILI detection results using MPCCs proved several key hypotheses. First, repeated drug dosing improved sensitivity for DILI detection without compromising specificity (i.e., no additional false positives compared to those with shorter durations of drug dosing in sandwich cultures). Second, secreted biomarkers such as albumin and urea were as sensitive for DILI detection as the more classical toxicity marker, ATP, which allows monitoring of the same well over time with repeated drug dosing and conserves the use of expensive and limited PHHs. Third, human MPCCs were more sensitive (65.7%) than their rat MPCC counterparts (48.6%) for human DILI detection. For an additional 19 drugs with the highest DILI concern as classified by the Food and Drug Administration (FDA) [19], human MPCCs displayed a sensitivity of 100% when at least 2 PHH donors were used for testing. Overall, human MPCCs improved the sensitivity by 2.3-fold compared to ECM-sandwich cultures (~28.6%) created using the same PHH donor and dosed with the same set of drugs.

In an interesting case study, MPCCs, but not sandwich cultures of PHHs, have been shown to detect the toxicity of fialuridine [20], a nucleoside analog drug for hepatitis B viral infection that caused the deaths of 5 patients in clinical trials due to lactic acidosis [21]. Such a drastic human DILI outcome was not predicted previously by studies in rats, dogs, or monkeys. As in the clinic, dosing human MPCCs with fialuridine for 4–21 days led to dose- and time-dependent toxicity as assessed by several endpoints such as mitochondrial activity, albumin, urea, and morphological alterations (Figure 2). The toxicity of fialuridine could be compared against its structural analogs towards enabling a structure-activity relationship (SAR) approach. On the other hand, rat MPCCs did not display the same extent of fialuridine toxicity as their human counterparts even after 28 days of dosing. Interestingly, urea secretion and CYP3A activity decreased in rat MPCCs with long-term fialuridine dosing, which is in agreement with a previous *in vivo* study [22]. Thus, MPCCs created using hepatocytes from different types of animals (i.e., mouse, rat, dog, and monkey) and humans can serve to elucidate key differences in species-specific DILI progression and thus allow for selection of the most appropriate species for FDA-required *in vivo* animal investigations.

Other groups have created different variations of micropatterned cocultures containing hepatocytes and stromal cells. For instance, Zinchenko et al. used photo- and soft-lithographic techniques to create micropatterned cocultures of primary rat hepatocytes and Kupffer macrophages [23]; however, this configuration displayed a decline in hepatic functions over 10 days, whereas the use of 3T3-J2 fibroblasts leads to stable functions for at least 4 weeks in both primary rat [15] and human hepatocyte cultures [14]. Cho et al. used 3T3-J2s as the stromal cell type but utilized PDMS stencils to culture rat hepatocytes on top of micropatterned fibroblast colonies [24]. The authors found higher functions in this "layered" configuration as compared to the configuration in which both cell types were in the same plane of culture. In a configuration containing three cell types, Liu et al. micropatterned electrospun fibrous scaffolds to in turn create micropatterns of rat hepatocytes, NIH-3T3 murine embryonic fibroblasts, and human umbilical vein endothelial cells [25]. Such tricultures led to the formation of hepatic spheroids that secreted albumin and urea as well as displayed CYP450 activities for 15 days. These tricultures were shown to be useful for prediction of drug clearance and for drug-mediated modulation of CYP450 activities; however, demonstration of their utility for drug toxicity detection is pending.

PHHs are considered the gold standard for constructing human liver models but are limited in the genetic diversity available to understand interindividual differences in DILI outcomes. On the other hand, induced pluripotent stem cells (iPSCs) can be derived from many patients, including those who have known susceptibilities to toxicity due to certain drugs. Furthermore, unlike PHHs, iPSCs are a renewable cell source for sustainable drug screening using the same set of donors. Protocols to differentiate iPSCs down the hepatic lineage use growth factors inspired from *in vivo* liver development as well as small molecules; however, adult liver functions remain low (<10%) when relying on such protocols alone [26]. We have shown that the MPCC platform is also useful to further differentiate and stabilize functions of iPSC-derived human hepatocyte-like cells (iPSC-HHs) for at least 4 weeks *in vitro* (Figure 1) [27]. Even though the iPSC-HHs in MPCCs were still not as differentiated as PHHs cultured

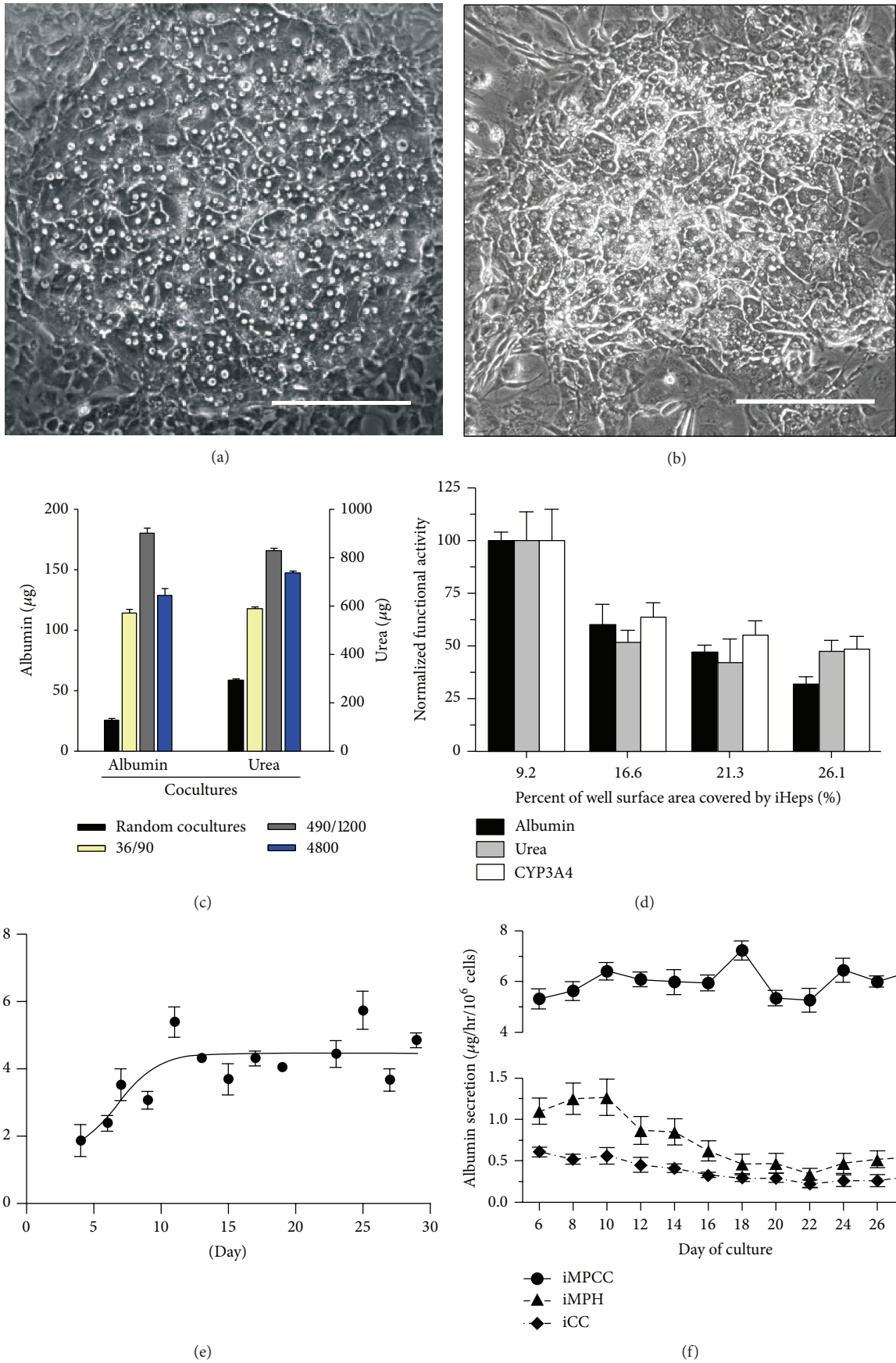


Figure 1: Continued.

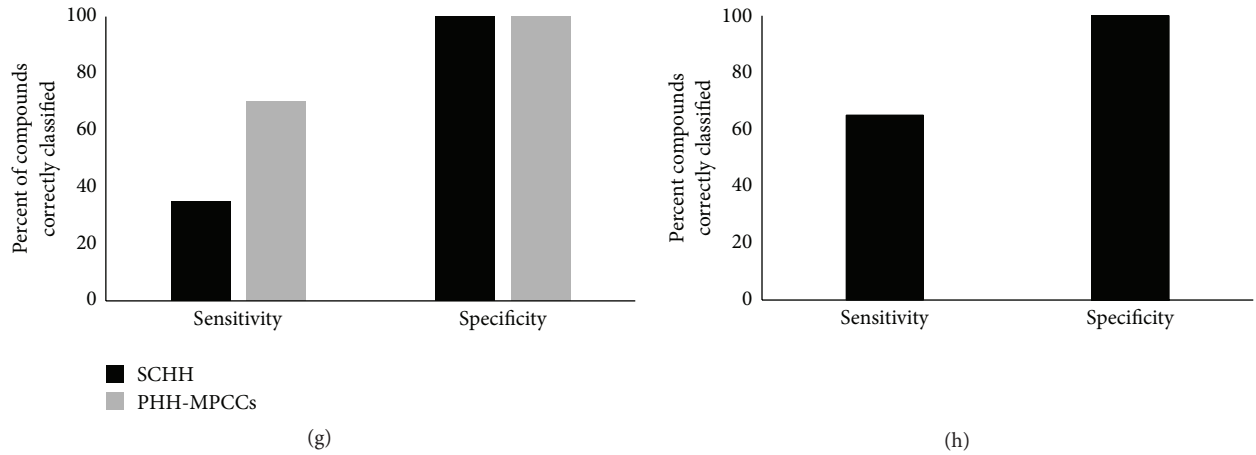


Figure 1: Micropatterned cocultures (MPCCs) containing primary human hepatocytes (PHH) or induced pluripotent stem cell-derived hepatocyte-like cells (iPSC-HH) with supporting 3T3-J2 fibroblasts. Phase contrast images of PHH-MPCCs (panel (a)) and iPSC-HH-MPCCs (panel (b)) display similar hepatic morphology with polygonal shape, formation of bile canaliculi, and distinct nuclei/nucleoli. Scale bars on images represent ~250 μm. The architecture (island diameter, center-to-center spacing, percent of a well's surface area covered by hepatocytes) affects functions in both PHH-MPCCs (panel (c)) and iPSC-HH-MPCCs (panel (d)). In panel (c), cell numbers and ratios were kept constant while changing the diameter (first number) and center-to-center (second number) spacing of the PHH colonies [14]. In panel (d), total well surface area covered by the iPSC-HHs (also called iHeps) was modulated by changing the island diameter and spacing [116]. Albumin secretion levels can be maintained for at least ~1 month in PHH-MPCCs (panel (e)) [117] and iPSC-HH-MPCCs (circles in panel (f), triangles: micropatterned iPSC-HHs without 3T3-J2 fibroblasts, diamonds: iPSC-HH conventional confluent cultures) [116]. Compared to ECM sandwich-cultured primary human hepatocytes (SCHH), both PHH-MPCCs (panel (g)) and iPSC-HH-MPCCs (panel (h)) display higher sensitivity and similar specificity for drug toxicity screening when cultures were dosed for 6–9 days with a panel of 47 drugs [27]. Sensitivity for drug toxicity detection was 65% for iPSC-HH-MPCCs and 70% for PHH-MPCCs for the chosen drug set, while it was 35% for SCHHs. Permission was obtained from Nature Publishing group to reproduce panels (a), (c), and (e). Permission was obtained from John Wiley and Sons to reproduce panels (d) and (f).

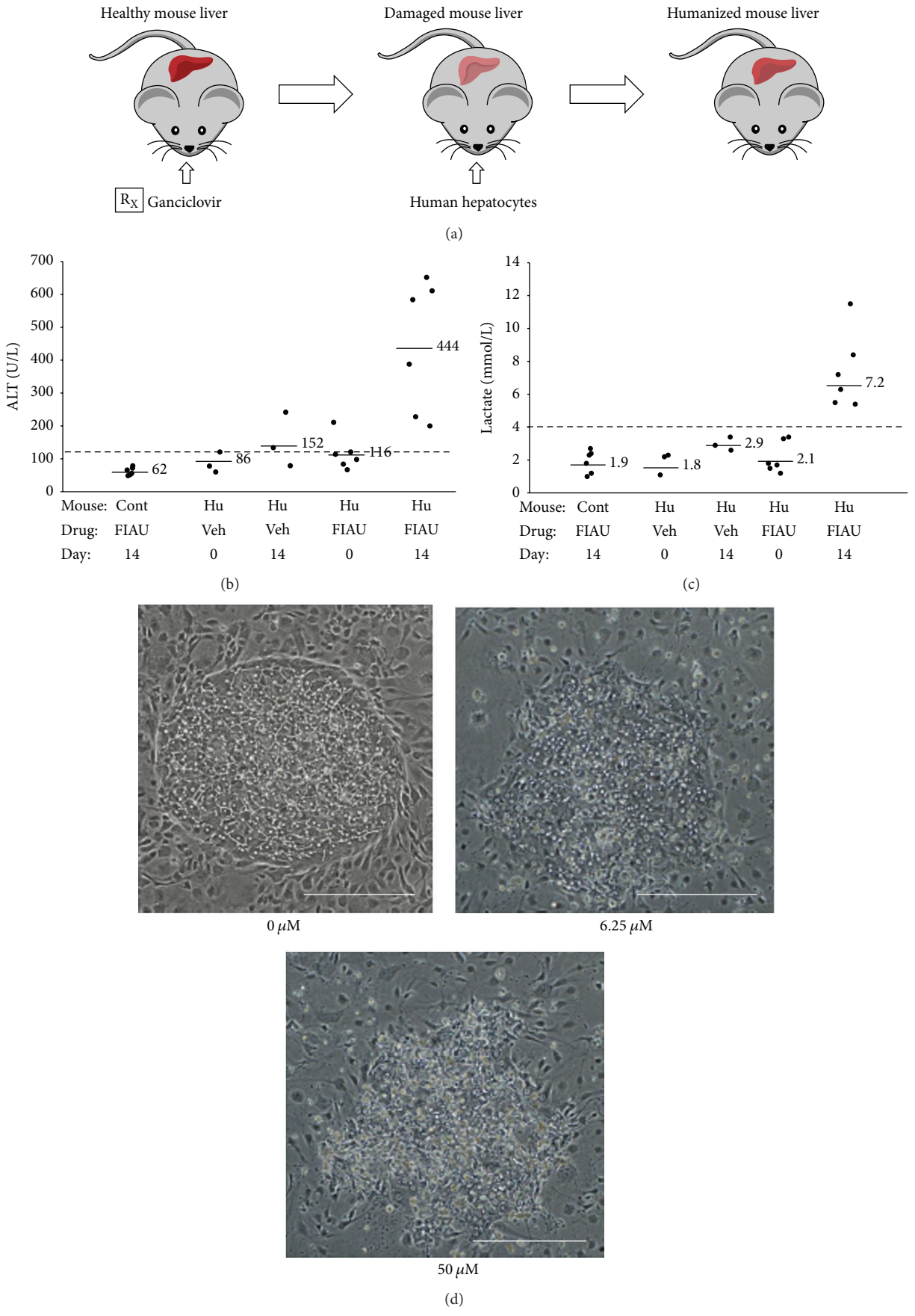
in the same system, we were nonetheless able to demonstrate iPSC-HH utility for DILI detection. In particular, iPSC-HH-based MPCCs dosed with a set of 47 drugs for 6 days yielded a sensitivity (65%) that was remarkably similar to sensitivity in PHH-based MPCCs (70%) dosed with the same drugs, while specificity in both models was 100% with a set of 10 non-liver-toxic drugs. These results suggest that iPSC-HH-based MPCCs may be ready for an initial drug toxicity screen during drug development; however, mechanistic inquiries into DILI outcomes will require further probing of active pathways within iPSC-HHs relative to PHHs.

4. Spheroidal and Bioprinted Cultures

Hepatocyte spheroids have shown improved functions over conventional 2D pure monolayers for drug screening applications, likely due to the establishment of homotypic cell-cell interactions and the presence of ECM within and around the spheroids [6]. Cell lines, primary hepatocytes, and iPSC-HHs cultured in spheroids have all shown utility for drug toxicity screening [28–33]. Additionally, PHH spheroids have been shown to replicate certain liver pathologies such as steatosis and cholestasis, allowing for the assessment of DILI in a diseased background [34].

Engineered scaffolds and channels can aid in the assembly of spheroids that are more consistent in size than is possible with random configurations. For instance, Kostadinova et al. first deposited a mixture of liver stromal cells onto a porous nylon scaffold, followed by seeding of PHHs [31]. Secretion of albumin, transferrin, and fibrinogen was maintained for ~77 days. This model detected clinically relevant drug toxicity, including species-specific drug effects with higher sensitivity than monolayer cultures. However, the gene expression profiles of this complex coculture changed over time, suggesting that it is not trivial to control the growth and interactions of the various cell types in a randomly distributed configuration. In another study, Tong et al. immobilized hepatocyte spheroids between a glass coverslip and an ultrathin porous Parylene C membrane that were both surface-modified with polyethylene glycol (PEG) and galactose for enhanced spheroid formation and maintenance [35]. In such a "constrained spheroid" configuration, loss of spheroids over time due to medium changes and/or perfusion was minimized.

A specialized plate has been developed for creating hanging drops of mixed liver cells such as PHHs, endothelial cells, and Kupffer macrophages that can lead to the formation of spheroids of controlled diameters (i.e., 253 $\pm$ 7.4 μm). These spheroids are then transferred to another multiwell plate for drug testing [36] (Figure 3). Such spheroids remain viable and secrete albumin for ~1 month. Dose-dependent toxicity of acetaminophen, diclofenac, and trovafloxacin has been observed in this platform. Trovafloxacin toxicity was further sensitive to activation of Kupffer macrophages via LPS. This system has been adapted to both hepatic



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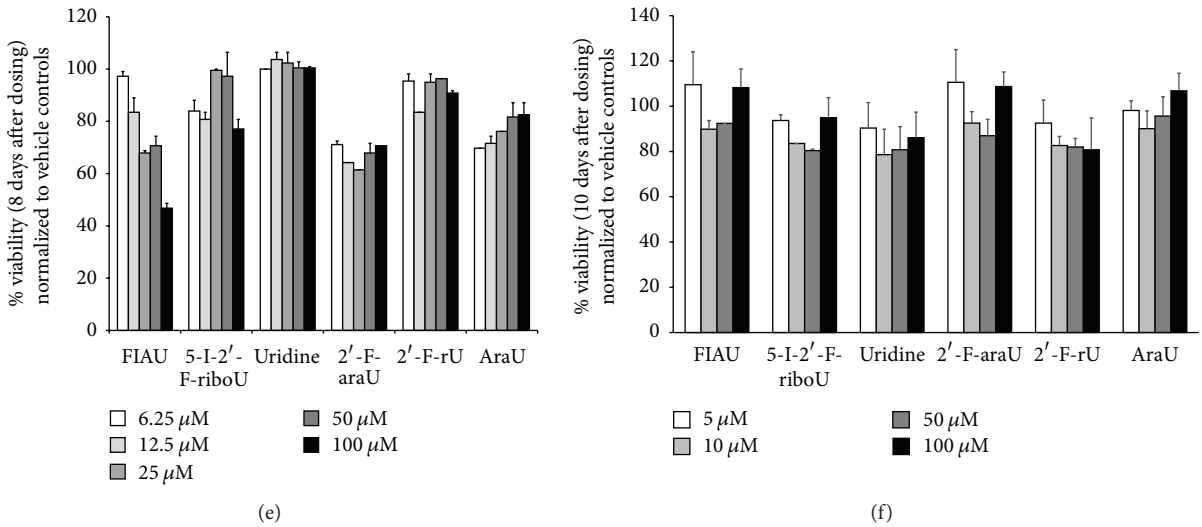


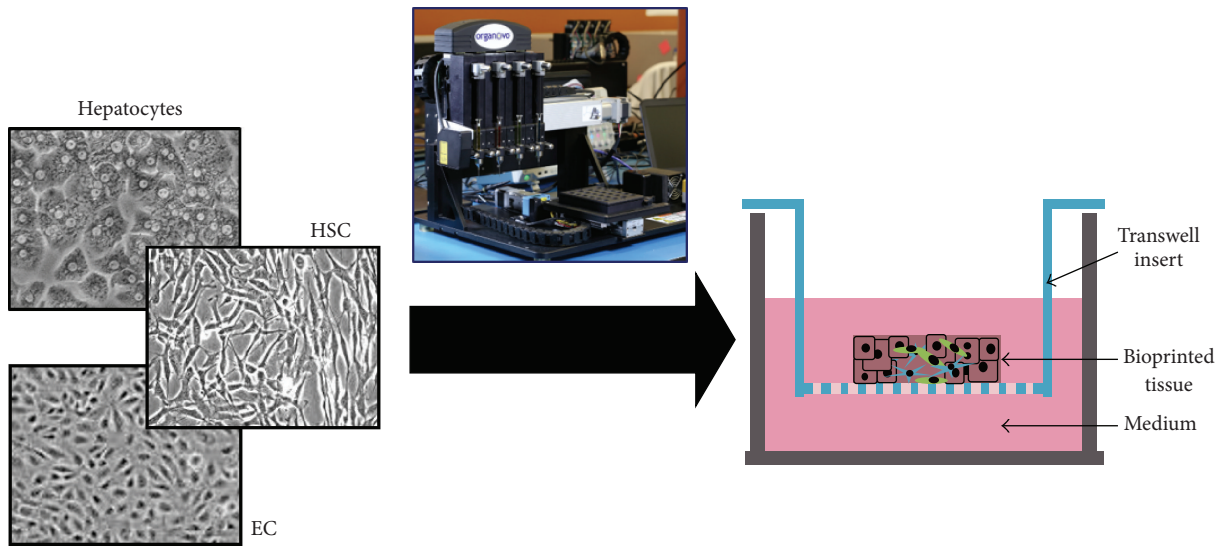
Figure 2: Fialuridine toxicity assessment in humanized rodents and micropatterned cocultures (MPCCs) containing either primary human hepatocytes (PHH) or primary rat hepatocytes. Mice were briefly exposed to a nontoxic dose of ganciclovir to ablate murine liver cells (panel (a)) [118]. PHHs were transplanted into 8-week-old mice and the humanized liver was established for 8 weeks prior to toxicology studies. Humanized and control nonhumanized mice were dosed with vehicle (0.5% dimethylsulfoxide) or 2.5 mg/kg/d fialuridine for 14 days by oral gavage. Plasma alanine aminotransferase or ALT (panel (b)) and lactate (panel (c)) levels were measured on days 0 and 14 [78]. Each dot in the graphs of panels (b) and (c) represents 1 mouse, the solid lines adjacent to the dots represent averages for each sample group, and the dashed line across each graph represents the upper limit of normal. PHH-MPCCs were dosed for 8 days with 0, 6.25, or 50 μM fialuridine and deteriorating hepatocyte morphology was recorded with increasing dose (panel (d)) [20]. Scale bars on images represent ~250 μm. In addition to fialuridine (5-I-2′-F-araU), PHH-MPCCs were dosed for 8 days with several doses of 5 other analog compounds. Mitochondrial activity was assessed using the MTT assay and normalized to vehicle only controls (panel (e)). Only fialuridine caused a dose-dependent toxicity in PHH-MPCCs. On the other hand, no dose-dependent toxicity was observed in MPCCs created using primary rat hepatocytes and dosed with the compounds for 8 days (panel (f)).

cell lines (i.e., HepG2) and PHHs. In another engineered platform, Miyamoto et al. utilized a Tapered Stencil for Cluster Culture (TASCL) device to form HepG2 spheroids [37].

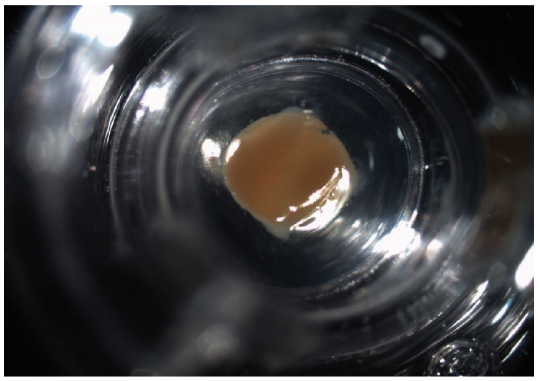
Culture of iPSC-HHs in spheroidal configurations, such as in collagen matrices, can also improve their functions relative to monolayer controls [32]. Takayama et al. utilized spheroidal cultures of iPSC-HHs created using a "nanopillar plate" to assess the toxicity of 24 drugs [38]. iPSC-HHs and HepG2 spheroids were exposed to the toxic drugs for 24 hours and cell viability was assessed using a WST-8 assay. iPSC-HH spheroids were found to be more sensitive to the toxins as compared to the HepG2 spheroids; however, the sensitivity of iPSC-HHs to the toxins was lower than that observed with plated primary hepatocyte monolayers.

Bioprinting can also be utilized to create spheroidal structures by positioning liver stromal cells (i.e., stellate cells, endothelial cells) relative to hepatocytes, which can lead to a compartmentalized architecture and microvascular networks (Figure 3). These scaffold-free "organoids" were shown to detect the toxicity of a drug that had been deemed safe in animal studies but caused human DILI (http://www.organovo.com/). Ma et al. utilized rapid, digital 3D bioprinting to print iPSC-HHs, endothelial cells, and adipose-derived stem cells in a microscale hexagonal architecture embedded in hydrogel that mimics the liver lobule architecture [39]. Whether such a complex architecture will lead to higher sensitivity for DILI detection than cells randomly distributed in a spheroidal structure has not yet been determined.

Naturally derived (i.e., alginate, chitosan, and cellulose) and synthetic biomaterials (i.e., PEG) can be utilized for embedding aggregated hepatocytes [6]. Use of biomaterials allows spatiotemporal tuning of mechanical and biochemical properties that the cells experience. For instance, the previously described MPCC platform can be first used to control cell-cell interactions between PHHs and stromal cells. Then, the entire micropatterned monolayer can be detached using collagenase treatment and embedded in PEG hydrogels that not only are biocompatible, but also provide control over mechanical properties via customization of chain length and biochemical properties by tethering active ligands such as growth factors [40, 41]. Micropatterned PHH/stromal cell clusters encapsulated in PEG displayed higher liver functions than encapsulated random cocultures. A microfluidic droplet generator can also be used to generate PEG-based hepatic microtissues, which are more amenable to high-throughput drug studies than bulk gels. In a study utilizing a naturally derived biomaterial, Tasnim et al. encapsulated human pluripotent stem cell-derived hepatocyte-like cells (hPSC-HLCs) in galactosylated cellulosic sponges [42]. The sponges promoted spheroid formation and the porous network served as a physical constraint to maintain spheroid sizes. The spheroid cultures were dosed with acetaminophen, troglitazone, and methotrexate and compared to conventional



(a)



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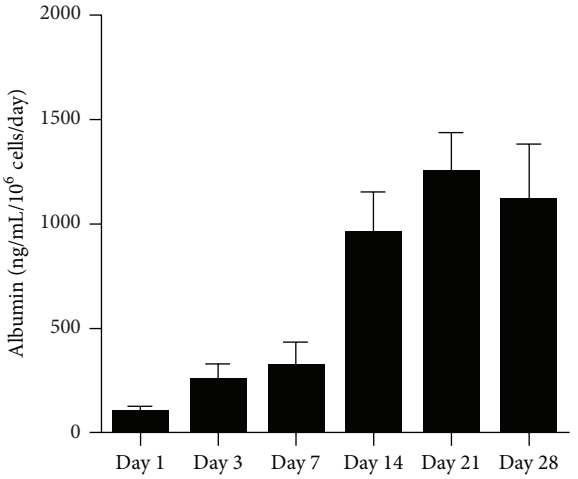

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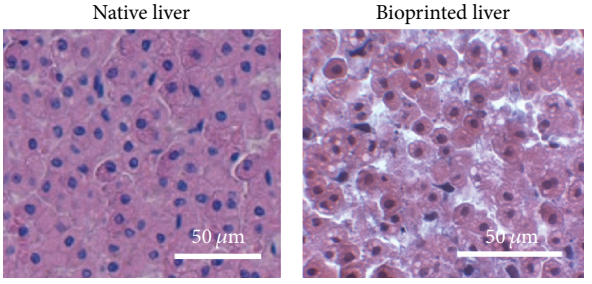

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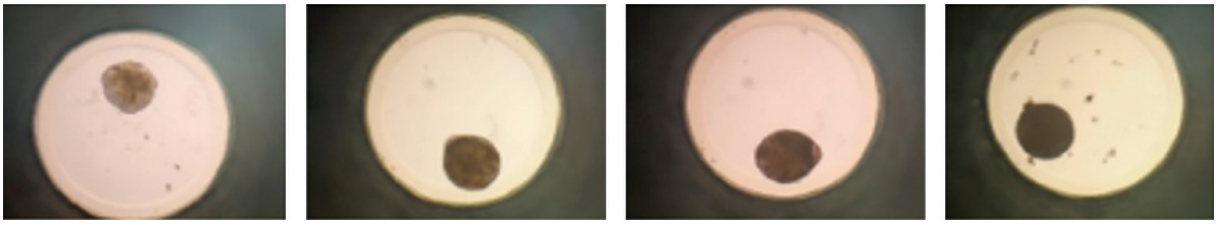

(e)

Figure 3: Continued.

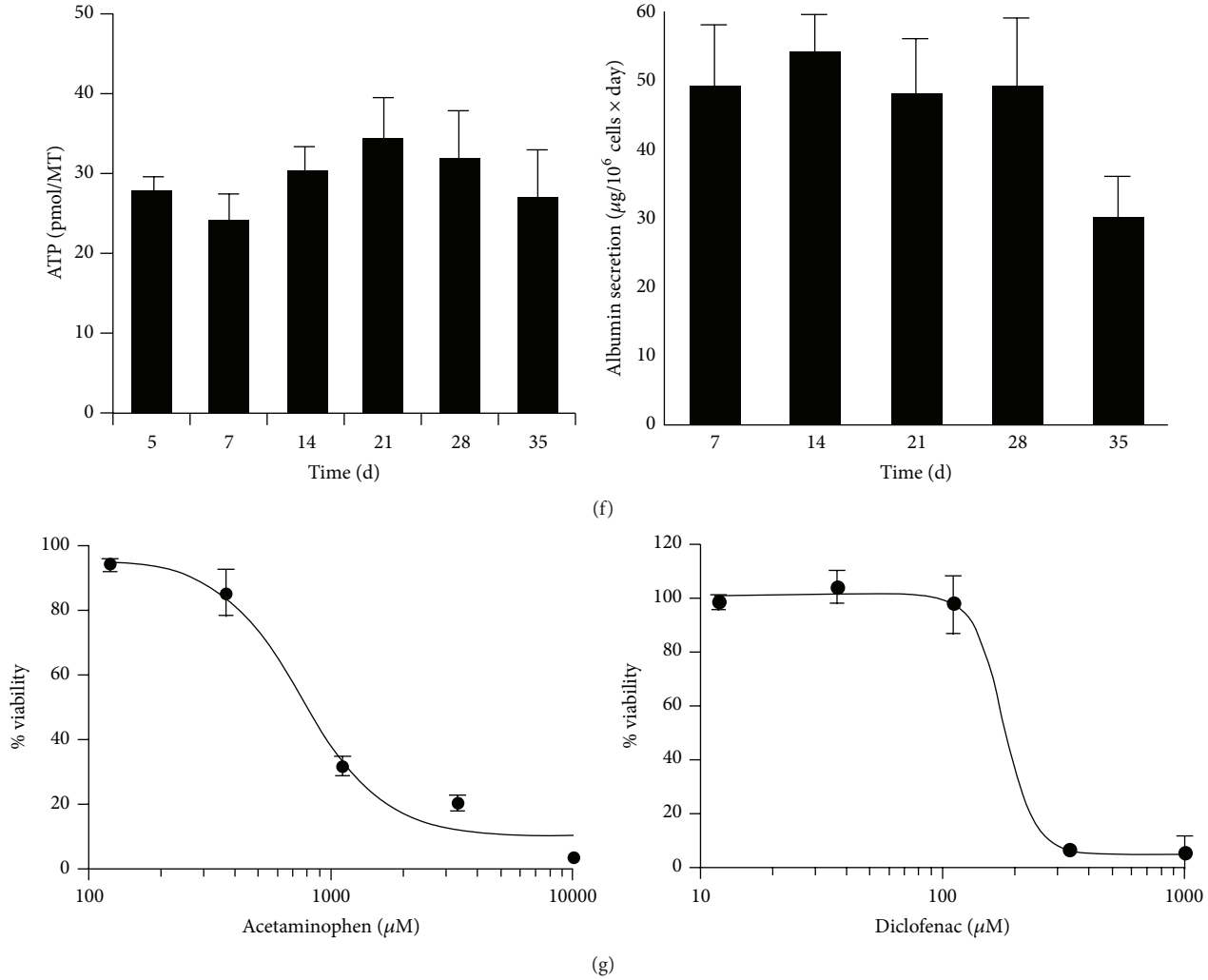

Figure 3: Spheroidal cocultures containing primary human hepatocytes (PHHs). (a) Schematic of transverse cross section of bioprinted liver tissue from Organovo containing PHHs, endothelial cells (ECs), and hepatic stellate cells (HSCs). Image of the 3D bioprinting instrument is shown as well. (b) Gross image of bioprinted human liver tissue with 2.5 mm diameter and 0.5 mm thickness. (c) Albumin secretion in bioprinted liver tissues over time. (d) Comparison of H&E stained native liver and bioprinted liver. Images and data for panels (a)–(d) were provided by Organovo, Inc. (e) Human liver spheroids from InSphero contain PHHs, ECs, and Kupffer macrophages and can maintain their size for at least 33 days *in vitro* [36]. MT indicates individual micro tissue. (f) Human liver spheroids maintain intracellular ATP content and secrete albumin for 35 days. (g) Utility of InSphero human liver spheroids for measuring dose-dependent toxicity of different drugs following an incubation period of 14 days.

2D cultures of both hPSC-HLCs and PHHs. hPSC-HLC spheroids were more sensitive to the toxins than the hPSC-HLC conventional cultures, and spheroid responses to the toxins were similar to that of PHHs.

5. Perfusion Systems

Even though hepatocytes in the liver are protected from flow-induced shear stress by the endothelial fenestrae, flow can cause gradients of oxygen, nutrients, and hormones, which have been shown to lead to zonation or differential functions in hepatocytes across the length of the sinusoid [43]. DILI can thus manifest itself with a zonal pattern dependent on the mechanism of action of the drug and its metabolism by specific isoenzymes in the hepatocytes. A parallel-plate bioreactor with oxygen gradients has been used to induce a zonal pattern of CYP450s in rat hepatocytes, which led to a zonal pattern in acetaminophen toxicity, particularly in low oxygen regions where CYP450 enzymes were expressed at higher levels than expression in high oxygen regions [44, 45].

In addition to inducing zonal hepatic functions, several investigators have postulated that flow can allow better nutrient exchange and removal of waste products, which can lead to higher hepatic functions than static cultures. Novik et al. observed production of drug metabolites at greater rates in PHH/endothelial cell cocultures subjected to flow as compared to static cocultures [46]. Sivaraman et al. perfused preformed hepatic aggregates adhered to

the collagen-coated walls of an array of microchannels and observed higher hepatic functions than static collagen sandwich cultures [47]. Esch et al. subjected multicellular cocultures of PHHs and stromal cells (fibroblasts, stellate cells, and Kupffer macrophages) to perfusion and found higher albumin and urea secretion than in static controls [48]. Instead of subjecting hepatocytes to shear stress via direct perfusion, Lee et al. subjected hepatic aggregates to nutrient exchange via flow in an adjacent channel that had through-holes similar to the fenestrae of the endothelial layer in the liver [49]. Perfusion in the channels was gravity-driven with the inlet and outlet reservoirs containing different volumes of culture media, which simplified the device by eliminating the need for external pumps. Other investigators are also incorporating gravity-driven flow into their liver devices [48, 50].

In addition to perfusion of culture medium, microfluidic devices can also be utilized to control the spatial arrangement of cells to yield the type of architecture (i.e., control over cell-cell interactions) that has been shown to improve liver functions (Figure 4). For instance, Kobayashi et al. utilized microfluidic and micronozzle devices to coculture HepG2 and Swiss 3T3 fibroblasts in a stripe-patterned hydrogel sheet, which allowed for the control of the direction of proliferation and the formation of arrays of rod-like organoids inside the hydrogel [51]. Skardal et al. mixed HepG2 cells with a hydrogel designed to mimic ECM prior to introducing the mixture into the parallel channels of a microfluidic device [52]. The cells were then exposed to ethyl alcohol and cell damage was assessed. Ma et al. also used a microfluidics-based biomimetic method to fabricate a 3D liver lobule-like microtissue [53]. The microtissue consisted of HepG2 cells and an immortal human aortic endothelial cell line to mimic the presence of liver endothelial cells and was able to metabolize acetaminophen, isoniazid, and rifampicin, and toxicity was assessed via fluorescein diacetate/propidium iodide costaining. Bhise et al. encapsulated HepG2/C3A spheroids in a photo-cross-linkable gelatin methacryloyl hydrogel and printed droplets in the cell culture chamber of a microfluidic bioreactor [54]. The encapsulated and perfused spheroids functioned for ~30 days as assessed by several markers, such as secretion of albumin and alpha-1-antitrypsin, and immunostaining for the tight junction protein, zona occludens-1. Furthermore, a 15 mM dose of acetaminophen induced a toxic response in the spheroids as expected from *in vivo* rat studies.

Microfluidic devices are inherently low-throughput for testing a large panel of drugs and are more difficult to set up and handle relative to industry-standard multiwell plates. Therefore, incorporation of real-time monitoring of toxicity biomarkers in microfluidic devices can not only aid in ease of usability, but also provide more rapid assessment of drug effects than is possible with conventional assays. For instance, Bavli et al. assessed mitochondrial function and glucose metabolism in real time on a liver-on-a-chip device where 3D aggregates of HepG2/C3A cells were exposed to rotenone and troglitazone for 24 hours [55]. Oxygen uptake dropped within a few minutes following drug exposure while the metabolic shift from oxidative phosphorylation to glycolysis occurred 3–6 hours later, coupled with a gradual change in glucose and lactate fluxes. Rennert et al. established a liver organoid consisting of human umbilical vein endothelial cells and monocyte-derived macrophages in the vascular plane and HepaRG and LX-2 stellate cells (immortalized line) in the hepatic plane with a membrane mimicking the space of Disse in a microfluidic perfused biochip [56]. Luminescent-based sensor spots were integrated in the chip to allow for real-time measurement of oxygen consumption levels. Finally, Vernetti et al. created a platform in which PHHs, EA.hy926 endothelial cells, U937 monocytes, and LX-2 stellate cells were sequentially layered in a microfluidic device that was continuously perfused and had fluorescent protein biosensors inside select PHHs [57]. Troglitazone and nimesulide toxicity were assessed whereas caffeine was used as a negative control. Increased toxicity of trovafloxacin was observed when cultures were costimulated with LPS. The model also demonstrated increased stellate cell migration and expression of alpha-smooth muscle actin and collagen in response to methotrexate, indicating fibrotic activation. The aforementioned multicellular culture models show the latest trends in liver-on-a-chip devices, which are being designed to include as many of the liver cell types as possible to allow for the crosstalk necessary (via paracrine signaling, ECM deposition, and cell-cell contact) to elicit more complex DILI outcomes than possible with PHH-only culture platforms.

Microfluidic perfusion is also useful to create organs-on-a-chip platforms in which the liver compartment is linked to compartments containing cells of other tissue types towards measuring how drug metabolism by liver cells affects other cell types [5]. Viravaidya et al. created one of the earlier organs-on-a-chip models in which cell lines were used to model lung, liver, and fat compartments that were linked with microfluidic flow to investigate the biodistribution of compounds [58]. Chouca-Snouber et al. created a microfluidic biochip that modeled the liver compartment with HepG2/C3a or HepaRG cell lines and the kidney compartment with the MDCK cell line [59]. The synergistic reaction between the two tissue types was demonstrated via ifosfamide dosing. HepaRG cells, but not HepG2/C3a, metabolized ifosfamide into a nephrotoxic metabolite. Materne et al. created a biochip consisting of HepaRG and primary human hepatic stellate cell spheroids and differentiated NT2 cell neurospheres [60]. After 2 weeks of repeated dosing with the neurotoxin, 2,5-hexanedione, the cocultures were more sensitive than the single-tissue cultures in the biochip. Esch et al. connected HepG2/C3a cells in a liver compartment on a biochip with an intestinal compartment containing Caco-2 (absorptive) and HT29-MTX (mucus-secreting) cells to investigate nanoparticle toxicity [61]. When both the liver and intestinal chambers were exposed to polystyrene nanoparticles, increased cellular damage was observed as compared to liver-only exposure. Other groups have used biochips to study liver-skin interactions in troglitazone-induced toxicity [62] and topical substance exposure [63]. Sung et al. created a microfluidic device that utilized 3D cultures of colon and liver cell lines to evaluate the toxic effects of anticancer drugs [64]. The results showed that, as compared to static 96-well cultures, the microfluidic device was able to more accurately

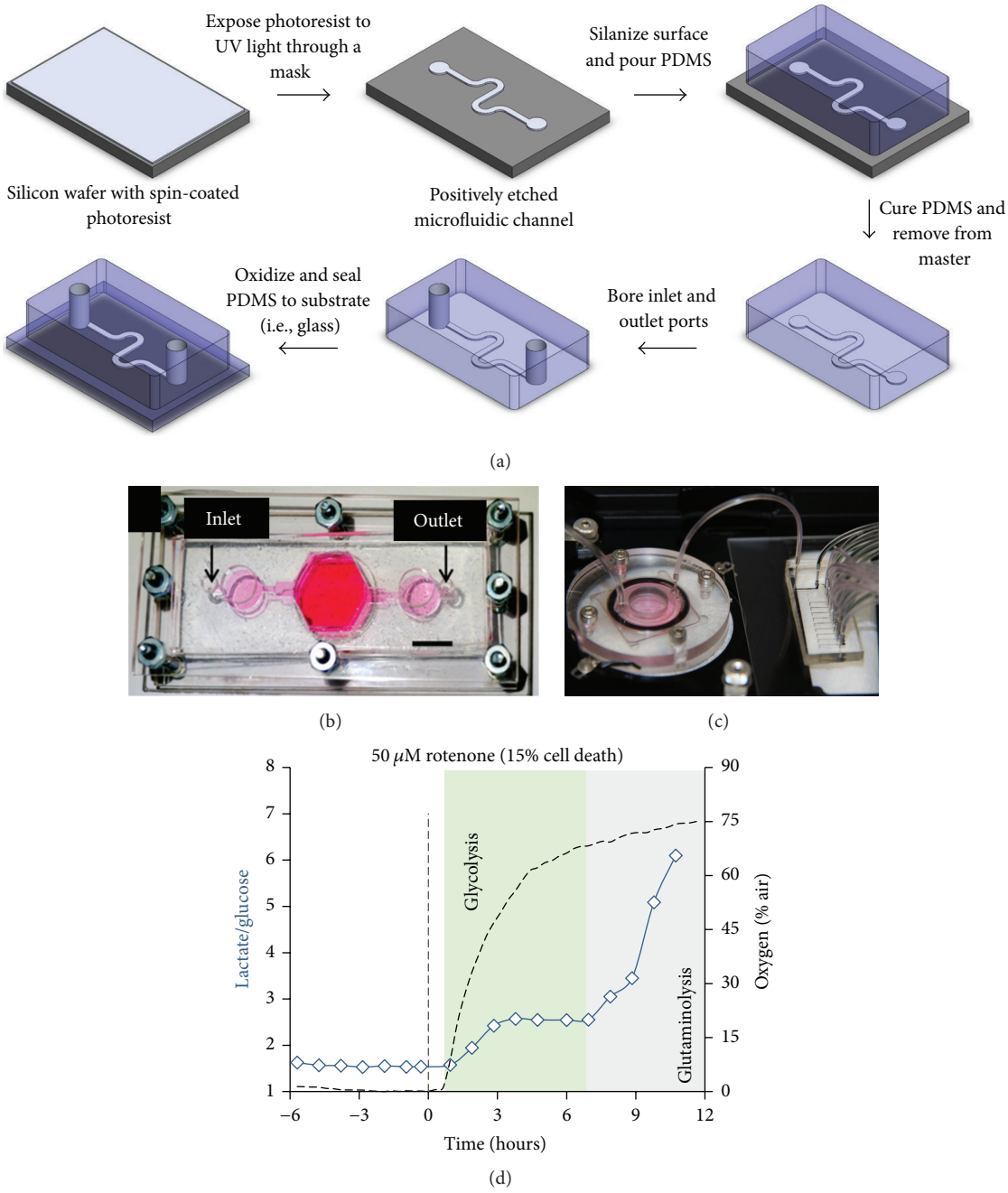

Figure 4: Liver-on-a-chip devices. (a) Soft-lithographic process utilizing photoresist-coated silicon wafers and molding of polydimethylsiloxane (PDMS) on the wafers to create microfluidic devices with channels for cell seeding and inlet/outlet ports for culture medium perfusion. (b) Top-view of an assembled bioreactor with inlet and outlet fluidic ports [54]. (c) Photo of a bioreactor hooked up to a switchboard that can be configured for real-time measurement of metabolites [55]. (d) Measurement of glucose uptake and lactate production in the device of panel (c) following dosing with rotenone. Permission was obtained from IOP Publishing to reproduce panel (b).

reproduce liver metabolism of the cancer drug, tegafur, to its metabolite, 5-fluorouracil, which caused expected toxicity to the cancer compartment. Finally, several groups have created four organ-chips for assessing multiorgan toxicity. Maschmeyer et al. combined intestine, skin, liver, and kidney modules onto a chip and assessed viability and functionality of the cells although no drug toxicity studies were carried out [65]. Oleaga et al. combined cardiac, muscle, neuronal, and liver modules and looked at doxorubicin, atorvastatin, valproic acid, acetaminophen, and N-acetyl-m-aminophenol toxicities [66].

6. Liver Slices

In contrast to many cell-based models, precision cut liver slices (PCLS) retain tissue architecture and contain all cell

types of the liver [67]. However, PCLS display a rapid decline in hepatic functionality when placed in a static culture medium [68]. Microfluidic devices have been used to extend the functional lifetime of PCLS. For instance, rat PCLS were embedded in Matrigel and placed in a microfluidic device to allow production of phase I drug metabolites after 72 hours of culture (~10% of metabolites produced relative to the day of isolation) [69]. van Midwoud et al. cultured human PCLS in a microfluidic device that was coupled with high-performance liquid chromatography (HPLC) for the detection of unstable metabolites [70]. While microfluidic perfusion can improve their longevity [71, 72], PCLS still do not display high levels of functions for more than a few days, which severely limits chronic drug dosing studies in which a stable liver phenotype is required for at least multiple weeks. Nonetheless, PCLS provide for the most intact *in vitro* human liver model for testing specific hypotheses in a 24–72-hour timeframe.

7. Humanized Rodent Models

In certain types of rodent models, the liver can be repopulated with PHHs such that human-specific drug metabolites and DILI can be measured in an *in vivo* context [73, 74]. In some instances, the repopulated humanized livers can express phase I and II metabolism enzymes and some transporters at *in vivo*-like levels [75, 76]. Several groups have subsequently used humanized rodent liver models to study mechanisms of drug toxicity. For instance, Yamada et al. used a humanized rodent model to supplement *in vitro* human hepatocyte data to assess the mode of action by which sodium phenobarbital produces tumors in rodents and the relevance of this data for human risk assessment [77]. Xu et al. showed that although control TK-NOG mice tolerated high doses of fialuridine, humanized TK-NOG mice showed dose-dependent toxicity by 3 days [78]. In particular, humanized liver regions showed vacuolar changes, the presence of enlarged fat vacuoles, and mitochondrial changes, which were present in human subjects (Figure 2). Additionally, the same group demonstrated dose-dependent toxicity of bosentan in humanized mice relative to the control mice [79]. Serum ALT (alanine aminotransferase) levels, bile acid accumulation, and the analyses of other liver plasma injury markers in humanized mice were consistent with findings in bosentan-treated human subjects.

Humanized rodent models in which only PHHs are used cannot recapitulate the types of human DILI where the adaptive immune system plays an important role. For example, although Kakuni et al. were able to recapitulate troglitazone toxicity in a chimeric mouse with a humanized liver, immune-mediated reactions associated with troglitazone toxicity could not be studied due to the use of an immunodeficient SCID mouse [80]. Therefore, more recent humanized rodent models have incorporated both human liver and human immune cells, though their utility for studying DILI is pending [81–83]. However, there remains room for improvement to better mimic the human immune system in these rodents.

Some groups have implanted PHHs in ectopic sites instead of directly transplanting them into a compromised rodent liver. For instance, Chen et al. first cultured PHHs and supportive stromal cells (3T3-J2 fibroblasts, immortalized endothelial cells) in PEG hydrogels before implanting these constructs in the subcutaneous or intraperitoneal regions of immune-competent mice [84]. The human constructs survived for up to 3 months in the intraperitoneal region. Finally, Ohashi et al. transplanted PHHs embedded in Matrigel in the kidney capsule with administration of an agonistic antibody against c-Met (also called hepatocyte growth factor receptor) to stabilize the PHHs for 3–6 months *in vivo* [85].

8. High Content Readouts

High content screening (HCS) of multiplexed fluorescent readouts can be used to obtain an understanding of mechanisms underlying DILI at the organelle level. HCS systems (i.e., Thermo-Fisher ArrayScan, Molecular Devices ImageXpress, GE Healthcare IN Cell Analyzer) couple automated and multispectral epifluorescent microscopy with software for real-time analysis of fluorescent intensities within individual cells. Here, we will focus on HCS studies in which toxicity tests were performed on human-relevant cells and will refer the reader to several other reviews that cover HCS technologies in greater detail [86–88].

HCS for DILI detection was initially implemented by O'Brien et al. using HepG2 cells [89] and later extended by Xu et al. to short-term ECM-sandwich cultures of PHHs [12]. Garside et al. then used HCS in a 384-well plate format to investigate the effects of 144 drugs on HepG2 cells cultured in the absence or presence of rat S9 fraction (for generating drug metabolites) and on cryopreserved PHHs [90]. The parameters assessed captured several mechanisms of DILI including cell number, reactive oxygen species, mitochondrial membrane potential, apoptosis, cell cycle arrest, cell stress response, phospholipidosis, and neutral lipid accumulation. HCS has also been applied to cocultures of PHHs and stromal cells by developing computational algorithms that can separate out the fluorescent intensities from multiple cell types based on nuclear size/shape or other cell type-specific signals [91]. The "Integrated Discrete Multiple Organ Coculture" (IdMOC) system was used to coculture PHHs and 3T3-L1 cells in separate wells that share culture media for the assessment of multiple endpoints after 4-aminophenol and cyclophosphamide exposure [92]. HCS has recently been adapted to monolayers of iPSC-HHs [93, 94]. Pradip et al. used HCS in human iPSC-HHs to evaluate drugs known to cause hepatotoxicity through steatosis and phospholipidosis and benchmarked them to the HepG2 cell line [95]. While the aforementioned HCS studies provide information on the effects of drugs on various endpoints, the overall sensitivity (~50–58%) is typically not improved significantly over non-HCS based assays (i.e., albumin, urea, and ATP), potentially due to the lack of other processes in such screens (i.e., transporters, interaction of hepatocytes with activated liver stromal cells, and innate and adaptive immune responses) that are relevant for the progression of DILI in the clinic. Nonetheless, the ability to probe DILI mechanisms using multiple endpoints is especially important when there is little to no information on the predicted or actual C_{max} of

a candidate compound in humans during the early stages of drug development.

Another type of high content readout involves toxicogenomics (TGx), which combines genomics (i.e., mRNA transcripts, microRNAs, DNA methylation patterns, and single nucleotide polymorphisms) and bioinformatics analyses to characterize genes and pathways underlying drugs' effects on cells [96]. Changes at the gene expression level may precede cellular damage and could thus be useful to identify the mechanisms by which a drug may cause injury following prolonged exposure. However, an FDA study showed that the human DILI potential of a drug can only be reasonably assessed using TGx analyses of *in vivo* studies in rats if the drug also produced significant elevation of ALT or TBL (total bilirubin) in the animal [97].

Human liver cultures can potentially complement TGx studies when no liver enzyme elevation is observed in animals. Rodrigues et al. exposed PHHs, HepaRG, HepG2, and human skin-derived precursor hepatic progenitor cells to acetaminophen [98]. Transcriptomics analysis showed comparable hepatotoxic effects among all the cell types except for HepG2, which did not show activation of liver damage. HepaRG was the most sensitive to liver damage, followed by human skin-derived precursor hepatic progenitor cells and PHHs. However, the culture method can lead to inherent gene expression changes in hepatocytes even in the absence of a drug stimulant. For instance, one study found significant gene expression changes when hepatocytes were cultured on collagen as opposed to Matrigel [99]. Ultimately, functionally stable engineered liver models may address such shortcomings so that TGx can be utilized during preclinical drug development for better prediction of clinical outcomes.

Gene expression can also be complemented with proteomics and metabolomics to allow for the study of DILI pathogenesis at multiple levels. We refer the readers to other reviews on the development and application of these tools for DILI detection [100, 101]. Here, it suffices to say that further validation using different drug sets across multiple laboratories with standardized data analysis schemes will be required before the aforementioned "-omics" technologies will be routinely employed in prospective drug development. Nonetheless, such tools provide a powerful means by which to study detailed molecular changes induced by drugs over time and we anticipate that their use with engineered human liver models will continue to grow.

9. *In Silico* Predictions

Quantitative structure-activity relationships (QSAR) can be used to determine whether any property of the chemical (i.e., structure) is an indicator of its potential to cause drug toxicity. There are multiple computational systems currently in use, some of which assess liver-specific toxicity [102]. For instance, Zhu et al. used 289 compounds to create *in silico* models based on chemical descriptors and *in vitro* toxicity endpoints and found that utilizing both the descriptors and the endpoints resulted in better toxicity prediction as compared to using the chemical descriptors alone [103]. Mulliner et al. have recently compiled a large set of *in vivo* hepatotoxicity data and used a machine learning approach to create models that are useful for the *in silico* safety assessment of new molecular entities during the early stages of drug development [104]. Another QSAR model that has recently been updated is the OpenVirtualToxLab, which has moved away from using "training sets" [105]. In this way, any biases that come from specific training sets are removed. The ToxCast project by the US Environmental Protection Agency (EPA) has assessed several different types of *in vitro* assays that provide information on diverse molecular pathways that are modulated upon dosing with industrial chemicals and reference pharmaceuticals [106].

Some groups are creating computational models that quantitatively integrate mechanistic pathways implicated in DILI. For instance, DILIsym software simulates pathways and progression of endpoints pertinent in DILI [107]. This software, when coupled with *in vitro* data, can model some species-specific aspects of mitochondrial effects, bile acid toxicity, and innate immune responses [108]. The "Virtual Liver" software by Strand Life Sciences can, in conjunction with *in vitro* assays, provide mechanistic insights into how a drug impacts known DILI pathways [109]. Finally, while retrospective validation of novel culture platforms for DILI prediction has traditionally used C_{max} values of drugs that have gone through human clinical trials, in a prospective drug screening campaign, physiologically based pharmacokinetic (PBPK) modeling can be useful to extrapolate between *in vitro* and *in vivo* exposure conditions [110, 111]. Such extrapolation can help establish a margin of safety (i.e., therapeutic index) for candidate compounds when comparing the concentration range that can cause toxicity relative to the concentration range that can bind to the molecular target of interest for potential efficacious effects.

10. Conclusions and Future Outlook

Human DILI is a major global health burden and it has become clear over many drug failures that animal studies are not sufficient to fully predict and understand human-relevant outcomes [3]. Furthermore, the idiosyncrasy of DILI in the clinic makes preclinical prediction even more challenging [1]. While the development of human liver models was initiated many decades ago with the isolation and culture of PHHs on ECM, the rapid functional decline of these cells outside of their native liver microenvironment limits the prediction of clinical DILI outcomes [12, 14]. Over the last few years, engineers have developed tools that now allow for more precise control over the microenvironment of PHHs such that functions can be stabilized for several weeks to months (Table 1). Initially, rat hepatocytes and cancerous/immortalized hepatic cell lines were used to test the utility of such tools, but now translation to PHHs is progressing rapidly with the realization that these cells are the closest representation of the human liver. Additionally, human liver models are being coupled with models of other organs/tissues to better predict and understand how drug metabolism in the liver affects toxicity in other tissue types. Such integration is being done both *in vitro* using microfluidic perfusion and *in vivo* with humanized rodent livers.

Table 1: Models for assessing DILI.

Model	Benefits	Limitations	Example references
Conventional cultures/cocultures	(i) High-throughput (ii) Sandwich cultures can maintain cell polarity	(i) Usually lack liver stromal cells (ii) Loss of drug metabolizing capacity occurs within hours	[6, 10–13]
Micropatterned cocultures	(i) Controlled architecture allows for higher functions for 1-2 months (ii) Coculture allows for the study of drug toxicity in a diseased background (iii) Compatible with high content imaging readouts	(i) Usually lack all liver stromal cells (ii) Use nonhuman supporting cells	[14, 17, 18, 20, 27]
Spheroidal cultures/cocultures	(i) Multicellular interactions (ii) Maintenance of major liver functions for 1–3 months	(i) Difficult to control disorganized cell type interactions over time (ii) Necrosis in center of larger spheroids (iii) Size variability (iv) Incompatible with standard high content imaging equipment	[30–38]
Bioprinted cultures/cocultures	(i) Precise control of cell placement (ii) Multicellular interactions	(i) Printing resolution does not allow placement of individual cells (ii) Low-throughput (iii) Heterogeneous distribution of drugs across the bioprinted tissues	[39, 53, 54]
Perfused biochips	(i) Dynamic fluid flow for nutrient and waste exchange (ii) Sustained functionality for at least 1 month (iii) Can be combined with module for real-time toxicity endpoint readouts	(i) Binding of drugs to tubing (ii) Large dead volume requiring higher quantities of novel compounds (iii) Low-throughput (iv) Shear stress may cause lower hepatic functions (v) Can wash away built-up beneficial molecules	[44–52, 55–66]
Precision cut liver slices	(i) Retains native liver architecture and all liver cell types (ii) Can be combined with flow to improve functional lifetime	(i) Low-throughput (ii) Rapid decline in liver functions (iii) Heterogeneous distribution of drug within the slice	[67–72]
Humanized rodents	(i) Human-relevant toxicity profiles *in vivo* (ii) Can look at organ-organ interactions with a humanized liver background	(i) Variability in human hepatocyte engraftment efficiency (ii) Low-throughput (iii) Residual murine liver cells can cause confounding results (iv) Interactions with murine organs	[73–85]

Over many years of research, the field of engineered liver models has come to realize some important considerations in the design of such models. First, PHHs can be functionally stabilized for many weeks even when the culture model does not always mimic the exact architecture or composition of the liver (i.e., disorganized 3D spheroids, cocultures with murine embryonic fibroblasts) [112]. Second, exercising control over cell-cell interactions, both homotypic and heterotypic with stromal cells in either monolayers or bioprinted tissues, is important to enhance PHH functions reproducibly across many experiments/donors and prevent premature decline. Third, inclusion of multiple liver cell types at physiologic ratios *in vitro* can be useful for modeling certain types of DILI where heterotypic cell-cell communication between two or more liver cell types is important. For instance, activation of Kupffer macrophages into a more inflamed state can downregulate certain CYP450s in PHHs, which can modulate the toxicity of drugs that are metabolized by those enzymes [17]. Additionally, drugs can activate hepatic stellate cells into becoming myofibroblasts that deposit excessive amounts of ECM and secrete cytokines which affect hepatocyte functions due to the changing microenvironment [57]. It is not yet clear how to incorporate biliary epithelial cells in liver models such that they interface with the bile canaliculi between hepatocytes and drain the canalicular contents. Such a directional flow would be important to properly determine drug disposition and toxicity to other organs.

The aforementioned technological developments have already and will continue to improve the sensitivity of human DILI detection and provide insights into the mechanisms of different types of DILI. However, with several models now available in the marketplace, selection criteria need to be applied to select appropriate models for specific phases of the drug development pipeline. In our view, the choice of the culture model is dependent on the hypotheses being tested and the confidence that the chosen culture model has the levels of sensitivity and specificity that are acceptable for the type of throughput desired. For example, renewable iPSC-HHs or even hepatic cell lines (i.e., HepaRG) when cultured in engineered platforms to improve their differentiated functions can be used to identify highly toxic compounds very early in the drug development pipeline with good specificity (i.e., low false positives) [27]. These compounds can then be subjected to medicinal chemistry to reduce or eliminate the severe toxicity with an appropriate safety margin. In the absence of $C_{\max}$ information for a compound, it is important to determine a safety margin using *in vitro* toxicity data and binding affinity of the drug to the molecular target of interest. PBPK modeling can also aid in extrapolating critical information on pharmacokinetic parameters that could be potentially important *in vivo* [110, 111]. In later stages of drug development, micropatterned cocultures (containing PHHs and liver stromal cells) and/or 3D aggregates of controlled sizes (created using engineered scaffolds or bioprinted) in multiwell plates can be used to further probe the toxic effects of lead candidate compounds following chronic dosing [18]. As a lead candidate progresses through the pipeline, organs-on-a-chip platforms could be used to determine how different tissue types interact to produce toxicity in one or more tissue types [113]. There is always a chance that a candidate drug is flagged as toxic only when the most complex/complete culture system such as an organ-on-a-chip is utilized; however, if a low-throughput but high content model like organs-on-a-chip were utilized in the early stages of drug development, it may create bottlenecks in testing many drug analogs in multiple drug classes. Thus, the needs for throughput and cost have to be balanced with the sensitivity/specificity of the culture model being utilized. Even with the need for such a balance, the aforementioned iterative use of progressively more complex human liver models still provides a significantly faster and cheaper tiered testing strategy than afforded by the slow and sometimes entirely misleading animal testing. Certainly, the expectation is that testing on human-relevant models will reduce attrition in clinical trials, which constitute a major cost center in the $3–5B and 12–15 years that it now takes to bring a successful drug to the market [114, 115].

Even with considerable progress in the development of increasingly complex human liver platforms, some key questions/issues will need to be addressed moving forward. First, it will be important to rely on similar endpoints and data normalization schemes (i.e., based on cell number, protein, and/or RNA levels) when showing functionality and stability of a given culture system so that the data can be compared across different laboratories using the same system and across different types of engineered systems. Some markers, such as albumin and CYP3A4 activity, are commonly employed for appraising PHH functions, but the community will need to agree upon which PHH markers are appropriate for specific applications and how best to demonstrate the phenotype of other cell types in the liver. Second, it is important to compare gene expression and functions of liver cells in culture over time to fresh tissue and freshly isolated cell counterparts (prior to any plating) from the same donor(s) in order to determine the extent to which cultured cells deviate from the *in vivo*-like phenotype and which pathways are affected more than others. With the increased use of commercially available cryopreserved cells, fresh tissues/cells are not always available. In that case, we believe that, at the very least, the gene expression of cultured cells over time should be compared against cells immediately after thawing. Finally, whether 3D architecture in the form of spheroids and bioprinted constructs will yield greater advances for DILI prediction than engineered 2D models is not yet clear. We anticipate that consortia led by pharmaceutical companies, which are already evaluating different engineered systems against their drugs in-house, would be highly beneficial towards addressing this question. Ultimately, *in vitro* liver culture, whether 2D or 3D, is likely not going to mimic the *in vivo* liver phenotype perfectly but the degree to which it does will determine its utility for testing specific hypotheses in drug development and mechanistic inquiries into DILI.

It would be highly beneficial to reach a consensus as to which biomarkers to utilize to validate the utility of a platform for predicting different forms of DILI. Typically, endpoints such as ATP, albumin, urea, ALT, and lactate dehydrogenase

(LDH) can be used to appraise the level of hepatic injury nondestructively in the same culture over time. Some combination of these endpoints used with stable PHH cultures can provide upwards of ~70% sensitivity for identification of drugs from several different classes as "toxic" [18]. HCS provides additional endpoints (i.e., mitochondrial membrane potential, reactive oxygen species, phospholipidosis, and lipid accumulation) to better elucidate mechanisms underlying DILI [12]. Furthermore, toxicogenomics, proteomics, and metabolomics can be used to provide indications of diverse molecular pathways that are affected by drug treatment even before overt cell injury. However, which of the aforementioned endpoints and analyses constitutes a "minimum essential set" for highly sensitive prediction of hepatotoxicity, especially during early drug development, is not yet clear. As more liver cell types are interrogated *in vitro*, we anticipate that consensus will also have to be reached on nonhepatic endpoints that are important for the prediction of those types of DILI in which heterotypic cell-cell communication plays an integral role.

Several studies utilizing cell lines, PHHs and iPSC-HHs, have shown that even some so-called "idiosyncratic" toxins (i.e., zafirlukast, troglitazone, diclofenac, and clozapine) can be detected using cellular stress markers [12, 89], potentially because such hepatic stress is a first step in the cascade of mechanisms that cause overt liver injury in specific patients with one or more covarying genetic (i.e., CYP450 polymorphisms) and environmental (i.e., coadministered drugs) factors. However, it is not currently possible to predict with *in vitro* approaches which *specific* individuals will go on to adapt to cell stress and which individuals will experience severe DILI. Creation of hundreds and even thousands of iPSC lines from different individuals, some with greater susceptibility to liver toxicity due to certain drugs, may ultimately be necessary to fully understand interindividual variations in DILI outcomes due to genetic makeup. Dosing iPSC-HH cultures with drugs under different diseased backgrounds (i.e., hepatitis B/C viral infection, steatosis, and inflammation) could also provide clues as to patient-specific DILI. However, iPSC-HH functions need to be further improved to be similar to PHHs before their potential for investigating DILI can be fully realized. Engineering tools have shown great promise in improving iPSC-HH functions, especially for DILI detection [27]; however, more progress needs to be made with not only further functional maturation, but also the use of standardized endpoints for appraising such maturity across different laboratories and culture systems.

In conclusion, different engineered human liver models can now be utilized in specific phases of drug development based on the posed hypotheses, throughput requirements, and budgetary constraints. Continued development and validation of such models will provide higher sensitivity in the prediction of different types of DILI and provide a better understanding of factors that can cause idiosyncratic DILI in certain patients. Ultimately, engineered human liver models will reduce the usage of animals in preclinical drug development and mitigate the risk of DILI to human patients in clinical trials and in the marketplace.

Competing Interests

There are no competing interests for Christine Lin. Salman R. Khetani holds stock in Ascendance Biotechnology (Medford, MA), which has exclusively licensed the MPCC platform from MIT for commercial pharmaceutical applications.

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Review Article

Key Challenges and Opportunities Associated with the Use of In Vitro Models to Detect Human DILI: Integrated Risk Assessment and Mitigation Plans

Franck A. Atienzar,[1] Eric A. Blomme,[2] Minjun Chen,[3] Philip Hewitt,[4] J. Gerry Kenna,[5] Gilles Labbe,[6] Frederic Moulin,[7] Francois Pognan,[8] Adrian B. Roth,[9] Laura Suter-Dick,[10] Okechukwu Ukairo,[11] Richard J. Weaver,[12] Yvonne Will,[13] and Donna M. Dambach[14]

[1] *UCB BioPharma SPRL, Chemin du Foriest, R9 Building, 1420 Braine-l'Alleud, Belgium*
[2] *AbbVie, 1 North Waukegan Road, North Chicago, IL 60064, USA*
[3] *Division of Bioinformatics and Biostatistics, National Center for Toxicological Research, US Food and Drug Administration (FDA), Jefferson, AR 72079, USA*
[4] *Merck KGaA, Frankfurter Strasse 250, 64293 Darmstadt, Germany*
[5] *Drug Safety Consultant, Macclesfield, Cheshire SK11, UK*
[6] *Sanofi, Bâtiment C. Bernard, 13 Quai Jules Guesdes, Zone B, BP14, 94403 Vitry-sur-Seine Cedex, France*
[7] *U.S. Food and Drug Administration, Center for Drug Evaluation and Research, Silver Spring, MD 20993, USA*
[8] *Novartis Pharma AG, Klybeckstrasse 141, 4057 Basel, Switzerland*
[9] *Hoffmann La-Roche Inc., 4000 Basel, Switzerland*
[10] *School of Life Sciences, University of Applied Sciences Northwestern Switzerland, Gründenstrasse 40, 4132 Muttenz, Switzerland*
[11] *Ipsen Biosciences Inc., 650 E Kendall Street, Cambridge, MA 02142, USA*
[12] *Institut de Recherches Internationales Servier (IRIS), 50 rue Carnot, 92284 Suresnes Cedex, France*
[13] *Pfizer R&D, Drug Safety Research and Development, Eastern Point Road, Groton, CT 06340, USA*
[14] *Genentech, 1 DNA Way, South San Francisco, CA 94080, USA*

Correspondence should be addressed to Franck A. Atienzar; franck.atienzar@ucb.com

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Drug-induced liver injury (DILI) is a major cause of late-stage clinical drug attrition, market withdrawal, black-box warnings, and acute liver failure. Consequently, it has been an area of focus for toxicologists and clinicians for several decades. In spite of considerable efforts, limited improvements in DILI prediction have been made and efforts to improve existing preclinical models or develop new test systems remain a high priority. While prediction of intrinsic DILI has improved, identifying compounds with a risk for idiosyncratic DILI (iDILI) remains extremely challenging because of the lack of a clear mechanistic understanding and the multifactorial pathogenesis of idiosyncratic drug reactions. Well-defined clinical diagnostic criteria and risk factors are also missing. This paper summarizes key data interpretation challenges, practical considerations, model limitations, and the need for an integrated risk assessment. As demonstrated through selected initiatives to address other types of toxicities, opportunities exist however for improvement, especially through better concerted efforts at harmonization of current, emerging and novel in vitro systems or through the establishment of strategies for implementation of preclinical DILI models across the pharmaceutical industry. Perspectives on the incorporation of newer technologies and the value of precompetitive consortia to identify useful practices are also discussed.

1. Introduction

Drug-induced liver injury (DILI) continues to be a major cause of clinical drug attrition. As such, identification of preclinical models to improve mitigation of this adverse event has continued to be a key focus area among pharmaceutical safety scientists [1–3]. DILI is the major cause of acute liver failure, accounting for ~14% of acute liver failure cases (excluding acetaminophen) with a mortality rate of up to 10% [4–6]. Hepatic injury is a potential clinical adverse finding for orally administered, small-molecule pharmaceuticals due to the anatomical location of the liver, which predisposes it to high transient drug concentrations ("first-pass effect"), and due to its role in xenobiotic metabolism and elimination. Therefore, continued efforts to improve preclinical models in terms of prediction and to better understand the translational implications of risk factors identified preclinically remain a major priority and challenge.

Intrinsic DILI typically occurs at a high incidence, will usually manifest in both animals and humans when a drug is taken at sufficiently high doses, and has an acute onset. As such, current preclinical models commonly detect drugs causing intrinsic DILI. The outcome is that severely hepatotoxic drugs are discontinued during discovery or early development phases, and those advanced to the clinic have safety margins that are considered acceptable for the intended indication. In contrast, idiosyncratic DILI (iDILI) occurs with less frequency ranging from an incidence of 1 in 100 patients (e.g., chlorpromazine) to the more typical incidence of 1 in 10,000 patients (e.g., flucloxacillin). Furthermore, iDILI does not follow a predictable dose-response relationship, is not related to the intended pharmacology, and often has an unpredictable or latent onset often occurring after weeks or months of dosing. Finally, iDILI is not reliably detected in preclinical models and thus is the major cause of late-stage clinical trial failures and marketed drug withdrawals [7, 8].

The pathogenesis of iDILI is not understood; however, a leading hypothesis posits that there is an initial, intrinsic insult caused by the drug followed by an adaptive response [9, 10]. According to this hypothesis, the initial insult is minimal and subclinical or transient in the majority of the population, whereas the insult is amplified or the adaptive response is inappropriate leading to severe toxicity in susceptible individuals [8, 11]. In particular, evidence suggests that intrinsic, drug-specific drivers of toxicity include drug exposure levels and inherent chemical properties, whereas factors that enhance susceptibility are specific to an individual and include a combination of physiological, environmental, and genetic risk factors [12]. The clinical manifestation of iDILI is related to some threshold concurrence of these independent factors [13, 14]. The physicochemical and structural features of a drug can cause toxicity through metabolic bioactivation and covalent binding to cellular components leading to cellular dysfunction or an immune response and/or by inhibition or alteration of cellular functions. The cellular processes that are commonly affected with DILI include mitochondrial functional impairment and initiation of apoptosis; alteration of protein function (e.g., enzymes or transporters); alterations in redox status; and activation of an immune or inflammatory response as illustrated in Figure 1 [9, 10, 15–21]. Susceptibility factors in individuals influence the adaptive responses to drug injury. The most common factors that have been identified include age, gender, nutritional status, comorbidities, drug-drug interactions, and genetic/epigenetic variability.

Specifically, several key risk factors have been identified through clinical epidemiological studies of drugs causing DILI as follows:

(1) Metabolism: drugs with extensive hepatic metabolism (≥50%) have a greater association with elevated alanine transferase (ALT) values (>3 × upper limit of normal), hepatic failure, and mortality [22].

(2) Dose: more than 75% of drugs that cause DILI are used at a daily dose ≥50 mg [22–25].

(3) Biliary elimination: drugs eliminated via biliary clearance have a higher incidence of jaundice [22].

(4) Gender and age:

  (a) cholestatic DILI occurs with a slight predominance of older age males;

  (b) hepatocellular (necrotic) DILI occurs predominantly in younger age females;

  (c) autoimmune-type DILI is reported to occur exclusively in women [24, 25].

(5) Hepatocellular DILI: hepatocellular DILI is the most common form to progress to liver failure [25].

(6) Genetic polymorphisms: genetic variants of metabolic pathways, inflammatory/immunological pathways, and mitochondrial functions have been reported; often multiple polymorphisms are present [25].

(7) Comorbid liver disease: diabetes and viral infections have been associated with enhanced susceptibility [26].

Given the pathogenic complexity of DILI, it is implicit that no single preclinical endpoint or model can predict its occurrence. Instead, preclinical hazard identification and risk assessment will require the integrated evaluation of several endpoints. However, the clinical risk factors and drivers of toxicity are still largely unknown, which hampers the development of predictive preclinical models. This is due, in part, to the fact that there is no definitive clinical diagnostic tool or set of risk factors which defines or predicts iDILI [12], and although various clinical causality-scoring criteria have been established, they are inconsistently used and cannot prospectively predict development of iDILI [26]. In addition, there is a poor correlation between results of animal studies, including rodent and nonrodent species, with the actual clinical outcome for DILI being documented [27]. Furthermore, animal studies are not statistically powered for the detection of low incidence events and are conducted using normal, young and healthy animals that are of similar age. As such, these in vivo studies may not cover many of

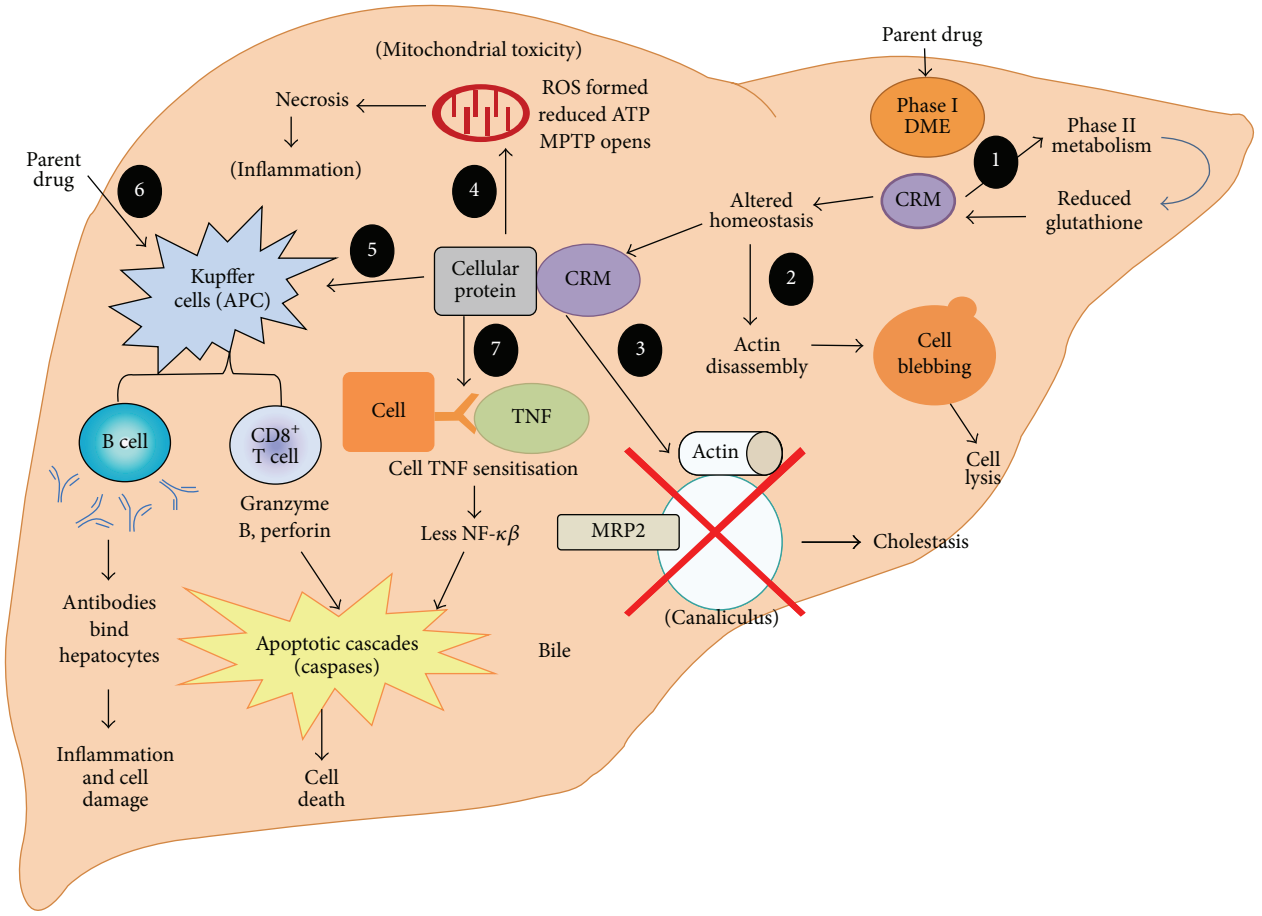

Figure 1: Overview of mechanisms of DILI. Figure extracted from Godoy et al. [21]. (1) Detoxification: conjugation with glutathione. (2) Altered calcium homeostasis. (3) Reactive metabolites may bind to transport pumps or actin around the bile canaliculi preventing bile export. (4) Reactive metabolites binding to mitochondrial proteins may reduce ATP formation, produce ROS, and open the MPTP causing apoptosis. (5) Immune stimulation via the hapten or prohapten mechanisms leading to either humoral (B cell) or cell-mediated (T cell) reactions. (6) Immune activation (PI mechanism with parent drug). (7) TNF receptor sensitivity may be heightened increasing responsiveness to TNF, leading to apoptosis. For more details, please refer to Godoy et al. [21]. Figure reproduced with permission.

the susceptibility factors that have been associated with the development of iDILI.

In vitro models can potentially address some specific limitations of in vivo models by leveraging, for example, cells with specific genetic polymorphisms or cells from patients with preexisting liver diseases or known DILI susceptibility. However, most of the currently used in vitro liver systems (e.g., monolayers of hepatic cell lines or primary hepatocytes) do not adequately reproduce the complex physiology of the liver and cannot reflect some mechanistic aspects or environmental conditions under which clinical DILI might occur. Furthermore, there has been no concerted effort at harmonization of current, emerging, and novel in vitro systems or the strategies for their implementation across the pharmaceutical industry. As a result, the knowledge of the utility and performance of the current in vitro systems is limited.

The recent breakthroughs in generating induced pluripotent stem cells (iPSCs) from selected populations may provide the variety of differentiated human liver cell types that will be needed for development of more physiologically relevant test systems [28], despite the current technical hurdles that affect reprogramming and differentiation of iPSC into mature phenotypes. Additionally, complex in vitro systems (e.g., 3D cultures containing hepatocytes and nonparenchymal cells) enable longer incubation times that may better reflect liver physiology [29, 30]. However, these systems still are not evaluated with respect to reproducing the intra- and extrahepatic variety of events (known and unknown) that ultimately lead to iDILI in patients. Future trends are moving toward the use of multiorgan cell culture systems to enhance the physiological relevance of cell cultures [31], as well as cell cultures obtained from diseased patients that may be susceptible to a specific compound. However, these advanced cell culture systems are still at an investigational stage.

The progression of preclinical assessment of DILI, in particular iDILI, will require continued mechanistic investigations both preclinically and clinically. This paper provides an overview of the key challenges for currently available in vitro preclinical models to assess DILI risk, practical considerations for improving the use of these models, and a

Table 1: Examples of in vitro assays used in DILI prediction.

Cell model	Endpoints assessed	References
HepG2 cells	High content screening of cell viability	[61, 70, 119]
HepG2 cells	Mitochondrial injury	[78, 154]
Human liver-derived cell lines expressing human P450s	Cell viability	[63, 155–157]
Isolated primary human hepatocytes	High content screening of cell viability	[60, 158, 159]
Isolated primary rat hepatocytes	High content screening of cell viability	[49]
Isolated rat or human primary hepatocytes	Biliary efflux inhibition	[160–162]
HepaRG cells	High content screening of cell viability, BC dysfunction, intrahepatic cholestasis, cell viability, steatosis	[106, 163–165]
Membrane vesicle expressing bile salt export pump (BSEP)	BSEP activity inhibition	[19, 166, 167]
Isolated human primary hepatocytes	Covalent binding of radiolabeled compounds to proteins	[48, 168, 169]
Human hepatocytes plus cytokines	Cell viability	[130]
Hepatocytes (various species cocultured with nonparenchymal hepatic cells)	Liver cell viability and function	[29, 62]
Micropatterned human or rat hepatocyte/accessory cell cocultures	Cell viability function	[71]
Human liver microtissues	Cell viability	[30]
Human liver cell 3D microfluidic liver model	Cell toxicity (multiparametric)	[32]

forward-looking perspective of the opportunities for the use of in vitro models including collaborative efforts to evaluate and standardize the use of these models.

2. Promises and Drawbacks of In Vitro Assays

2.1. Introduction. A variety of cellular models have been described and illustrative examples are summarized in Table 1. These include relatively simple cell systems that use liver-derived cell lines which express metabolic activity (HepaRG) or have limited (HepG2) or no (THLE) metabolic capacity, transfected cell lines which express physiologically relevant human cytochrome P450 (CYP450) activities, primary hepatocytes cultured in a static monolayer configuration, hepatocytes cocultured with nonparenchymal liver cells or other accessory cells, human liver microtissues that contain multiple cell types in physiologically relevant 3D configuration, and 3D multicellular culture formats exposed to shear stress using microfluidic devices. All of the cellular models can be used as high volume routine assays, apart from human hepatocyte covalent binding studies (which require availability of radiolabeled drugs) and the 3D microfluidic human liver models [32]. Among the cell lines, HepaRG cells represent a highly differentiated model of liver metabolism and transport function for the study of many intracellular events associated with drug toxicity [33, 34].

Based on our current understanding of DILI mechanisms, it is reasonable to assume that an optimal discovery test cascade could require routine high volume use of several assays in parallel, thereby concurrently investigating key mechanisms that may cause DILI. Use of multiple assays that explore individual mechanisms is resource intensive but is essential to develop the required scientific understanding and to enable project teams to explore and understand potential structure-toxicity relationships that can aid rational design of nonhepatotoxic drugs. Such assays are also valuable for exploring and understanding mechanisms by which drug candidates cause liver injury in humans or animals and potentially to enable selection of alternative compounds that do not exhibit such liabilities.

2.2. From Patients to In Vitro Early Screening: The Promise of hiPSCs. The generation of functional hepatocytes from human induced pluripotent stem cells (hiPSCs) continues to pose a major challenge. Although iPSC-derived hepatocytes have been generated, these remain neither fully characterized nor validated, and currently these cells cannot be produced on a large scale. Nevertheless, cardiomyocytes derived from human cells are currently in use and provide valuable insight into the usefulness to the pharmaceutical industry of differentiated cells derived from hiPSCs.

The classical preclinical methods for detecting cardiotoxicity have relied on genetically modified cell lines, which do not accurately simulate human cardiomyocytes. Recent technological advancements permit the generation of hiPSCs from the skin, which can then be used to produce patient-specific cardiomyocytes (CMs) under in vitro conditions. This means that each hiPSC generated from a patient's fibroblasts carries the relevant genetic information from that individual, thereby providing a huge opportunity to better understand many human disorders through "disease in a dish" modelling. For example, hiPSCs have been used to

recapitulate disease phenotypes of genetic cardiac diseases such as long QT syndrome (LQT [35]), familial hypertrophic cardiomyopathy (HCM [36]), and familial dilated cardiomyopathy (DCM [37]). Patients suffering from LQT, HCM, and DCM syndromes are particularly sensitive to cardiotropic drugs and are vulnerable to fatal arrhythmias [38]. Recently, a library of hiPSC-CMs derived from patients with LQT, HCM, and DCM was characterized and screened against a panel of drugs known to affect cardiac ion channels [39]. Liang and collaborators [39] recapitulated drug-induced cardiotoxicity profiles for healthy subjects and LQT, HCM, and DCM patients at the single cell level for the first time. The data obtained revealed that healthy and diseased individuals display different susceptibilities to cardiotoxic drugs [39]. In other words, cohorts of disease-specific hiPSC-CMs have produced distinct pathological phenotypes associated with clinical presentations of LQT, HCM, and DCM. Finally, Liang et al. [39] revealed that hiPSC-CMs could detect drug-induced cardiac toxicity more accurately than the classical preclinical assays mandated by regulatory authorities.

These investigations using iPSCs clearly illustrate the ability to use these models for lead optimization and exemplify the concept of personalized medicine using in vitro assays, which enable assessment of the genetic susceptibilities of distinct individuals to better predict clinical outcomes. This aspect is especially valuable, because the majority of cardiotoxic drugs have a low incidence of harmful effects for the general population (similar to DILI) and are often toxic to specific patient populations with determined genetic traits [39]. Taken together, these findings strongly support the use of hiPSC-CMs to better select and develop promising compounds devoid of cardiotoxic effects.

2.3. Generation of Human In Vitro Data to Predict Clinical Data: A Case Study with Fialuridine. Second generation nucleoside analogues, such as fialuridine (FIAU), have been used as potential drugs to treat hepatitis B. Preclinical studies in mouse, rat, dog, and monkey showed no sign of DILI at doses up to 1000-fold the human therapeutic dose [40, 41]. In a clinical trial, fifteen patients with chronic hepatitis B received FIAU at a dose of either 0.10 or 0.25 mg kg/day for 24 weeks and were monitored every 1 to 2 weeks by means of physical examination, blood tests, and testing for hepatitis B virus markers [42]. Unfortunately, seven patients developed severe hepatotoxicity, with progressive lactic acidosis, worsening jaundice, and deteriorating hepatic synthetic function [42]. Five patients died and two survived after liver transplantation. These toxic effects were probably caused by mitochondrial damage and were not predicted by animal studies [42]. In vitro investigations using hepatocytes in a micropatterned coculture model (Hepregen Corporation) revealed that FIAU was significantly more toxic to human hepatocytes (IC_{50}: ~5 μM) as compared to rat hepatocytes (IC_{50} > 100 μM), while its diastereoisomer was not toxic (IC_{50} > 100 μM) in either species [43]. These data illustrate the added value of using human relevant models as a part of the selection of drug candidates because in vivo preclinical studies do not always predict clinical outcome. A large multinational pharmaceutical company survey, which evaluated animal toxicity data and human adverse effects observed in clinical trials of 150 candidate drugs, revealed a true positive human toxicity concordance rate of 71% for rodent and nonrodent species [44]. Toxicity studies in nonrodents alone were predictive of 63% of the 221 human toxicities that were observed, while studies in rodents alone were predictive of 43%. Furthermore, DILI and hypersensitivity/cutaneous reactions in humans were the most difficult target organs to predict based on animal studies [44]. Therefore, there is a substantial opportunity for data provided by well-validated in vitro models to improve human DILI prediction.

2.4. Drawbacks and Limitations of In Vitro Assays. Useful in vitro assays should focus on detection of known mechanistic risk factors for DILI in humans. An important use of these assays is to flag and enable deselection of compounds exhibiting a high human DILI propensity, thereby aiding the selection of drug candidates with low propensity to cause DILI. It is now generally accepted that interpretation of data provided by in vitro assays requires knowledge of in vitro drug potency (typically expressed as EC_{50} or IC_{50}) and can be improved when human drug exposure is available [45–47]. Typically, steady state drug concentrations in plasma (C_{ss}) or maximum plasma drug concentrations (C_{max}) are used. Ideally, obtaining in vitro intracellular drug concentrations would be useful when analyzing the data; however, this is usually not known. Knowledge of in vitro hepatocyte concentrations would add important information for understanding exposure-effect relationships, so this limitation is an important consideration.

While the modest DILI sensitivity of individual assays is not surprising since liver injury can occur by different mechanisms, it highlights the limitations of these in vitro models. Development of DILI in patients is a complex consequence of multiple contributory biological processes, all of which are not reproduced by the currently available in vitro methods. Notable omissions include limited or no metabolic capacity, which may result in underestimating toxic effects of metabolites or the potential for detoxification, limited bile formation and excretion, and no adaptive immune responses. Consequently, several groups have explored whether improved sensitivity of DILI prediction can be obtained by combining data provided by several assays, each of which address differing mechanisms. This approach has yielded very encouraging results (e.g., [48]), as have approaches that combine in vitro assay data with physicochemical properties of drugs and/or in vivo plasma exposure data (e.g., [47, 49]).

The cell types utilized in assays are also an important consideration. Primary cells are considered to be the more relevant cell type because they more closely mimic the normal hepatocyte in vivo with regard to expression patterns and functions. Primary cells usually are less abnormal in their overall biology compared to transformed cells lines, which are derived from tumors and continue proliferating even after reaching confluency in monolayers [50]. However, because primary cells do not divide, their supply can be limited and there is a high degree of donor variability with regard to gene expression and function caused by underlying diseases,

as well as life style (alcohol abuse, smoking, and chronic drug treatments). Many different types of immortalized liver-derived cell lines are readily available and can be used to study hepatotoxicity. Most hepatocyte cell lines are derived from hepatocellular carcinomas and exhibit abnormal karyotypes and expression patterns that change after passaging of the cells. For example, HepG2 cells display a highly abnormal hyperdiploid karyotype with 55 chromosome pairs (http://www.hepg2.com/) and a long list of genetic mutations [51]. Thus, transformed cells are considered to least represent the normal hepatocyte in vivo and this must be considered when utilizing these cells [50]. Liver cell lines can also be generated from primary liver cells, which can be engineered to become immortalized [52].

Traditional static in vitro cell systems use single cell types and so lack interactions between different cell types (e.g., nonparenchymal and immune) and exposure to immune, hormonal, and humoral factors that together alter liver function [53, 54]. The interaction with the immune system often plays a key role in human iDILI [8] and typically does not occur in toxicity studies undertaken in animals or in traditional monoculture in vitro models. In addition, hepatocytes require key interactions with extracellular matrix components for normal function. This is demonstrated by hepatocytes taking on a pseudo-3D shape and forming functional bile canaliculi when cultured in matrix sandwich configuration but not when cultured in a standard monolayer configuration [55, 56]. Other important factors that impact the use of in vitro models include the choice of dose range and duration of treatment of cells with test compounds, often markedly different from those that occur when patients are dosed with drugs [57], and the physiological cell status, specifically with regard to oxygen tension, which can have important consequences on cell behavior [58].

3. Lack of Standardization in the DILI In Vitro Field

A current critical hurdle is the lack of standardization of these models, which limits our understanding of how to best utilize them and the need for validation for potential use in regulatory submissions [59]. The following sections address important parameters which need to be standardized, to facilitate comparison across in vitro DILI studies and thus to maximize scientific knowledge and the potential for industry wide acceptance. Finally, in order to be widely used by the industry, the developed assays will need to be of reasonable throughput, reliable, robust, easy to handle, reproducible, sensitive, specific, cost-effective, and easy to interpret (i.e., with a minimal amount of ambiguity in the data generated).

3.1. Compound Classification. The foundation for establishment of an in vitro tool to predict DILI should ideally rely on a well-defined set of compounds, which have been tested in vivo (animal and/or clinical data, depending on what endpoint the in vitro tool is aiming to predict) and where the severity and frequency of observed toxicity are described consistently. For DILI, a key challenge is the need to take account of both intrinsic (acute, short-term) hepatic injury and iDILI. Drugs causing human iDILI are especially difficult to classify because preclinical toxicity data are often not available in the scientific literature and there is only limited knowledge about their clinical adverse effect if available, due to the very low number of patients affected. Furthermore, different investigators may classify the available data in markedly different ways (see Section 5.1 for more details). The following example illustrates the challenge when attempting to classify iDILI. Tacrine was the first centrally acting cholinesterase inhibitor approved for the treatment of Alzheimer's disease, but its use was discontinued in the US in 2013 due to hepatotoxicity concerns. Tacrine has been classified by different investigators as nonhepatotoxic [60], moderately hepatotoxic [61], or highly hepatotoxic [62, 63]. In the Liver Toxicity Knowledge Base (LTKB), it is classified as $^{\text{V}}$Most-DILI-concern with a DILI severity score of 7 [64], because rare cases of liver toxicity associated with jaundice, raised serum bilirubin, pyrexia, hepatitis, and liver failure have been reported in Tacrine exposed patients (LTKB data). This example demonstrates the conflicting information available for compound classification (for more details please refer to Chen et al. [65], Figure 2 and Section 5.1). Classification of the type of hepatotoxicity is also important to consider, especially when investigating mechanisms of action, as there are many different liver pathologies caused by drugs [66] (e.g., liver hypertrophy, bile duct hyperplasia, cholestasis, steatosis, and phospholipidosis). The link between the liver specific pathologies and mechanism of actions is largely unknown now, but its exploration will be important to help better understanding and prediction of hepatotoxicity. Finally, it is important to recognize that the DILI classification of a given drug may evolve with time as new information becomes available.

The current lists of DILI drugs used for the validation of in vitro models contain a mixture of compounds with high and very low incidence for DILI, as well as intrinsic and idiosyncratic toxicants [67]. This mixture of incidence and type of DILI confounds the predictive power of these assays. A more realistic approach for assessing the predictive value of a new assay would be to separate model compound sets based on their incidence of injury [67]. To achieve this, a collaborative effort is required to obtain and share incidence data and to determine cut-offs for inclusion of compounds as positive or negative controls [67]. It is important to use a reliable and recently updated system that allows for classification of drugs. The LTKB was developed with the specific aim of enhancing our understanding of DILI ([65, 68] and Section 5.1). It is recommended that current and future investigators use the LTKB to aid their compound selection and data interpretation wherever possible, thereby enabling improved comparison between different models. It is also proposed that researchers use a well-balanced selection of reference drugs spanning a wide range of targets and chemical structures, in order not to bias the training set. The chemical space in drug development has dramatically evolved over time and many of the new drug entities in industry display properties which are potentially not represented in reference sets of well-characterized classic hepatotoxic drugs

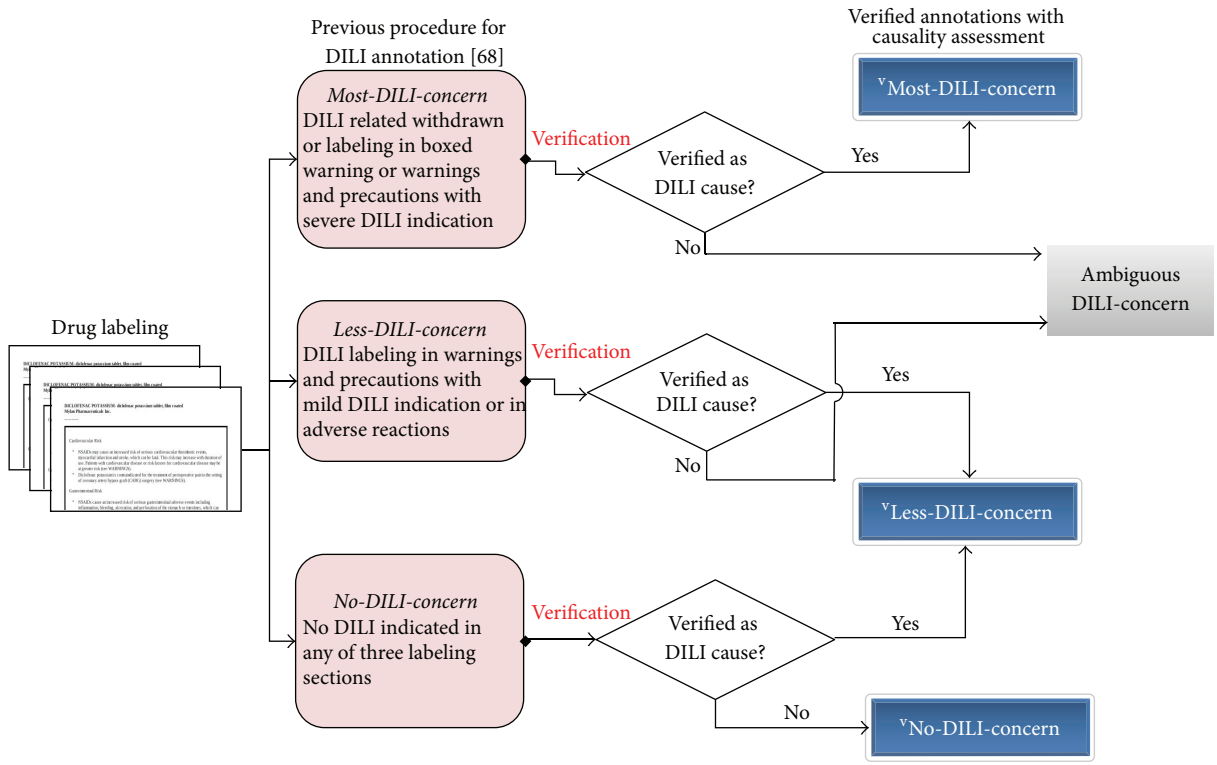

FIGURE 2: The schema to refine the drug labeling based DILI annotations by weighting the causality evidence. Three verified categories ([v]Most-, [v]Less-, and [v]No-DILI-concern) and one "Ambiguous DILI-concern" group were classified in the new schema. For more details, please refer to Chen et al. [65]. Figure reproduced with permission.

developed decades ago. This poses a risk for both under- and overprediction of hepatotoxic potential.

3.2. Concentrations and Cut-Off Selection to Evaluate the Predictivity of In Vitro Models. The translation of exposure-effect relationships from in vitro to in vivo is a major challenge for drug testing. For creation of a reference set with well-known drugs, clinical exposure data should be incorporated to mimic liver drug load as closely as possible. As outlined below, some published approaches make use of such concentration estimates. What needs to be taken into account is the fact that, at the stage of development, where a DILI assay typically would be applied, human exposure data is not available, and in most cases animal exposure data are not available. In vitro pharmacology data and ADME parameters can be used to estimate human exposure, with the caveat that these estimates have a significant degree of uncertainty, which, in turn, limits the conclusions that can be drawn about the translatability of a toxicity signal at a given concentration.

Scientists usually assess assay performance in terms of sensitivity and specificity. The sensitivity (true positive rate) is defined as the ability of a test system to predict the positive outcome under evaluation (i.e., hepatotoxicity). The specificity (true negative rate) represents the ability of a test system to predict the negative outcome under evaluation (i.e., nonhepatotoxicity). It is clear that such parameters depend greatly on the concentrations and cut-offs used in the experiments. Some studies have used fixed concentrations to study drugs in the ranges 0.1–100 μM [69], 100 μM [61], 1–500 μM [63], and 1–1000 μM [70] and/or multiples of plasma C_{max} (the therapeutically active average plasma maximum concentration value upon single-dose administration at commonly recommended therapeutic doses): 30-fold [61], 1–100-fold [71], 12.5–100-fold [62], and 100-fold [60]. In addition, different concentration criteria have been used to classify drugs as hepatotoxic: 10 μM [69], 100 μM [63], 100 and/or 1000 μM [70], 30-fold [61], or 100-fold C_{max} [60, 62, 71]. All together, these data illustrate the diversity in the strategies in terms of concentrations and cut-offs. They may also reflect an attempt to set thresholds that best fit the experimental data to obtain the most favorable predictivity in terms of specificity and sensitivity outcomes. However, these adjusted thresholds may not hold true with a different set of data. Hence, it would be helpful to reach a consensus particularly when reference drugs are used. Xu and collaborators [60] reported that the 100-fold C_{max} scaling factor represented a reasonable threshold to differentiate safe versus hepatotoxic drugs. This calculation takes into account different scaling factors: 6 × (for population C_{max} variability), 6 × (for higher drug exposure to the liver), and 3 × (for drug-drug or drug-diet interactions) = 108 C_{max} which has been approximated to 100 C_{max} [60]. For screening activities, in absence of known C_{max} values, fixed concentrations therefore should be used. When analyzing and interpreting data obtained for drugs which have been evaluated in the clinic, it is more logical to use multiples of C_{max} up to 100-fold as this is scientifically

justified. However, one limitation of using a C_{max}-based testing approach is that it does not take into account potential drug accumulation in the liver or protein binding (see Section 5). Nevertheless, C_{max} values can be easily measured and are easily accessible for reference compounds.

3.3. Endpoint Selection. Liver injury is certainly challenging to predict because many mechanisms can induce hepatotoxicity (Figure 1) and what finally results in DILI may be the interplay of genetic disposition of the patient age and disease state and a chain of cellular effects triggered by drug treatment leading to multiple events. The types of DILI cellular events can be very diverse (see introduction part for more details). So before using any in vitro models it is important to determine which mechanisms can be detected, particularly when these are used as a part of investigative studies.

Many diverse endpoints have been measured such as ATP [48, 62], LDH [72], 5-carboxyfluorescein diacetate acetoxymethyl ester [73], albumin [74], impedance (label free approach) [62], glutathione [62, 71, 75], reactive oxygen species [75], mitochondrial toxicity [76–78], phospholipidosis [79], transporter inhibition [48], or a mixture of parameters using high content analysis [55, 70, 80, 81] as illustrated in Figure 3. Screening compounds using high content imaging of cells have the advantage of measuring multiple parameters simultaneously. For instance, Persson et al. [80] presented the validation of a novel high content screening assay based on six parameters (nuclei counts, nuclear area, plasma membrane integrity, lysosomal activity, mitochondrial membrane potential, and mitochondrial area). Multiple parameters can also be measured with other approaches. For example, glutathione and ATP levels as well as albumin and urea secretion were measured in micropatterned coculture models [71]. In this study, it was reported that albumin secretion was the most sensitive parameter (10/10), followed by urea secretion and ATP levels (9/10) and GSH levels (7/10). Consequently, nondestructive measurement of albumin and urea in medium could be sufficient for an initial toxicity assessment, whereas parameters such as GSH could be used subsequently for probing specific mechanisms [71]. In another study, Porceddu and collaborators [82] developed a high-throughput screening platform using isolated mouse liver mitochondria and measured multiple mitochondrial endpoints such as inner and outer membrane permeabilization as well as alteration of mitochondrial respiration driven by succinate or malate/glutamate.

It may not be possible to reach a consensus on a list of markers to use to measure hepatotoxicity in vitro. One may also question the relevance of measuring general cytotoxicity markers in comparison to more mechanistic endpoints. Nevertheless, scientists are encouraged to use endpoints that cover as many mechanisms as possible in a logical and hypothesis-driven manner as illustrated by Thompson et al. [48]. Next to technologies allowing parallel measurements in one experiment such as high content imaging, approaches incorporating a battery of assays run in parallel and taking into account exposure aspects have been recently published and show promising performance with respect to DILI prediction [48, 83].

3.4. Other Parameters Influencing the Predictivity of DILI In Vitro

Length of Exposure. Short-term, high dose, single exposure in vitro studies are often performed but they have missed a number of hepatotoxic drugs in humans. One reason could be that the exposure time is restricted to days while liver injury can occur 1–6 months after initiating therapy [16]. With the emergence of novel in vitro models that can be cultured for weeks, in vitro studies with repeated administrations are now more common [62, 71, 84]. For instance, Khetani et al. [71] reported that more hepatotoxic compounds were detected in coculture models after 9 days of dosing (four repeat drug administrations in total) compared with 5 days of dosing (two repeat drug administrations in total). In another study, the use of label-free technologies allowed longitudinal assessment of cell behavior from attachment to the end of experiment and after compound additions [62]. The next step will likely be to expose in vitro models to low doses of drugs for longer time to better mimic the human situation. Finally, the selection of endpoints, the duration of exposure, and the number of repeat drug administrations chosen for these in vitro models is also a matter of debate. One may consider that, in a screening mode, single administration and 24–72 h exposure may be enough to rank compounds, whereas multiple administrations over long periods may be required for mechanistic studies to better compare to in vivo data.

Culture Conditions. The objective here is certainly not to describe all factors that influence the data but simply to remind scientists that a simple change in culture conditions may have a strong impact on the data generated. For instance, the presence of serum may not only decrease drug free concentrations due to protein binding but also enhance the long-term culture of coculture models [71]. In addition, most cell media contain high concentrations of glucose. As a consequence, ATP is mainly generated via glycolysis despite the presence of oxygen and functional mitochondria in cells. Unfortunately, such anaerobically poised cells are resistant to xenobiotics that impair mitochondrial function [85, 86]. To better allow the detection of drug-induced mitochondrial effects, it is important to force cells to rely on mitochondrial oxidative phosphorylation rather than glycolysis by substituting glucose with galactose in the growth media forcing cellular use of glutamate through the Krebs cycle [86]. Another important parameter to consider is the solvent used to solubilize test materials and especially the concentration of solvent in order not to interfere with cell functionality. For example, while DMSO is a commonly used solvent, it is known that above a certain concentration DMSO may have an effect on mitochondria and CYP activities and may modify cellular responses [87], as well as being an antioxidant which may also hinder the effect of reactive oxygen species [88].

In Vitro Models. There are a large number of in vitro and ex vivo models available (e.g., 2D, 3D, with or without nonparenchymal cells, static, microfluidic, microtissues, liver slices, and perfused liver), but currently there is no clear consensus on which models most accurately predict human

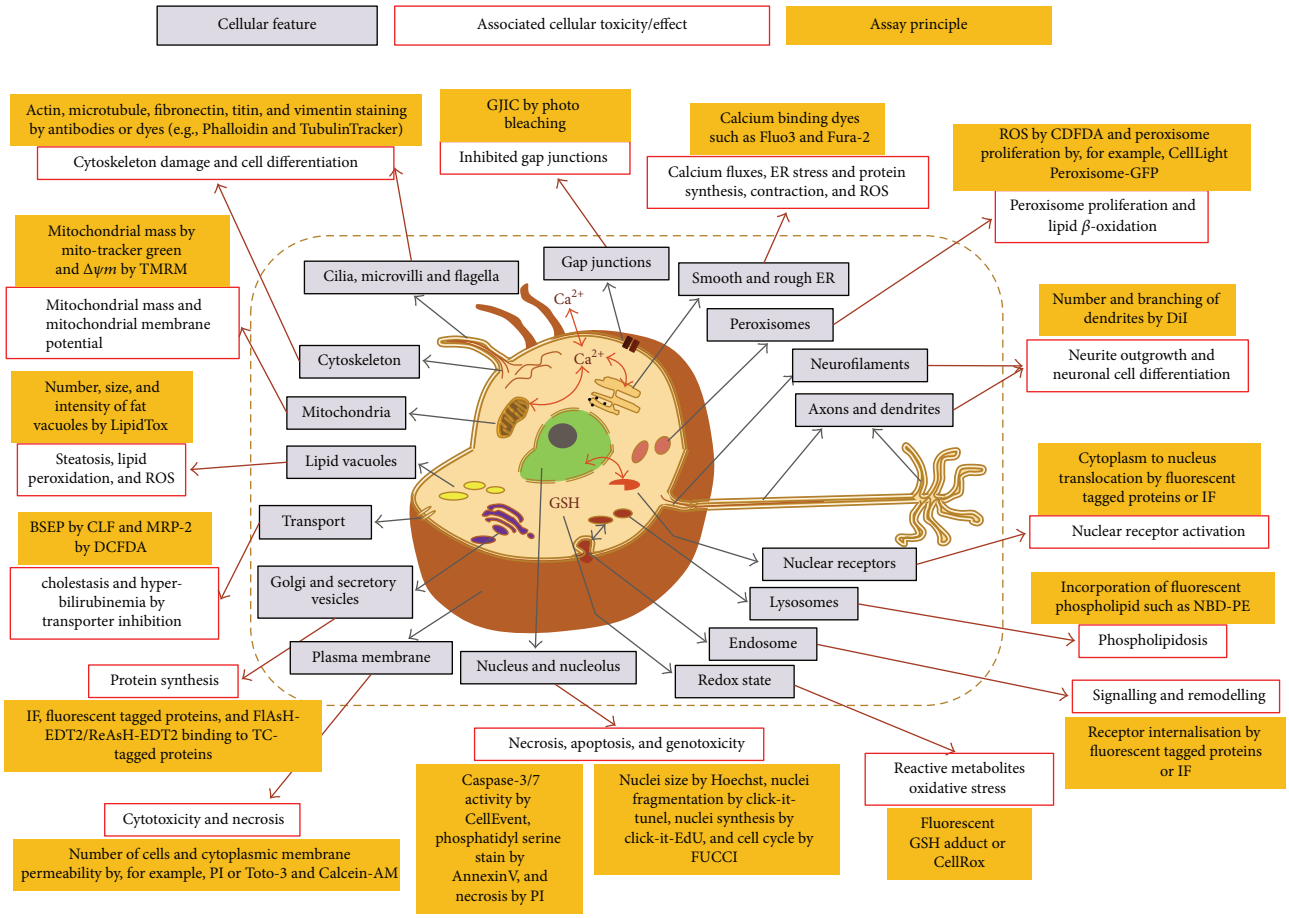

Figure 3: HCI assay examples for assessment of specific cellular functions and toxicity. For more details, please refer to Uteng et al. [81]. Figure reproduced with permission.

hepatotoxicity. While many systems such as liver slices and isolated perfused livers have been developed to investigate mechanisms of liver toxicity, technical, economical, and reproducibility issues limit their use in drug discovery where reliability and throughput are key factors. All models have strengths and weaknesses and one may argue that a particular model may be better to detect some of the DILI mechanisms, but none of the models address all mechanisms. Furthermore, there is a lack of agreed upon controls in such experiments and descriptions of experimental parameters such as oxygenation (see Section 2.4 for more details) and cellular functionality, including target expression and metabolism (xenobiotic metabolism and energy metabolism), are often not provided; these should be included in the experimental design. Even if the same cells are used across different studies, cells may not remain in the same experimental state, which may partly explain the differences in results reported by different investigators [89].

Three-dimensional and dynamic, microphysiological models are believed to be more physiologically relevant since they more closely reproduce the structure of the organs and physiologic conditions, such as blood flow. The same arguments were used for organ slices and perfused organs, with the difference that ex vivo organ slice cultures come with significant levels of inflammation and tissue necrosis as a consequence of the preparation process. The newer 3D models also have challenges, particularly with regard to the level of oxygen, as hypo/hyperoxygenation may generate toxic artifacts in cells and tissues [90–92]. The prediction of iDILI is even more challenging as it may require individualized in vitro models, as well as a substantial number of tests [93]. Finally, it is important to identify the right model depending on the pathology of interest. For example, prediction of hepatic fibrosis is often based on stellate cell cultures but metabolism-based fibrosis may not be detected in this cell system [94].

When setting up new in vitro assays, the assumption is that the selected in vitro model can recapitulate the mechanism of toxicity leading to DILI, but this may not always be the case. It is now generally accepted that transformed cell lines insufficiently represent hepatocytes. Also, primary hepatocytes cultured for just 24 h in monolayers only partly display the complexity of biological interactions of native liver. Cocultures and 3D cultures of hepatocytes that permit long-term compound exposure as well as inclusion of other liver nonparenchymal cell fractions increase the chances of detecting liver toxicants which typically escape conventional testing systems [21, 29, 30, 95]. The choice of a particular

model may be based on short-term versus long-term culture and the ability to study the toxicity of parent compounds or metabolites [96]. Finally, another aspect is how to address genetic variability in the patient populations. This is clearly challenging and may not be addressed to full satisfaction even when using different hepatocyte donors.

The development of in vitro models representing a pathological state would be highly desirable to enable better prediction of DILI in humans. Of interest in this regard is the use of hiPSC-derived hepatocytes from healthy volunteers as well as DILI patients. Such approach could help the scientific community to better predict human DILI and understand the role played by genetic predisposition. In addition, this may open new opportunities to develop assays using patient-derived hiPSC particularly to better understand individual differences in iDILI susceptibility. Nevertheless, some technical challenges need to be resolved particularly regarding the activity of drug-metabolizing enzymes, as well as the generation of mature and fully functional hiPSC-derived hepatocytes [28].

3.5. Concluding Remarks. The scientific community can fuel progress by establishing consensus around reference drugs with recommended test concentrations and cut-off criteria for conducting and interpreting in vitro studies. This would enable comparison of the performance of many in vitro models. Such approaches have already been applied in the field of in vitro genotoxicity (refer to Section 6.3 for more details). Furthermore, a clearer guidance is needed for the classification of reference drugs as severely toxic, less severely toxic, or nonhepatotoxic associated ideally with clear mechanisms of actions and specific histopathology lesions.

4. In Vitro Model Characterization and Validation: The MIP-DILI Consortium

Many private and public initiatives have invested extensive efforts toward the development of research tools for the early and safe prediction of human DILI. These efforts have provided biomedical tools for use preclinically and methods for detection and monitoring of DILI in the clinic. Among the many research initiatives are those which have contributed to recent developments of novel biomarkers for use in the identification of DILI [97], novel preclinical animal models [98, 99], and preclinical diagnostics, including "omic" technologies [100, 101] for the early deployment and detection of chemical risk assessment during preclinical R&D. Despite much research in the field of human DILI, little, if any, progress has been made toward a thorough understanding of which of the different in vitro systems that are routinely employed in pharmaceutical research and development are more suited for the detection of certain types of hepatocellular injury [29, 102–104]. To address these questions in conjunction with still poorly understood mechanisms of human DILI, a 26-partner consortium was formed under IMI's EU Industry-Academic Partnership Programme on Drug-Induced Liver Injury and Mechanism-Based Integrated Prediction of DILI [105]. The overarching and primary objective of MIP-DILI is to specifically address the a priori need for an improved panel of in vitro assays for the prediction of human DILI risk of drug candidates during the lead optimization and preclinical candidate selection phases of drug discovery.

MIP-DILI broadly comprises four principal work streams: the evaluation of existing and novel in vitro cell models, biomarkers of cell injury, and mechanistic studies complemented by mathematical modelling approaches for the improved understanding of human DILI. The evaluation of in vitro cell models comprises the quantitative pharmacological, toxicological, and physiological phenotypes of primary human hepatocytes and cell lines, HepaRG and HepG2, in routine use by industry. In addition, novel cell models, such as hiPSC-derived hepatocytes in 2D and 3D cell platforms, are being evaluated in parallel with the overall aim of identifying which of these cell models are more appropriate for the detection of certain types of hepatocellular injury. Biomarkers assessed for use as endpoint measurements of hepatocellular injury include those commonly employed by industry, such as cytotoxicity and mitochondrial dysfunction, alongside novel biomarkers indicative of hepatocellular stress, necrosis, and apoptosis [97]. The quantitative evaluation of toxicological readouts for each of these cell models are supported by use of evidence-based selection of drugs (training compounds) known to cause clinical DILI, together with prevailing mechanisms by which these drugs are believed to cause liver injury. Of the mechanisms currently described, training compounds are grouped according to mitochondrial and lysosomal impairment, intrahepatic cholestasis, immune response, and cytotoxicity. These well-described training compounds are further complemented by a larger set of test compounds to validate the selection of cell models and endpoints.

The combined efforts of interlaboratory ring-trials are enabling in-depth evaluation of different test systems [106] and their comparative sensitivity and selectivity for the detection of certain forms of human DILI. These yet vitally important ring-trials are beginning to provide the industry with important comparative bench-marking of the simplest 2D test systems and direct quantifiable measures of gain-of-physiological and pharmacological function. In addition, any improved sensitivity and selectivity for the detection of chemical risk in more complex 3D formats are being assessed. An important contribution toward the efforts of establishing an improved panel of in vitro assays is efforts by the consortium toward understanding intrahepatic and extrahepatic events leading to hepatocellular injury. These activities include the mapping of primary gene signalling pathways, proteomic and transcriptomic studies, and the direct and indirect effects of drugs on hepatobiliary function. These mechanistic studies, coupled to modelling activities, are helping underpin the characterization of cell models and the pathologies associated with drug toxicities.

A major gap in the current panel of preclinical models available to industry is a test system that affords the detection of immune-mediated human DILI, which is believed to be a central tenant of idiosyncratic drug toxicities. Both the innate and adaptive immune systems are believed to play a role in both the initiation and attenuation of iDILI [107, 108]. The complexity of immune-mediated DILI cannot be

underestimated with intra- and extrahepatic signalling and genetic diversity bringing with them important challenges to the development of meaningful test systems for drug safety assessment [105].

Ultimately, the MIP-DILI consortium aims to help defining which test systems are more amenable for use in the detection of certain types of drug-induced hepatocellular toxicities and when to use these test systems during the drug discovery process. To provide solid bench-marking and exploit novel test systems to offer an important step-change for the improved risk assessment of new drug entities prior to preclinical regulatory and early clinical research programmes will be a key deliverable for the project. Now in its 5th year (in 2016), the work performed by MIP-DILI continues to make significant contributions toward a better understanding of the in vitro models most likely to improve pharmaceutical research and development and to define a concrete, tiered roadmap for future research in this field. The MIP-DILI consortium is supported by the Innovative Medicines Initiative Grant Agreement number 115336.

5. Practical Considerations

5.1. Drug Annotation/Classification. A reference list of drugs annotated for DILI risk in humans is required for the development of in vitro predictive models. The performance of the developed predictive models is subject to the quality of DILI annotations. By DILI annotation we refer here to the classification of drugs based on DILI risk observed in human populations treated for various diseases and reflects the frequency, causality, and severity of DILI for each drug [68]. Information on mechanisms of actions and effects at pathological level are also key parameters to take into account but this level of information is often unknown for the majority of the compounds used in the in vitro investigations. Unfortunately, there is no "gold standard" for defining DILI risk and no consensus on drug classification for DILI. Some authors classify a drug as DILI positive or negative according to the availability of DILI case reports retrieved from the literature [63, 109, 110] or FDA's adverse event reporting system (FAERS) [111–113], while others utilize information summarized in the drug compendiums such as Physicians' Desk Reference [114]. Inevitably, inconsistent annotations based on different approaches are reported for some drugs (as already reported in Section 3.1). For example, buspirone is an anxiolytic psychotropic drug that is used to treat generalized anxiety disorder. The compound has been classified as both nonhepatotoxic [60, 61, 70, 71] and mildly hepatotoxic in humans [62, 63]. Additionally, buspirone was classified as a ^{v}less-DILI-concern drug in the Liver Toxicity Knowledge Base (LTKB) with DILI information only found in the label section of "Adverse Reactions" with the query "infrequent increases in hepatic aminotransferases were found during premarketing trial" [68]. Thus, the variability in published DILI annotations, each utilizing different schema and data sources, is an impediment for the development of predictive in vitro models.

The classification of DILI negative compounds is even of more concern. Most published approaches that define a drug as DILI negative depend on search results from PubMed or other databases [60, 61, 70, 71, 113]. In some studies [115], drugs were labeled DILI negative if they simply were without searchable results for a specific DILI adverse event (e.g., acute liver failure). However, due to the diverse manifestations of clinical DILI and the severe underreporting of DILI cases [68], these approaches may miss the information necessary to designate a drug as DILI negative. In a recently published survey, 7.9–41.8% of drugs defined as DILI negative in published datasets were verified as the cause of DILI in case reports in which causality had been fully justified [65]. The high percentages of misclassification highlight the importance of selecting appropriate DILI annotation. Chen et al. [68] published a DILI annotation approach based on FDA-approved drug labeling and classified 287 drugs into three categories (i.e., ^{v}Most-DILI-concern, ^{v}Less-DILI-concern, and ^{v}No-DILI-concern). Recently, the authors refined the drug labeling based approach by incorporating causality evidence collected from the literature and further classified 1036 FDA-approved drugs into three verified categories (i.e., ^{v}Most-DILI-concern, ^{v}Less-DILI-concern, and ^{v}No-DILI-concern) and one "Ambiguous DILI-concern" category (Figure 2) [65].

These drug labeling based DILI annotations are recommended for the development of in vitro DILI models. Firstly, although it is not perfect, the FDA-approved drug labeling is the authoritative document in which drug safety information is summarized through the systematic assessment of data from clinical trials, postmarketing surveillance, and literature publications. The comprehensive information contained in drug labels is especially useful for limiting false negative DILI compounds [65]. Secondly, the procedures of the DILI-label based annotation approaches are transparent and reproducible, and the data source (i.e., drug label and causality evidence) can be updated with the advance of DILI knowledge. Thirdly, the annotations and dataset have been extensively applied to develop in silico [64, 116], in vitro [49, 62, 63, 71, 117–120], and in vivo models [121–124] and were also recommended as the standardized list for model validation [95].

5.2. Endpoints. Many pharmaceutical companies have implemented or are developing screening paradigms to decrease hepatotoxicity-related attrition. While some companies try to address this aspect during series selection, others put more emphasis during lead optimization and compound selection. Screens for series selection require the following attributes: appropriate throughput, utility in hazard identification, and utility for rank-ordering of compounds. In a recent publication by Aleo et al. [118] a strong correlation was found between DILI in humans and compounds exhibiting mitochondrial toxicity as well as inhibition of the bile salt export pump (BSEP). Compounds exhibiting both liabilities were more likely to be associated with more severe clinical DILI than compounds with only one of these two liabilities. These data suggest that adding mechanistic endpoints could be useful to decrease hepatotoxicity-related attrition.

Triage of compounds using high-throughput cytotoxicity, sometimes followed or complemented by additional

mechanistic endpoints, represents a rather common general approach used by pharmaceutical companies. However, approaches across companies are extremely variable, such that it is not possible to identify the optimal approach. It is noteworthy that the use of liver-derived cell (THLE and HepG2) lines and simple cytotoxicity readouts is likely not sufficient to predict DILI accurately and may be more representative of generalized cell health [125]. Only more liver specific mechanistic endpoints can provide specificity toward prediction of hepatotoxicity.

During hit selection and lead optimization, some companies utilize high content technologies for assessment of specific cellular functions and toxicity (Figure 3). Caution must be taken when utilizing this technology for the assessment of organ toxicity and particularly liver toxicity. The combined measurement of multiple mechanistic endpoints (e.g., mitochondrial membrane potential, ROS formation, ER stress, lipid accumulation, and DNA damage) has limitations for the prediction of liver injury. For example, many compounds known to induce cardiac toxicity will be equally positive using this approach [126]. Hence, to increase predictively toward liver injury, true liver specific endpoints should be assessed, such as transporter inhibition (e.g., BSEP or MRP) [55].

Analysis of new chemical entities (NCEs) using either cytotoxicity assessment [46, 127] or single mechanistic endpoints such as mitochondrial toxicity [78, 82, 128, 129], BSEP [19, 118], or high content analysis [60, 61, 81] is a hazard identification approach that requires exposure information for appropriate risk assessment. Hence, this approach is most useful for comparing and rank-ordering compounds, especially when assessing compounds with similar physicochemical properties. Finally, this strategy is predicated on establishing in vitro systems that could mimic the in vivo biology for functional or morphological events but does not take into account the concentrations needed to achieve them in vitro.

5.3. Concentrations, Duration of Exposure, and Culture Conditions. In vitro systems can reproduce in vivo observations, but a correlation between in vitro concentrations and in vivo systemic exposure levels (C_{max}, AUCs, and bound or free fraction) may not exist. Indeed, drug concentration in the portal vein and liver can be much higher than plasma concentrations and human subjects differ in their drug-metabolizing capabilities and hepatic transporter content/function. Therefore, studies that have been aimed at establishing predictivity for human outcome have tested the compounds at concentrations between 30 and 100 times higher than human plasma C_{max} [47, 60–62, 130, 131]. No matter the final endpoints, either straight cytotoxicity or more relevant functional parameters, this approach remains very empirical and arbitrary as many factors can offset the relevant concentration range from in vivo to in vitro settings. First, plasma concentrations may be either significantly above or below tissue concentrations [132]. The drug volume of distribution can indicate tissue accumulation, but assessment of individual tissue exposure to drugs and eventually to their metabolites is rarely performed. In addition, in tissues like liver, drug concentrations can also be vastly different between the various parts of the liver (e.g., centrilobular versus periportal) [133] or the cell types (e.g., hepatocytes versus nonparenchymal cells). This is particularly true for compounds inducing phospholipidosis where cationic amphiphilic drugs with similarity to bile acids [134] can accumulate up to mM range in cells like cholangiocytes while hepatocytes could remain mostly in the nM to μM range. Furthermore, C_{max} values are not always the relevant concentrations to be considered when the overall AUC might drive the observed toxicity [135]. In that case, the duration of treatment in vitro should be more relevant than the actual compound concentration, or, more accurately, the combination of both. Increasing the length of treatment in vitro by a few days may not be an issue with cell lines that divide and survive on plastic but is more challenging with primary cells. Finally, a major drawback of a C_{max} or AUC-based concentration methodology for "predictive" in vitro screening approaches is that clinical exposures are rarely well estimated at this stage of compound optimization and selection.

The question of the significance of free- versus bond-fraction of the compound is of great importance. In vivo, the drug will have a relatively constant protein binding rate and, most likely, only the free fraction will be the active part for both the pharmacology and toxicology aspects [135]. It is therefore logical to consider protein binding when setting an in vitro dosing range. However, there is great uncertainty of how in vivo free fraction levels translate in vitro. The use of a serum-free culture medium and very well-controlled conditions should be preferred for the following reasons. Firstly, since compounds bind to albumin and other serum proteins, the use of a serum-free medium removes the need to correct for protein binding. However, overall protein concentrations used in vitro are different from the in vivo situation, the binding rates of compounds to fetal calf serum albumin may be quite different from those to human adult albumin [136], and protein binding differs among compounds, especially from different chemical series, limiting compound differentiation. Secondly, compounds can bind the plastic of culture vessels (i.e., dish and tubing of fluidics systems) [57, 137] and this can result in a vast difference between the estimated concentration and the actual medium concentration. Thirdly, as the field moves toward the use of more complex cell culture environments with extracellular matrices [57] (e.g., 3D architectures, cocultures, and fluidics stations), control of compound concentration becomes even more important. Finally, all the above considerations are relevant for chemicals passively diffusing in and out of the cells. For actively transported compounds, the biology of the cells in vitro adds yet another layer of complexity and may skew their exposure, either up or down [132]. Primary hepatocytes typically have lower export transporter and CYP450 function compared to the liver, which may result in overexposure to test articles with some important variations with time [138]. In contrast, many cell lines are transformed tumor-derived cells that may overexpress export pumps such as MDR1 and others, resulting in lower exposures to compounds. Such confounding factors that are only very rarely checked by investigators, as they add a fair burden on the speed and cost of experiments, can lead to erroneous conclusions about the respective cytotoxicity potency of chemicals.

6. Examples from Other Disciplines That Could Help to Better Guide Investigations in the In Vitro DILI Domain

6.1. The Comprehensive In Vitro Proarrhythmia Assay (CIPA) Initiative. In the early 1990s, six drugs approved by FDA induced cardiac arrhythmia in humans. Consequently, the drugs were withdrawn from the market and international regulatory authorities (US, EU, and Japan) released three guidance documents: two nonclinical (ICH S7A and S7B) and one clinical (ICH E14). Since the implementation of these guidelines, no drugs have been withdrawn from the market due to cardiotoxic events. Nevertheless, these specialized clinical studies add time to development and are very costly [139, 140]. In addition, it is believed that such guidelines prevented some potentially efficacious drugs to reach the market because of false positive signals. In 2013, a workshop was organized to change the current applied cardiac safety arrhythmia guidance paradigm [141]. The proposed paradigm would shift the emphasis from the present approach that strongly relies on QTc prolongation and would obviate the need for the clinical Thorough QT study during later drug development. The Comprehensive In Vitro Proarrhythmia Assay (CIPA), an integrated nonclinical in vitro/in silico paradigm, was initiated toward these aims [142]. CIPA consists of three components aiming to (1) test the effect of compounds on different cardiac ion channels, (2) develop in silico models on cardiac action potential by integrating the ion channel dataset, and (3) measure action potential in human stem cell-derived ventricular cardiomyocytes.

What Can Be Learned from the Current CIPA Initiative to Help the In Vitro DILI Field? International consensus on assay protocols, method standardization, and validation will need to be implemented in a new guideline [142]. For instance, a first objective of the three CIPA core assays is to rapidly achieve ICH regional (Europe, Japan, and USA) consensus on best practice protocols (e.g., stimulation rate, holding potential, and specific ion concentration in the pipette solution). The topics of discussions also include the use of either hiPSCs or human embryonic stem cells, cell purity (e.g., proportion of atrial, ventricular, and nodal cells), maturity of the ventricular cells, known limitations of the cells, electrophysiological characteristics of the cells, endpoints (i.e., technology to use), and risk predictability [142]. Although there are currently a high number of in vitro models to predict DILI (e.g., 2D, 3D, stem cells, and liver on a chip) compared to the CIPA initiative, some of the concerns (e.g., cell characterization, optimized protocols, advantages, and limitations of cellular models) highlighted in the CIPA initiative can be directly translated to the in vitro DILI field.

6.2. Past Microarray Initiatives. DNA microarrays emerged in the public scientific domain in the early 1990s. Such technology enabled study of the expression of thousands of genes in a single experiment. Initially, no major concerns were described with regard to data analysis, validation, and comparison but the situation changed in the early 2000s. Indeed, the scientific community started to question the influence of many parameters to interpret microarray data, as well as the lack of comparison among different studies [143].

Brazma et al. [144] presented a proposal, the Minimum Information About a Microarray Experiment (MIAME), which described the minimum information required to ensure that microarray data could be easily interpreted and that results derived from its analysis could be independently verified. MIAME has not only facilitated data sharing but also guided software development [145]. In 1999, the Microarray Gene Data Expression Society (MGED) was founded with a basic aim to standardize the field [146]. In addition, MGED asked for the depositary of primary experimental data into a permanent public database. In 2002, the MGED society convinced high impact scientific journals such as Nature, The Lancet, and Cell to require MIAME for publication of microarray results [146].

What Can Be Learned from the MIAME Initiative to Help the In Vitro DILI Field? In 2006, a thorough analysis of widespread microarray platforms by a multicenter consortium demonstrated intraplatform consistency across test sites, as well as a high level of interplatform concordance in terms of genes identified as differentially expressed [147]. Since the majority of scientific journals require that raw and normalized microarray data be accessible to the public at the time of publication, a significant number of datasets are publicly available [148]. The technology has been successfully used for disease diagnosis and prognosis, human disease subtype classification, and therapeutic treatment selection.

Overall, the efforts to standardize the microarray field enabled microarray-based gene expression profiling to evolve into a mature, high-throughput, analysis approach that has been extensively applied in biomedical and clinical research for more than 20 years [149]. We believe that the efforts provided in the microarray field could also be used as a relevant example for the in vitro DILI community.

6.3. Strategies to Reduce Rate of False Positive in the In Vitro Genotoxicity Field. In vitro genetic toxicology tests are performed for regulatory purposes to predict carcinogenic potential of drugs, chemicals, food additives, and cosmetic ingredients. If a chemical is positive in one of the battery of assays, in vivo genotoxicity studies are often performed to better assess carcinogenic risk for humans. Kirkland et al. [150] evaluated the performance of a battery of three in vitro genotoxicity assays to discriminate rodent carcinogens and noncarcinogens from a large database of over 700 chemicals and found that 93% of rodent carcinogens were detected by the assay battery. Nevertheless, approximately 80% of the 177 noncarcinogenic compounds tested gave a false positive result in at least one in vitro test [150]. The low specificity data highlighted the need for more meaningful in vitro genotoxicity tests or practical interpretation of current positives. In order to address the high rate of false positive results, a 2-day workshop was hosted and sponsored by the European Centre for the Validation of Alternative Methods (ECVAM) in 2006 [150].

What Can Be Learned from the ECVAM Initiative to Help the In Vitro DILI Field? The recommendations of the experts were to use cell systems that are p53 and DNA-repair proficient, with defined phase I and phase II metabolic capacities, and to reduce the top concentrations and the maximum level of cytotoxicity to reach [150]. A few years later, Kirkland et al. [151] published recommendations on chemicals that would be appropriate to evaluate the sensitivity and specificity of new/modified mammalian cell genotoxicity tests, in particular, to avoid misleading positive results.

Some of the recommendations expressed in the vitro genotoxicity field could be also applied to the DILI vitro domain. In particular, it would make sense to recommend a list of human hepatotoxicants as well as nonhepatotoxicants to be tested in the different cellular models. A range of concentrations to test per compound would also certainly facilitate the comparison of in vitro DILI studies. In addition, metabolic data on the most relevant cytochrome P450 as well as phase II, phase III, and transporter enzymes could help to better evaluate the relevance of the in vitro models to detect hepatotoxic metabolites.

6.4. Refinement of DMPK Tools to Better Predict Clinical Outcomes. The dramatic improvements in clinical attrition rates due to poor pharmacokinetics (PK)/absorption, distribution, metabolism, and excretion (ADME) properties of small-molecule compounds that have been documented in the last two to three decades are an excellent opportunity to reflect on what may make initiatives successful [152]. These improvements were the combined results of significant investments in the field of ADME profiling and formulation, the more consistent inclusion of PK measurements in animal studies, the earlier integration of relevant ADME endpoints in discovery testing funnels (now routinely conducted in parallel to potency measurements), the development of targeted in silico filters, and some early initiatives to reach a consensus among scientists in academia and industry around optimization of ADME properties. High-throughput ADME profiling is now widely adopted within drug discovery R&D organizations or even provided by specialized contract research organizations [153]. Over the years, these ADME profiling platforms have been refined in terms of quality of assays and timing of assay execution, as well as by regular addition to the testing battery of additional assays with proven utility.

While the ADME profiling experience is worth mentioning and learning from, it should however be pointed out that significant differences exist with DILI prediction and these differences highlight the complexity behind DILI prediction, in particular iDILI. Firstly, in silico or in vitro ADME prediction can rapidly be validated in relevant animal models at reasonable cost and sufficient throughout in contrast to most DILI cases. Likewise, interrogation of PK in the clinic is rapid and simple, such that compound PK characterization and selection can occasionally take place in the clinic (one of the arguments for the use of exploratory INDs). This rapid feedback allows for the generation of in vitro-in vivo (IV-IV) correlations that markedly strengthen the validity of and confidence in in silico or in vitro predictions. Secondly, the basic mechanisms and principles behind ADME mechanisms are relatively well understood and this contrasts with the complexity, lack of full characterization or understanding, and diversity of mechanisms of DILI. A better alignment around the fundamentals of a biological phenomenon should clearly facilitate consensus reaching in a scientific field, as well as the definition of what endpoint or property is relevant to interrogate for prediction. Regular interactions and argumentations around mechanisms of DILI at scientific venues illustrate the state of our current knowledge of DILI: it is clearly difficult to efficiently predict a phenomenon that one does not comprehend totally.

Keeping these limitations in mind, it is noteworthy that some assays or models designed to predict DILI could be much better understood in terms of performance if precompetitive evaluation and standardization of experimental conditions and dosing paradigms would occur. This is one of the aspirational objectives of some recent initiatives such as MIP-DILI (see Section 4 for more details). The positive outcome would not be limited to the better conduct and interpretation of early high-throughput assays that could be conducted in parallel to ADME profiling; it could also demonstrate the lack of utility of tests currently used by some R&D organizations or lead to a better positioning of tests within a discovery testing cascade. For example, there is still quite a lot of debate around the utility and timing of tests for reactive metabolite formation or effects on mitochondrial function. Finally, evaluating new technologies is time consuming and often libraries of test articles in individual companies are too limited in size to generate meaningful testing and validation sets. Development, evaluation, and interrogation of these novel technologies would be much more efficient in the context of precompetitive efforts.

7. Concluding Remarks

Since DILI is a major cause of attrition during early and late-stage drug development, there is a need to develop reliable in silico, in vitro, and in vivo assays for better predicting hepatotoxicity in both animals and humans early in drug development. The present paper identifies some of the key opportunities and challenges that the pharmaceutical industry is facing with a focus on the in vitro DILI field. Scientists from academia and industry need to work closely together to standardize the use of the most promising tools, taking into account some of the practical considerations highlighted in this paper. Successful initiatives in other domains and, in particular, ADME, genetic toxicology, and microarray, should be used to guide future efforts and help to harmonize current and emerging models as well as strategies such as integrated risk assessment and mitigation plans at early stages of drug development. The current evolution in in vitro technologies stemming from decades of previous experience is opening an optimistic window on the future and authorizes hope for the alleviation of many of the limitations described in this review. After all, "*the future depends on what you do today,*" Mahatma Gandhi.

Disclosure

The opinions expressed by the authors do not reflect the opinions or policies of their respective institutions. Any statements in this paper should not be considered present or future policy of any regulatory agency.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Expression of miRNA-122 Induced by Liver Toxicants in Zebrafish

Hyun-Sik Nam,[1,2] Kyu-Seok Hwang,[3] Yun-Mi Jeong,[3] Jeong-Im Ryu,[4] Tae-Young Choi,[4] Myung-Ae Bae,[4] Woo-Chan Son,[5] Kwan-Hee You,[3] Hwa-Young Son,[6] and Cheol-Hee Kim[3]

[1]*New Drug Development Center, Osong Medical Innovation Foundation, Chungbuk 28160, Republic of Korea*
[2]*Graduate School of New Drug Discovery and Development, Chungnam National University, Daejeon 34134, Republic of Korea*
[3]*Department of Biology, Chungnam National University, Daejeon 34134, Republic of Korea*
[4]*Department of Drug Discovery Platform Technology, Korea Research Institute of Chemical Technology (KRICT), Daejeon 34114, Republic of Korea*
[5]*Department of Pathology, Asan Medical Center, University of Ulsan College of Medicine, Seoul 05505, Republic of Korea*
[6]*College of Veterinary Medicine, Chungnam National University, Daejeon 34134, Republic of Korea*

Correspondence should be addressed to Kwan-Hee You; khyou@cnu.ac.kr, Hwa-Young Son; hyson@cnu.ac.kr, and Cheol-Hee Kim; zebrakim@cnu.ac.kr

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MicroRNA-122 (miRNA-122), also known as liver-specific miRNA, has recently been shown to be a potent biomarker in response to liver injury in mammals. The objective of this study was to examine its expression in response to toxicant treatment and acute liver damage, using the zebrafish system as an alternative model organism. For the hepatotoxicity assay, larval zebrafish were arrayed in 24-well plates. Adult zebrafish were also tested and arrayed in 200 mL cages. Animals were exposed to liver toxicants (tamoxifen or acetaminophen) at various doses, and miRNA-122 expression levels were analyzed using qRT-PCR in dissected liver, brain, heart, and intestine, separately. Our results showed no significant changes in miRNA-122 expression level in tamoxifen-treated larvae; however, miRNA-122 expression was highly induced in tamoxifen-treated adults in a tissue-specific manner. In addition, we observed a histological change in adult liver (0.5 μM) and cell death in larval liver (5 μM) at different doses of tamoxifen. These results indicated that miRNA-122 may be utilized as a liver-specific biomarker for acute liver toxicity in zebrafish.

1. Introduction

During the process of drug development, many compounds fail somewhere along the pipeline due to drug-induced toxicity. Half of these compounds are removed due to hepatotoxicity, including necrosis, steatosis, cholestasis, proliferation, inflammation, and bile duct hyperplasia [1]. Drug-induced liver injury (DILI) has been established as a major cause of acute liver failure in the United States. DILI further represents a major reason why approved drugs are restricted or removed from the market [2, 3]. Thus, rapid and accurate detection of hepatotoxicity remains a vital issue in new drug development. Over the years, various model systems have been developed to enable detection of potential liver toxicity of chemicals and drugs [4]. The strategy of screening large groups of chemical compounds in cell culture for their effects on specific cellular characteristics has already been well established; however, many biological processes cannot be reproduced in cultured cells and often require the three-dimensional environment of cells to determine their function. Furthermore, metabolism of compounds in whole organisms may be profoundly different from the processes at work in vitro. It is desirable therefore to screen large numbers of small molecule compounds for biological activity in whole organisms with high throughput as early as possible in the screening process [5, 6]. Due to transparent embryos and rapid organogenesis, zebrafish have been recognized as a tool for developmental biologists and researchers in a wide variety of fields. In comparison to rodents and nonhuman primates, there are numerous advantages to the use of zebrafish as a toxicological model species

[7, 8], and the number of publications utilizing zebrafish has increased dramatically in recent years. The main benefits of zebrafish as a toxicological model over other vertebrate species are well established regarding size, morphology, and easy and cost-effective husbandry and maintenance conditions [9].

MicroRNAs (miRNAs) are small noncoding RNAs that posttranscriptionally regulate gene expression [10]. It has been estimated that over 60% of human protein-coding genes are regulated by miRNAs [11]. In addition, miRNAs released from cells are capable of regulating gene translation in distant cells in a process similar to cell-to-cell communication by cytokines and other soluble factors [12]; however, the roles of miRNAs are not yet fully understood [13]. As a liver-specific miRNA species, miRNA-122 has recently shown potential as a predictor of liver injury and thus could serve as an addition to the repertoire of standard hepatic injury biomarkers used in humans [14] and rodents [15]. Studies have been performed to assess whether the cellular release of miRNA-122 is associated with specific types of liver injury [16–18] and, due to their numerous functions and cellular specificity, miRNAs are potential biomarkers for particular conditions and cell types.

In the present study, we investigated the expression of miRNA-122 during the initiation and progression of acute liver injury induced by liver toxicants. In addition, we assessed the usefulness of the zebrafish assay model in early-stage preclinical liver toxicity screenings.

2. Materials and Methods

2.1. Maintenance of Zebrafish. Zebrafish (*Danio rerio*) were maintained and raised under standard conditions as previously described [9]. All experiments were approved by the Institutional Animal Care and Use Committees (IACUC) of Chungnam National University (CNU-00620).

2.2. Liver Toxicants Treatment. Tamoxifen (T5648, Sigma-Aldrich) or acetaminophen (A7085, Sigma-Aldrich [19]) was dissolved in DMSO. For the hepatotoxicity assay, 4 dpf (day after fertilization) zebrafish larvae were arrayed in 24-well plates (five individuals per well) containing 1 mL embryonic medium that was diluted from 1000x stock solutions of NaCl (29.4 g/100 mL, Sigma-Aldrich), KCl (1.27 g/100 mL, Sigma-Aldrich), $CaCl_2{\cdot}2H_2O$ (4.85 g/100 mL, Sigma-Aldrich), and $MgSO_4{\cdot}7H_2O$ (8.13 g/100 mL, Sigma-Aldrich), as these solutions can be autoclaved and stored at room temperature. The larvae were exposed to various doses of liver toxicants for 24 hours. For imaging, larvae were anesthetized with tricaine (MS-220, Sigma-Aldrich) and mounted on 3% methyl cellulose (Sigma-Aldrich). Mounted larvae were imaged with a Leica MZ APO stereomicroscope and DC300 FX (Leica, Japan). In addition, 3-month-old zebrafish were arrayed in 200 mL cages (3 individuals per cage) and exposed to liver toxicants for 24 hours. To induce nervous system-specific toxicity, we used the nitroreductase/metronidazole system in combination with the neuron-specific transgenic line. Nitroreductase converts the nontoxic prodrug, metronidazole (Mtz), into cytotoxic agents [20].

2.3. Blood Concentration Analysis of Liver Toxicant. Blood (7–10 μL/fish) was collected from adult zebrafish by tail cutting. Each zebrafish blood sample (3 μL) was placed in a 1.5 mL microfuge tube and mixed with 72 μL of solution (5 ng/mL disopyramide in ACN) to precipitate plasma proteins and protein from cells such as red blood cells. The mixture was vigorously mixed for 10 min using a vortex mixer, followed by centrifugation at 10,000 ×g for 10 min at 4°C using a temperature-controlled microcentrifuge (Eppendorf, Hamburg, Germany). After the mixture was transferred to a fresh vial, an aliquot of 50 μL of each supernatant was directly injected into the LC-MS/MS system for analysis.

A 1200-series HPLC system coupled to an Agilent 6460 triple-quadrupole mass spectrometer (Agilent, CA, USA) was operated under positive-ionization mode for the LC-MS/MS analysis. Spectrometry was performed using multiple-reaction monitoring mode. The LC chromatograph was equipped with a Hypersil Gold C18 column (100 × 2.1 mm; i.d., 3 μm; Thermo, Waltham, MA, USA) and maintained at 35°C. The mobile phase for sample analysis consisted of acetonitrile/10 mM ammonium formate in water (80 : 20, v/v) at a flow rate of 0.3 mL/min.

2.4. Quantitative Real-Time RT-PCR of miRNA-122. miRNA-122 stem-loop sequences of vertebrates obtained from microRNA database (http://www.mirbase.org/) were aligned and analyzed using ClustalX (version 2.0) and GeneDoc (version 2.7.000) software. Small RNAs were isolated from the livers of drug-treated adult zebrafish using the mirVana^{TM} miRNA Isolation kit (AM1560, Ambion). Subsequently, we synthesized cDNA from purified small RNAs using the TaqMan MicroRNA Reverse Transcription Kit (4366596, Applied Biosystems). Specific primers targets were hsa-miRNA-122 (stem-loop accession MI0000442) and U6 snRNA (NCBI accession #NR_004394). U6 snRNA was used as an endogenous control for normalization. For quantitative RT-PCR, we used TaqMan Universal PCR Master Mix II and No UNG (4440043, Applied Biosystems) and performed quadruplication per cDNA. The relative expression of the target gene was calculated using a 7500 Fast Real-Time PCR System and its associated software (version 2.0.1, Applied Biosystems) [15].

miRNA-122 expression was analyzed in the liver, brain, heart, and intestine, to verify the sensitivity and specificity of miRNA-122 as a liver injury biomarker.

2.5. Histopathology. After tamoxifen treatment, 3-month-old zebrafish were fixed using 10% neutral-buffered formalin and sectioned longitudinally for histopathologic evaluation. The livers were embedded in paraffin and sectioned at 5 μm thickness and stained with hematoxylin and eosin.

2.6. Statistical Analysis. Data of miRNA-122 expression was indicated as mean ± standard error (SE). Data of DMSO- and toxicants-treated groups were analyzed in a one-way analysis of variance (ANOVA). The significance of differences between DMSO- and tamoxifen-treated groups was assessed by Dunnett's multiple comparison tests using GraphPad Prism for Windows (version 6.0). Significance between

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hsa: CCUUAGCAGAGCUGUGGAGUGUGACAAUGGUGUUUGUGUC-UAAACUAUCAAACGCCAUUAUCACACUAAAUAGCUACUGCUAGGC :85
rno: CCUUAGCAGAGCUCUGGAGUGUGACAAUGGUGUUUGUGUC-CAAAACAUCAAACGCCAUCAUCACACUAAACAGCUACUGCUAGGC :85
mmu: ---------AGCUGUGGAGUGUGACAAUGGUGUUUGUGUC-CAAACCAUCAAACGCCAUUAUCACACUAAAUAGCU---------- :66
bta: CCUUAGCAGAGCUGUGGAGUGUGACAAUGGUGUUUGUGUC-CAAACUAUCAAACGCCAUUAUCACACUAAAUAGCUACUGUUAGGC :85
ssc: CCUUAGCAGAGCUGUGGAGUGUGACAAUGGUGUUUGUGUC-CAAACUAUCAAACGCCAUUAUCACACUAAAUAGCUACUGUUAGGC :85
xtr: --------GAGCUAUGGAGUGUGACAAUGGUGUUUGUGUC-AGAGCUAUCAAACGCCAUUAUCACACUAAUGAGCUAC-------- :69
fru: ---------AGCUAUGGAGUGUGACAAUGGUGUUUGUUUC-CUUUCUUUCAAACGCCAUUAUCACACUAAAUAGCU---------- :66
tni: ---------AGCUGUGGAGUGUGACAAUGGUGUUUGUGUC-CUUUCUUUCAAACGCCAUUAUCACACUAAAUAGCU---------- :66
dre: ----UCCAGAGCUGUGGAGUGUGACAAUGGUGUUUGUAUCAUCUGUCGUCAAACGCCAUUAUCACACUAAAUAGCCACGG------ :76
                                  Mature miRNA-122
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Figure 1: Alignment of miRNA-122 stem-loop sequences among vertebrates. hsa: *Homo sapiens* (MI0000442); rno: *Rattus norvegicus* (MI0000891); mmu: *Mus musculus* (MI0000256); bta: *Bos taurus* (MI0005063); ssc: *Sus scrofa* (MI0002413); xtr: *Xenopus tropicalis* (MI0004824); fru: *Fugu rubripes* (MI0003315); tni: *Tetraodon nigroviridis* (MI0003316); dre: *Danio rerio* (MI0001965).

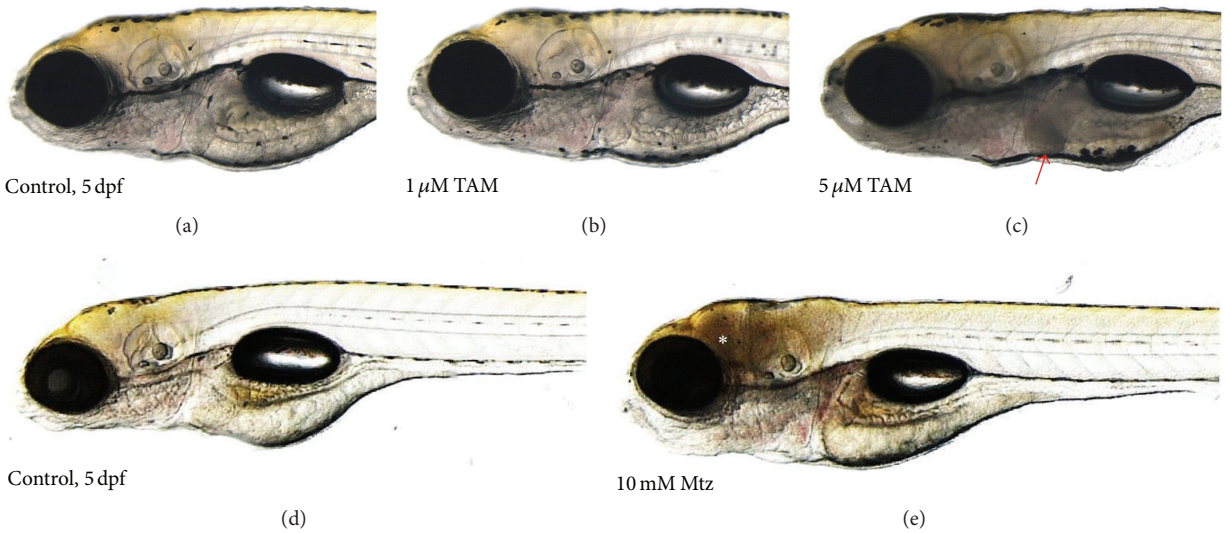

Figure 2: Tissue-specific cell death in the zebrafish larvae treated with tamoxifen (TAM) or metronidazole (Mtz). (a) 0.1% DMSO-treated control (5 dpf) and (b) 1 μM and (c) 5 μM TAM-treated zebrafish larvae. (d) 0.1% DMSO-treated control (2 dpf) and (e) 10 mM Mtz-treated zebrafish larvae. Liver-specific cell death was visualized by reduction of transparency in the TAM-treated zebrafish larvae (red arrow), compared to brain-specific cell death in the Mtz-treated larvae (white asterisk). For Mtz experiments, the transgenic zebrafish system, having neuron-specific nitroreductase expression, was used [20].

DMSO- and toxicants-treated groups is indicated as $^{*}p < 0.05$ or $^{**}p < 0.01$.

3. Results

3.1. Toxicity in Zebrafish Larvae and Adult Induced by Liver Toxicants. The liver-specific miRNA-122 sequence was shown to be highly conserved between various species (Figure 1). Zebrafish larvae (4 dpf) were utilized to test the toxicity in the liver. In our previous report [20], severe cell death in zebrafish was visualized as a reduction of transparency following metronidazole treatment. This method allowed simple detection of the damaged tissue or organ in toxicant-treated larvae. When larvae were treated with 5 μM tamoxifen for 24 hours (h), a reduction of liver transparency was observed which indicated liver toxicity (Figure 2). To explore the effects of treatment duration in tamoxifen-induced liver toxicity, zebrafish larvae were treated with 5 μM tamoxifen for up to 24 h. Tamoxifen-induced liver toxicity in the larvae was visually detected as early as 12 h (Figure 3). In addition to larvae, 3-month-old adult zebrafish were treated with 0.5 μM tamoxifen for 24 h. Although tamoxifen-treated liver exhibited vacuoles in hepatocytes, we could not detect dead and/or dying cells (Figure 4) suggesting that tamoxifen induced liver damage but did not cause cell death in 0.5 μM tamoxifen-treated adult zebrafish.

3.2. Quantitative Expression Levels of miRNA-122. To measure the effect of liver toxicants on miRNA-122 expression, we carried out quantitative RT-PCR from whole larvae samples treated with 5 μM tamoxifen for 2- to 24-hour time duration. Tamoxifen-treated zebrafish larvae showed no increase in miRNA-122 expression levels (Figure 5). miRNA-122 expression levels were also examined in adult zebrafish. Adult zebrafish were treated with various doses of liver toxicants (tamoxifen or acetaminophen) for 24 hours and analyzed for miRNA-122 expression levels using quantitative RT-PCR on dissected livers. The data showed miRNA-122 expression levels gradually increasing in acetaminophen- and tamoxifen-treated animals at low doses and gradually decreasing at high doses (Figure 6(a)). To confirm the tissue-specific pattern of miRNA-122 expression, quantitative

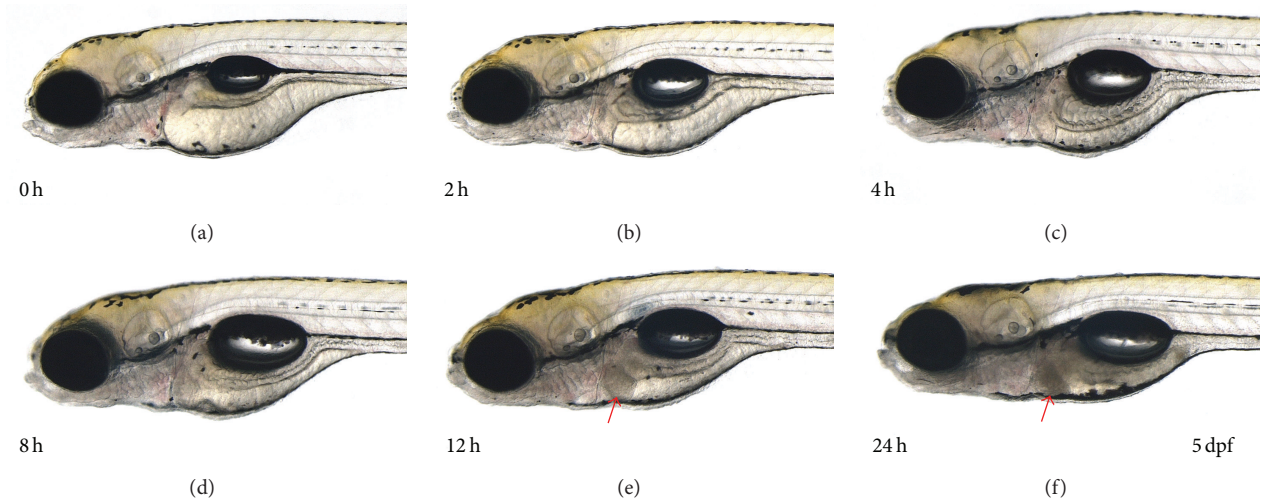

Figure 3: Duration-dependent changes induced by exposure to 5 μM tamoxifen in zebrafish larvae. (a) Pretreatment at 4 dpf and (b) 2-hour exposure, (c) 4-hour exposure, (d) 8-hour exposure, (e) 12-hour exposure, and (f) 24-hour exposure. After 12 hours, tamoxifen induced cell death in zebrafish larvae liver.

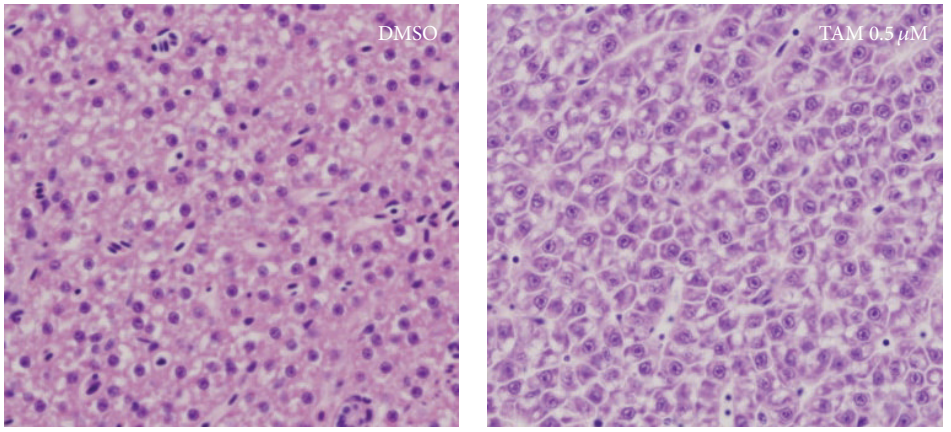

Figure 4: Histopathology of the adult liver treated with 0.5 μM tamoxifen for 24 hours. (a) Control untreated zebrafish liver and (b) 0.5 μM tamoxifen-treated zebrafish liver. Any significant cell death was not detectable in the 0.5 μM tamoxifen-treated adult liver, but vacuole formation was detected in hepatocytes. H&E staining.

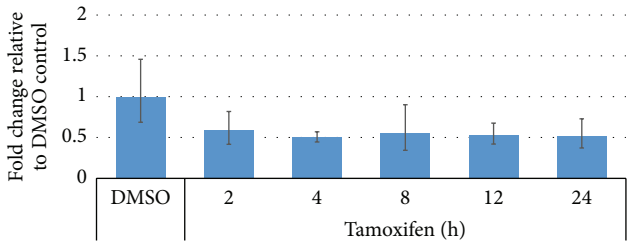

Figure 5: Quantitative expression levels of miRNA-122 in zebrafish larvae (5 dpf). Expression of miRNA-122 did not change as a result of 5 μM tamoxifen in zebrafish larvae. Error bars indicate minimum and maximum values of relative quantification.

RT-PCR was performed on various dissected tissues including liver, brain, heart, and intestine of 0.5 μM tamoxifen-treated adult zebrafish. We found that miRNA-122 exhibited a liver-specific expression pattern while being only marginally detected in the brain, heart, and intestine of tamoxifen-treated zebrafish (Figure 6(b)).

3.3. Blood Concentration of Liver Toxicant. Bioanalysis using LC-MS/MS was performed to correlate the induced phenotype with the actual concentration of the tested compound. The tamoxifen-treated group showed a dose-dependent increase of tamoxifen concentration in blood (Table 1).

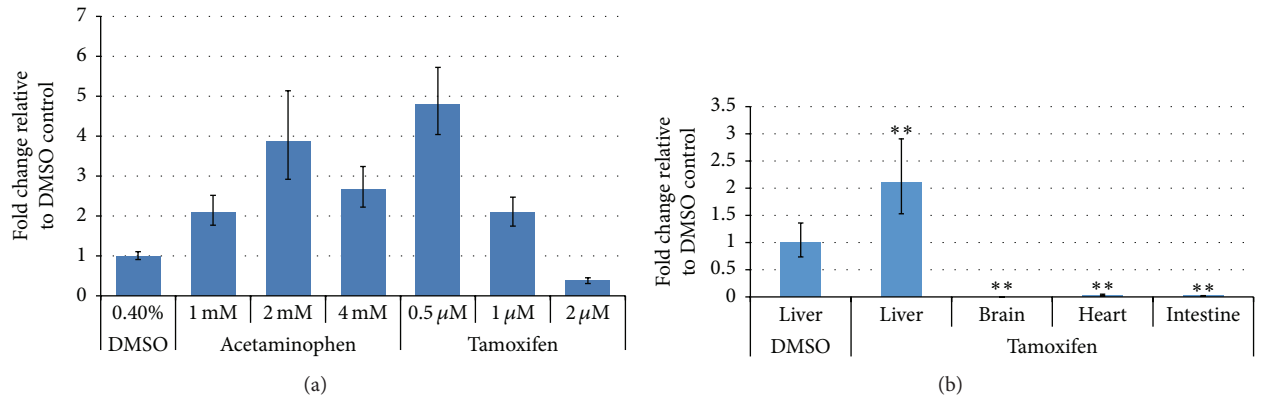

Figure 6: Quantitative expression levels of miRNA-122 in liver toxicant-treated adult zebrafish. (a) Quantitative expression levels of miRNA-122 in tamoxifen- (or acetaminophen-) treated adult zebrafish livers. miRNA-122 expression was upregulated at 0.5 μM and 1 μM and downregulated at 2 μM tamoxifen. miRNA-122 expression was gradually upregulated at 1 mM and 2 mM and decreased at 4 mM acetaminophen. (b) Tissue-specific expression of miRNA-122. miRNA-122 was expressed in the liver of tamoxifen-treated zebrafish, but not in the brain, heart, and intestine at 0.5 μM. Error bars indicate minimum and maximum values of relative quantification ($^{**}P < 0.01$).

Table 1: Blood concentration of tamoxifen-treated zebrafish.

Group	Blood concentration Average ± SE (fold change between water and blood concentration)
Control	0.00 ± 0.0 μM
Low group (0.5 μM)	8.01 ± 0.5 μM (~16-fold)
Middle group (1 μM)	20.4 ± 1.3 μM (~20-fold)
High group (2 μM)	34.1 ± 4.6 μM (~17-fold)

4. Discussion

New drug discovery and development are a protracted and uncertain process. Many drugs fail during development and are rejected due to unacceptable toxicity. Thereby, predictive assays for toxicity are an important part of the drug development process. Due to the practical and ethical concerns associated with human experimentation, animal models have been essential in evaluating drug safety. Indeed, animal and in vitro models serve as an important resource for toxicity and safety information, but emerging alternative translational approaches may eventually provide an important link between in vitro studies and mammalian animals models [21]. In vivo zebrafish assays have proven to be a rapid, cost-efficient, and reliable toxicity model to serve as the intermediate step between cell-based and mammalian testing [22]. In addition, the high homology of miRNA-122 among human, rodent, and zebrafish (Figure 1) supports the utilization of the zebrafish model as a suitable alternative to mammalian species in liver toxicity screenings.

The liver is involved in a number of vital activities including metabolism, detoxification, and maintenance of homeostasis. Drug-induced liver injury is a leading cause of drug attrition during drug development and postmarketing drug withdrawal [23–25]. Hepatotoxicity is a potential adverse side effect of the drug tamoxifen [26] which is an estrogen-receptor antagonist used in the treatment and prevention of hormone-dependent breast cancer. Tamoxifen causes hepatic steatosis in a significant number of patients that can progress toward steatohepatitis [27]. In this study, we analyzed the concentration of tamoxifen in zebrafish and found that the blood concentration of tamoxifen increased in a dose-dependent manner in the tamoxifen-treated group. Tamoxifen may continually accumulate in the blood for up to 24 hours after treatment. Initially, we considered whether or not chemically amended water was a suitable method of treatment exposure for zebrafish but the results demonstrated that tamoxifen could indeed enter the zebrafish via water (Table 1).

Macroscopically, larvae showed tissue-specific cell death (pale discoloration) in the liver at 5 μM tamoxifen (Figure 2). At the same concentration, the liver of tamoxifen-treated larvae showed duration-dependent pale discoloration (Figure 3), starting at 12 h. However, we could not find evidence of cell death in adults (Figure 4). These results were inconsistent with the results of the miRNA-122 assay and blood concentration measurements and may suggest that zebrafish absorbed tamoxifen from the water but that low doses and short-term application were not sufficient to induce morphological changes in the target organ of adult zebrafish. In adults, miRNA-122 expression was significantly increased at 0.5 μM (Figure 6(a)) and this increase was isolated to the liver (Figure 6(b)). Thus, miRNA-122 expression changes were detected for short-duration and low dose of toxicants

prior to the onset of morphological changes in the injury process. High dose (2 μM) significantly downregulated miRNA-122 expression, while low and medium doses (0.5 μM and 1 μM) upregulated expression. It is possible that induction of miRNA-122 expression levels in 0.5 μM tamoxifen-treated adults is caused by hepatocytes damage in the liver and not by cell death, suggesting that an increase in miRNA-122 expression in the liver may accompany hepatocyte damage. This warrants further mechanism studies which are currently underway in our laboratory.

Recently, many studies have shown that miRNAs are differentially expressed in various tissues during normal physiology, injuries, or diseases. The cellular level of a given miRNA may be increased or decreased following drug exposure, leading to corresponding changes in the amount of miRNA released from the cells. miRNA release processes involving microparticles, exosomes, and protein complexes may be altered during exposure. miRNA expression patterns identified to date have not been subject to rigorous qualification and thus remain unproven [28–30]; however, it has been found that miRNA-122 expression levels in the liver [31] and circulating levels of miRNA-122 are significantly increased in mice after treatment with acetaminophen [31], *d*-galactosamine, or ethanol [14].

In this study, the change of miRNA-122 expression was easily and rapidly detectable. Assessment of zebrafish larvae showed that the tamoxifen-treated group did not exhibit increased miRNA-122 expression (Figure 5). In developing zebrafish embryos, miRNA-122 is required for normal hepatic cell differentiation [32]; however, miRNA-122 expression was noted as being consistently high in the liver during exposure to tamoxifen without exhibiting dose-dependence. This finding also correlated with the blood concentration data of tamoxifen. The increase of miRNA-122 expression in adult zebrafish treated with tamoxifen was likely due to hepatocellular damage in the liver. Also, the tamoxifen-treated zebrafish demonstrated increased expression in the liver, but not in the brain, heart, and intestine (Figure 6(b)), indicating the potential of miRNA-122 as a sensitive liver-specific biomarker to assess liver toxicity. Increased miRNA-122 expression at low dose of toxicants also suggests that miRNA-122 may serve as an early biomarker of liver injury prior to the onset of morphological changes.

Interestingly, we found a very high level of tamoxifen concentration in the blood (Table 1) which was 16~20-fold higher than concentration administrated in the water environment of fish tank. Currently, we have no clear explanation of this unexpected high accumulation of chemical compound in the blood of treated adult zebrafish; however, we speculate that this actual concentration in the body may explain the different effective concentrations in liver toxicity between early zebrafish larvae (5 μM tamoxifen, Figure 2(c)) and adult zebrafish (0.5 μM tamoxifen, Figure 6(a)). In addition, this result may also explain why induced miRNA-122 expression begins to decline from the relatively low concentration of 0.5 μM tamoxifen (8 μM in the body) to higher doses of 1 μM and 2 μM (20 μM and 34 μM in the body, resp.) (Figure 6(a)). However, to verify these speculations we will need to conduct further experiments in following mechanism studies.

5. Conclusion

The objective of this study was to measure miRNA-122 expression in zebrafish during acute liver toxicity in order to determine the value of miRNA-122 expression as a biomarker for liver injury in this species. After the exposure of toxicants in the zebrafish, miRNA-122 was specifically expressed in the liver. The findings support miRNA-122's potential as a biomarker for acute liver toxicity applicable to the assessment of liver toxicity during drug development. Also, the zebrafish system demonstrated viability as an alternative animal model in the assessment of drug toxicity.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Hyun-Sik Nam and Kyu-Seok Hwang contributed equally to this study.

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Research Article

Ultrastructural Mapping of the Zebrafish Gastrointestinal System as a Basis for Experimental Drug Studies

Delfine Cheng,[1] Gerald J. Shami,[1] Marco Morsch,[2] Roger S. Chung,[2] and Filip Braet[1,3,4]

[1] *School of Medical Sciences (Discipline of Anatomy and Histology), The Bosch Institute, The University of Sydney, Sydney, NSW 2006, Australia*
[2] *Faculty of Medicine and Health Sciences, Macquarie University, Sydney, NSW 2109, Australia*
[3] *Australian Centre for Microscopy & Microanalysis (ACMM), The University of Sydney, Sydney, NSW 2006, Australia*
[4] *Charles Perkins Centre, The University of Sydney, Sydney, NSW 2006, Australia*

Correspondence should be addressed to Delfine Cheng; delfine.cheng@sydney.edu.au

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Research in the field of gastroenterology is increasingly focused on the use of alternative nonrodent model organisms to provide new experimental tools to study chronic diseases. The zebrafish is a particularly valuable experimental platform to explore organ and cell structure-function relationships under relevant biological and pathobiological settings. This is due to its optical transparency and its close-to-human genetic makeup. To-date, the structure-function properties of the GIS of the zebrafish are relatively unexplored and limited to histology and fluorescent microscopy. Occasionally those studies include EM of a given subcellular process but lack the required full histological picture. In this work, we employed a novel combined biomolecular imaging approach in order to cross-correlate 3D ultrastructure over different length scales (optical-, X-ray micro-CT, and high-resolution EM). Our correlated imaging studies and subsequent data modelling provide to our knowledge the first detailed 3D picture of the zebrafish larvae GIS. Our results provide unequivocally a limit of confidence for studying various digestive disorders and drug delivery pathways in the zebrafish.

1. Introduction

Zebrafish (ZF, *Danio rerio*) exhibit a high degree of resemblance in their genetic profile (69% of the their genes have at least one human ortholog [1]), molecular mechanisms, cell development, and organ physiology to humans [2]. Their small size and translucent nature make them easy to manipulate and observe as a whole animal [3], which contribute to their attractiveness as model organisms for biological experimentation. Accordingly, ZF have been successfully employed to study various physiological and pathophysiological processes in embryogenesis, organogenesis, genomics, and cancerogenesis, as well as research in toxicology and drug studies (for reviews, see [4–6]).

Although ZF have been shown to be a beneficial complementary model to rodent in many research fields, they lack some of the typical mammalian organs such as lung, prostate, skin, and mammary glands. On the other hand, as vertebrates, ZF possess organs and tissues, such as heart, kidney, liver, pancreas, intestinal tract, and brain that display analogous structures and functions to those found in humans [7]. Furthermore, with the advances in genetics, any human tumour type has also been successfully genetically developed in the ZF with similar morphology, gene expression, and signalling pathways [8, 9]. Consequently, despite their infancy in research fields, ZF have already gained distinctive popularity in the fields of genetics and development [10, 11]: they have become a complementary platform to rodent experimental models in preclinical screening studies in the field of translational drug research, in particular, in the assessment of drug compounds delivered to the gastrointestinal system (GIS). These attributes further extended the use of ZF to include the fields of metabolic organ diseases, including cancer [4, 12–14].

The ZF digestive system organogenesis [15–18] and morphogenesis [19–21] have been described by a few, and histological information is available on FishNet [22], the Zebrafish

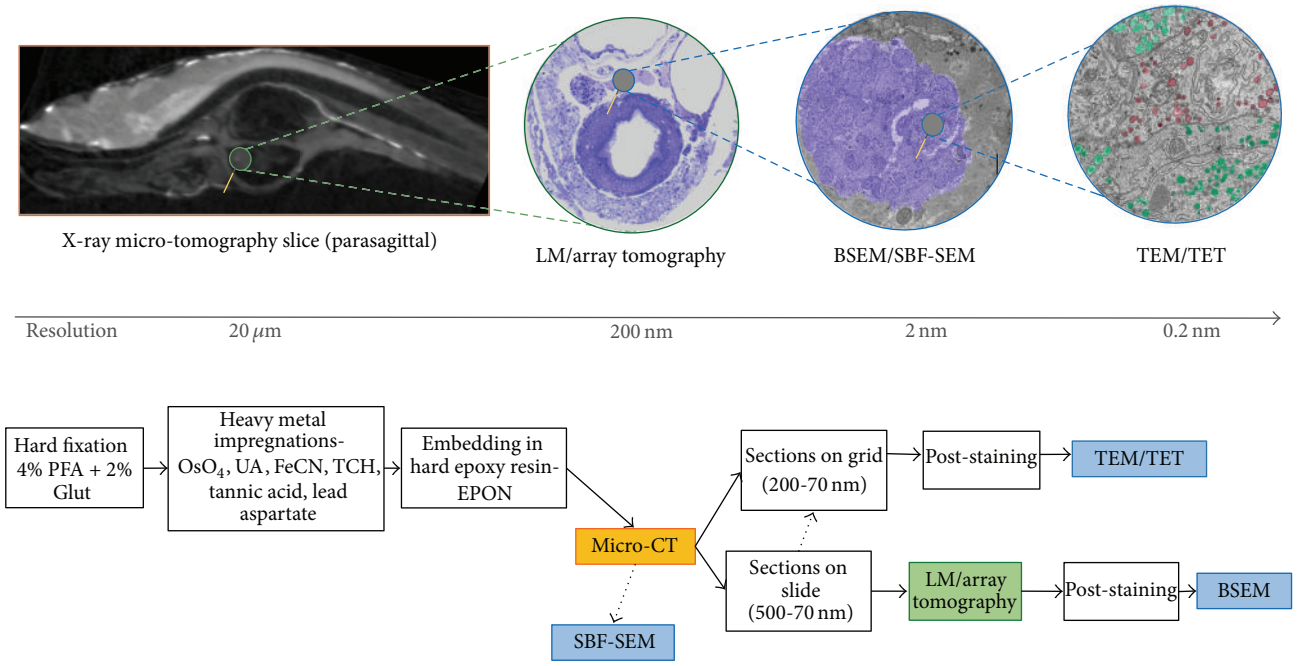

Figure 1: Sample preparation and imaging workflow used for the observation and ultrastructural data correlation of a single zebrafish sample compatible with X-ray micro-CT, LM, and EM imaging modalities. LM imaging modality includes the array tomography technique, whereby serial sections are collected onto a glass slide and imaged using LM and back-scattered EM (BSEM). EM includes TEM, transmission electron tomography (TET), BSEM, and SBF-SEM. This sample preparation protocol not only allows for the sample to be compatible with all the different microscopy platforms but also provides superior ultrastructural preservation of the zebrafish larvae, compared to conventional protocols used for EM.

Atlas [23], and The Zebrafish Atlas of Macroscopic and Microscopic Anatomy [24]. However, to-date, the literatures available regarding comparative imaging studies that explore the ZF GIS with different imaging modalities and fine ultrastructural studies of the ZF microarchitecture are particularly scarce, mainly limited to advanced light-laser optical microscopy approaches. Hence, we present here the first full image sets of a 12 dpf ZF larvae from the macro- to the nanometre scales, including detailed information on cellular and subcellular features of the digestive system organs and a comprehensive comparative analysis of ZF GIS, weighted against published literature from rodents.

2. Materials and Methods

Firstly, a dedicated sample preparation and imaging workflow was prepared after assessing different experimental approaches, allowing for the subsequent swift imaging of one sample across different microscopy platforms, including X-ray microcomputed tomography (micro-CT), light microscopy (LM), and electron microscopy (EM) (Figure 1, top panel). Secondly, following this workflow, we designed the subsequent sample manipulation processes so the entire sample was retained for whole-mount investigation, allowing for the ability to image multiple areas, multiple times, and across different beam-lines (Figure 1, lower panel).

2.1. Zebrafish Animal Model. Zebrafish (wild-type, *Danio rerio*) were maintained at 28°C in a 13 h light and 11 h dark cycle. Embryos were collected by natural spawning and raised at 28.5°C in E3 solution according to standard protocols [25]. Note that, from previous organogenesis studies of the ZF digestive system, it was determined that from 6 days after fertilisation (dpf), when the yolk is exhausted, the digestive functions are comparable to those of an adult fish [15, 19, 21]. Therefore, we studied ZF larvae aged 12 dpf to map the ultrastructure of the GIS. This timeframe is of particular importance as we can take the advantages including the optical translucent properties of the animal and the small size of the fish to facilitate the different sample preparation steps needed, inherent to correlated biomolecular microscopy (i.e., staining and fixation). The sample can be processes as a whole animal (i.e., whole mount), which excludes dissection artefacts and is large enough to easily differentiate the internal organs in LM.

2.2. Sample Preparation. For all microscopy examination purposes, ZF larvae at 12 dpf were collected and fixed in 4% paraformaldehyde + 2.5% glutaraldehyde in cacodylate buffer (4% sucrose + 0.15 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer), overnight at 4°C. The samples were prepared following a protocol modified from Deerinck et al. [26] and Tapia et al. [27], whereby whole animals were exposed to the following solutions of heavy metal stains and mordanting agents: 2%

osmium tetroxide (OsO_4) + 1.5% potassium ferrocyanide (KFeCN) for 2 h at 4°C, 1% thiocarbohydrazide (TCH) for 20 min, 2% OsO_4 for 20 min in the dark, 1% aqueous uranyl acetate at 4°C overnight, and Walton's lead aspartate for 30 min at 60°C. The samples were thoroughly washed with distilled water in between staining steps, then dehydrated through a series of ethanol, gradually infiltrated with a series of EPON (hard grade) dilutions over a period of 3 days, and incubated in pure resin over 2 days. Finally, each ZF was carefully positioned, head-down, at the bottom of a BEEM® capsule (bottleneck, size 00), filled with fresh resin, and polymerised at 60°C overnight.

2.3. X-Ray Microcomputed Tomography (Micro-CT). Micro-CT imaging was performed on resin-embedded ZF samples using a Skyscan 1072 system (Bruker microCT, UK), with no filter, operating at 40 kV and over 180 deg rotation angle. The individual X-ray images were reconstructed using NRecon (Bruker microCT, UK) and volumetric data were processed and analysed using Avizo (FEI Software) and IMOD (Boulder, Colorado, USA).

2.4. Light Microscopy (LM) and Array Tomography. ZF larvae were orientated sagittal to minimise the block face surface. The head was trimmed off to just behind the eyes and the block was carefully faced up to just behind the inner ears or to the start of the pharyngeal pad, just prior to exposing the oesophagus and the liver. Excess resin around the tissue is trimmed away with a razor blade and a mix of Welwood® glue and xylene (1 : 2) was applied to the sides of the block, following the methods developed by Micheva and Smith [28] and Blumer et al. [29] for array tomography sectioning. We found that applying glue only to the bottom of the trapezoid was sufficient and preferable, as the dried layer of glue from the top and the sides frequently does not cut well and builds up and interferes with the sectioning. This method successfully generated long, uninterrupted serial sections with minimal section loss. A ultramicrotome (Ultracut 7, Leica Microsystems, Heerbrugg, Switzerland) and a histojumbo diamond knife (Diatome, USA) were used to create ribbons of 50 serial sections 0.5 μm thick, which were placed, in order, onto a glass slide previously placed inside the knife boat (Supplementary information 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/8758460).

After every ribbon, the slide was dried, stained with 0.5% toluidine blue, and observed with LM for orientation purposes. Once the entire GIS had been sectioned, each section containing the GIS was sequentially imaged using a light microscope (DM6000, Leica, Germany). The ImageJ plugin *StackReg* (Biomedical Imaging Group, EPFL, Lausanne, Switzerland [30]) or the IMOD plugin *midas* (Boulder Lab for 3D Electron Microscopy of Cells, Colorado, USA) was used to either automatically or manually align the images relative to a reference image chosen within the stack (Supplementary information 2). The aligned stack was then used to create 3D models of the GIS organs using 3dMod (IMOD plugin), whereby volumetric and morphometric analysis can be generated. Counting sections from the array tomogram allows for precise localisation (within 0.5 μm) of the different organs, their size, and relative positions with each other.

2.5. Back-Scattered Scanning Electron Microscopy (BSEM). Following LM imaging, the glass slides were carbon-coated and mounted on a stub in preparation for Scanning Electron Microscopy (SEM) imaging. Two lines of silver paint were applied from the top surface of the slides to the stubs to increase conductivity. Sections were then imaged by detection of back-scattered electrons using a SEM (Sigma VP FEG SEM, ZEISS, Germany) operating at 3.8 kV. Consecutive sections were imaged, aligned, and modelled following the same method described previously for array tomography with LM. In this way, dozens or even hundreds of consecutive sections can be imaged over multiple areas, multiple times.

2.6. Transmission Electron Microscopy (TEM). At any time during sectioning for array tomography, ultrathin sections of 70 nm can be collected on 200 mesh copper grids for TEM observation. Typically, after every 50 sections, the ribbons of sections were checked. When an area of interest is apparent on the LM sections, an ultrathin section can be collected at this point which is adjacent to the last section from the last ribbon. Next, retrieved sections on grids were poststained with 2% aqueous uranyl acetate and Reynold's lead citrate solutions for 10 min each and imaged with a TEM (1400 TEM JEOL, Tokyo, Japan), operating at 120 kV.

3. Results and Discussion

Currently, ZF have gained exponential momentum as an experimental animal model in biomedical research fields [31–33]. Although the ZF model has shown to be a viable addition to other animal models (e.g., pigs and dogs), and even rodents, they lack some of the typical mammalian organs as outlined earlier. Providentially, ZF possess a GIS that displays an analogous function to humans, including such organs as a liver, pancreas, and gallbladder, with the notable addition of a swim bladder [7]. This unique aspect of the ZF, together with their relative small size and translucent properties—directly benefiting sample processing and imaging—make them very appealing model in the fields of gastroenterology and hepatology. The importance of these features have been convincingly demonstrated throughout the literature, such as for liver development [34] and regeneration [35], hepatocellular carcinoma [36], hepatic nanoparticle-targeting [37], hypertriglyceridemia-mediated pancreatic organ abnormalities [38], drug-induced liver injury [39], intestinal inflammation [40], and gut-associated nutritional programming [41].

Despite the fervent application of ZF in the investigations of various functional aspects of liver and pancreas- and gut-associated diseases, their fine micro- and nanoanatomical structures are relatively unexamined. Further, there is no detailed comparison of ZF GIS to its mammalian and human counterparts. While many teams only apply state-of-the-art live-cell biomolecular optical imaging techniques, limited studies have subsequently verified their findings at the nanoscale, throughout multiple dimensions (i.e., X, Y,

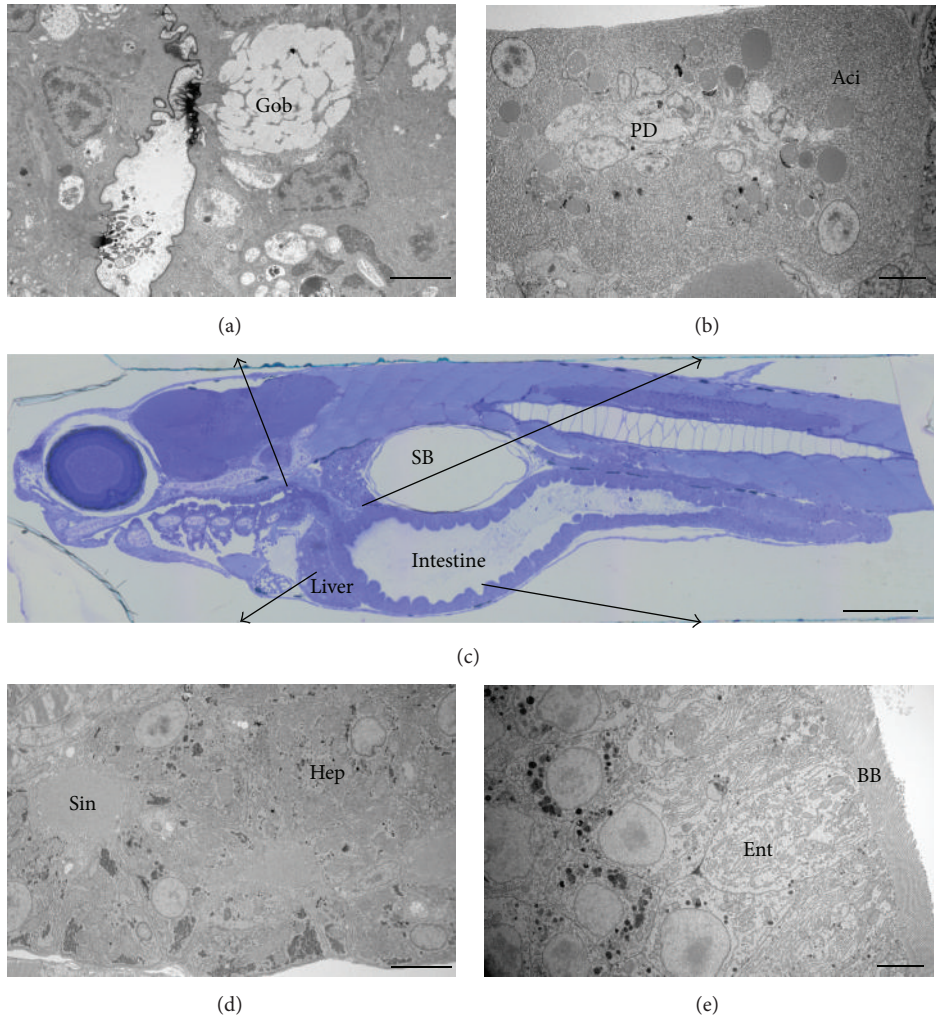

Figure 2: Parasagittal section of a 12 dpf ZF larvae stained with toluidine blue and imaged with light microscopy, showing the different components of the digestive system (c). Corresponding EM images of the different regions include the oesophageal area, rich in goblet cells (Gob) (a), the pancreas, with a pancreatic duct (PD) in the middle and surrounded by acinar cells (Aci) rich in zymogen granules (b). The liver and its hepatocytes (Hep) surrounded by sinusoids (Sin) and its network of bile ducts are shown in (d), as well as the intestine lined with enterocytes (Ent) rich in villi forming the intestinal brush border (BB) in (e). (SB) is the swim bladder. Scale bar = 20 μm (LM) and 5 μm (EM).

and Z). Indeed, high-resolution microscopy is the only way to provide important complementary histological information for many subcellular processes observed using optical microscopy [42–44].

In this contribution, we outline an alternative multimodal imaging route to fill in the missing histological pieces of the ZF GIS puzzle (Figure 1). By doing so, we firstly illustrated the unique overall ultrastructural resemblance of the ZF GIS to the mammalian GIS as examined by standard TEM (Figure 2). Next, we expanded those results by employing contemporary three-dimensional (3D) microscopy techniques (Figures 3–5) and finally reviewed our fine structure findings, including our morphometric data, against the existing literature (Table 1).

The sample preparation protocol used in this study was modified from Deerinck et al. [26] and Tapia et al. [27] and was originally developed for the preparation of biological samples for serial-block face sectioning and back-scattered SEM imaging. Although the sample processing time is significantly longer (6-7 days) than that of a conventional sample preparation protocol for EM (3-4 days), we have still opted to base our protocol on the two protocols mentioned previously: the aforementioned series of heavy metals stains and mordanting agents have shown great contrast improvement and conductivity of biological tissues, critical for high-resolution EM imaging, without interfering with conventional LM staining. The use of reduced osmium (RO) with potassium ferrocyanide not only improved membrane preservation but also contrasted glycogen granules in hepatocytes [45, 46]. The addition of sucrose and $CaCl_2$ to the buffer further improved the ultrastructural preservation by stabilising the osmolarity [47] throughout the processing. X-ray imaging was used here as a relative quick way to image the GIS in its entirety. Due to the small size of the animal (in average

Table 1: Comparison between the zebrafish and the rat digestive systems, including the GIT and the accessory organs. The information on the ZF digestive system presented in the table below mainly result from the data analysis obtained in this study, complemented by information providing various literature sources [20, 24, 40, 54–56, 60–63]. The information on the rat ZF are mainly compiled from a review of the literature, including the following references [64–70].

(a)

Zebrafish liver		Zebrafish pancreas		Zebrafish gut	
Size	*12 dpf*: volume = 5 061 840 μm^3. Transverse diameter = 349 μm. Anterior-posterior diameter = 520 μm *Adult*: volume = 0.535 mm^3. Transverse diameter = 2.08 mm. Anterior-posterior diameter = 1.64 mm. (In males, ~2.10% of total body weight. In females, ~4.51% of total body weight)	Size	*12 dpf*: Volume = 1 738 670 μm^3. Transverse diameter = 290 μm. Anterior-posterior diameter = 500 μm *Adult*: volume = 0.3 mm^3. Transverse diameter = 1.9 mm. Anterior-posterior diameter = 2.5 mm	Size	Long tube of about 400 μm. The interior is also folded and lined with a layer of simple columnar epithelium
Location	Posterior to inner ears and pharyngeal pad, anterior to intestinal bulb	Location	Between the intestine and the swim bladder. The head starts right posterior to the liver	Location	From esophagus to anus
Shape	3 lobes, boomerang shape. The left lobe, the largest, crosses the midline, under the swim bladder and the right lobe extends ventrally towards the head of the pancreas	Organisation	The pancreas is very diffused and acinar cells are scattered	*Oesophagus*	Located under the trachea. Short and muscular
Organisation of hepatocytes	Tubular	*Endocrine tissue*	One principal islet (or Brockman body) in the head (50 μm diameter). 2-3 secondary islets surround the principal islet	Composition	Mostly goblet cells and a few enterocytes
Composition	Hepatocytes, endothelial cells, bile duct epithelial cells. Kupffer cells seem to be absent	Beta cells	Insulin granules (160 nm)	Role	First place of enzymatic digestion
Hepatocytes	65% of total liver volume	Alpha cells	Glucagon granules (182 nm)	*Intestinal bulb*	Dilated, up to 80 μm in diameter
Size	Polygonal, 14–17 μm in size. Hepatocytes form plates, lined with sinusoids and biliary ducts	Other-cells	Delta-cells produce somatostatin	Composition	Enterocytes with very long microvilli at the beginning (up to 7.5 μm long and 115 nm in diameter) and then shortened to 2-3 μm long
Organelles	Nucleus, rER, mitochondria, Golgi apparatus, glycogen, lipid droplets, lysosomes	*Vascular system*	Rich vasculature (12% of the islet)	Role	Fat absorption
Biliary system	18% of liver volume	*Exocrine tissue*	Production of pancreatic digestive enzymes	*Midintestine*	Narrow, folded 3 times on itself and measures 120–140 μm in diameter

(a) Continued.

Zebrafish liver		Zebrafish pancreas		Zebrafish gut	
Bile canaliculi	Presence of microvilli on the surface. Diameter = 2.22 μm	*Acinar cell*	Polyhedral, 8–10 μm in size, surrounding a central acinar duct	Composition	More goblet cells than in the intestinal bulb. Enterocytes have shorter microvilli (2-3 μm long, 115 nm in diameter)
Bile ducts	Intrahepatic, and extrahepatic ducts	Organelles	Zymogen granules (2.30 μm), nucleus, ER, mitochondria	Role	Proteins absorption
Vascular system	17% of liver volume	*Pancreatic system*	Main pancreatic ducts through the middle of the pancreas head	*Posterior intestine*	Narrow and measures 80–90 μm in diameter
Endothelial cells	Fenestrated, average diameter = 130 nm.			Composition Role	Rare or no microvilli Osmoregulation

(b)

Rat liver		Rat pancreas		Rat gut	
Size	Transverse diameter = 7.5–8 cm, superior-inferior diameter = 2.8–4.2 cm, anterior-posterior diameter = 2.2–2.5 cm. 5% of total body weight, mean weight = 13.6 g	Size	Weight = 804 mg	Size	The long tube measures up to 2 m in length
Location	Upper right portion of the abdomen, beneath the diaphragm and above the stomach. A small portion extends into the upper left quadrant	Location	In the cranial abdominal cavity, between the stomach and the small intestine	Location	From mouth to anus
Shape	4 lobes: left, middle, right, and caudate	Organisation	Diffused, lobulated in appearance and divided into 3 parts: biliary, duodenal, and gastrosplenic	*Oesophagus*	Long tube from mouth to stomach, approx. 20 cm long
Organisation of hepatocytes	Lobular	*Endocrine tissue*	2–3.5% of the pancreas. 5000 islets of Langerhans scattered within the organ	Composition	Stratified squamous epithelium
Composition	Hepatocytes, endothelial cells, bile duct epithelial cells, Kupffer cells	Beta cells	65–80% of the islet. Production of insulin granules (207 nm)	Role	Transport food from mouth to stomach
Hepatocytes	80.6% of total liver volume	Alpha cells	15–20% of the islet. Production of glucagon granules (172 nm)	*Stomach*	*Functions are comparable to the ZF intestinal bulb*, approx. 6 cm long
Size	Polyhedral, 27 μm in diameter. Overlapping plate-like sheets (trabeculae) form the three-dimensional structure of the liver lobule	Other-cells	Delta-cells (3–10% of the islet) produce somatostatin. PP-cells (3–5% of the islet) produce pancreatic polypeptides	Composition	Enterocytes are the main cell types (90% of the villi surface)

(b) Continued.

	Rat liver		Rat pancreas		Rat gut
Organelles	Nucleus (25% are binucleate), mitochondria, rER, sER, lysosomes, Golgi apparatus, peroxisomes, lipid droplets, free ribosomes, lipoproteins, glycogen, polyosomes	*Vascular system*	Rich vasculature (10% of the islet)	Role	Storage of food, start of enzymatic digestion
Biliary system	0.2% of liver	*Exocrine tissue*	95% of the pancreas	*Small intestine*	*Functions are comparable to the midintestine*, approx. 1–1.5 m long
Bile canaliculi	Presence of microvilli on the surface. Diameter = 1.5 μm	*Acinar cell*	Polyhedral, 10 μm in diameter, surrounding a central acinar duct.	Composition	Divided into 3 parts: duodenum, jejunum, and ileum. Enterocytes are the main cell types
Bile ducts	Intrahepatic, interlobular, and extrahepatic bile ducts	Organelles	Zymogen granules (500–800 nm), nucleus, ER, mitochondria	Role	Absorption of nutrients
Vascular system	19.2% of liver volume	*Pancreatic system*	Anterior pancreatic duct (main duct) occupies 50–60% of the pancreas. Draining the pancreatic juice to the stomach and small intestine	*Large intestine*	*Functions are comparable to the posterior intestine*, approx. 22–26 cm long
Endothelial cells	Fenestrated, average diameter = 6.5 μm			Composition Role	Enterocytes Absorption of water and left over digested nutrients

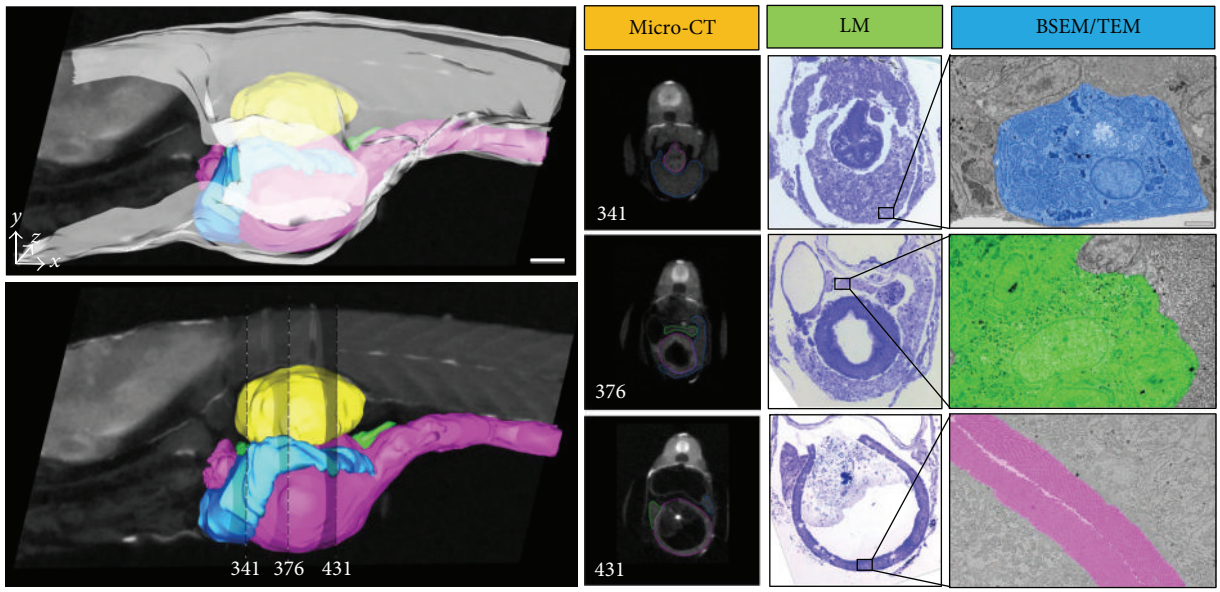

Figure 3: Zebrafish larvae (12 dpf) digestive system imaged using X-ray, LM, and EM (BSEM and TEM). At any positions (here, sections 341, 376, and 431 are shown as examples), micro-CT images and model can be viewed as cross-sections. Corresponding LM images of toluidine blue stained sections (500 nm) can be retrieved by mean of measuring distances from recognisable organs in the X-ray data. Back-scattered SEM images are generated from the same sections as the LM sections. TEM images are generated from adjacent sections from the LM ones. Colour code for micro-CT model: swim bladder (yellow), pancreas (green), intestine (pink), and liver (blue). Colour code for EM images: hepatocyte (blue), islet of Langerhans (green), and intestinal brush border (pink). Scale bars = 100 μm (micro-CT) and 2 μm (TEM).

5 mm × 700 μm), a full scan of the GIS usually takes 2 hours while it would take a few days to scan an entire rat at a comparable resolution. Gross organ sizes and their relative positions within the fish body can be observed. The small sample size and the enhanced contrasting protocol used in this work also made the segmentation and modelling of the internal organs relatively easy. In fact, ZF samples processed with the protocol herein displayed superior contrast relative to samples processed using a standard protocol involving staining with osmium tetroxide only. In fact, previous studies have shown that soft tissue contrast can be enhanced for X-ray micro-CT imaging by heavy metal staining [48, 49]. Imaging of the sample embedded in resin also exhibits a higher signal-to-noise ratio compared to sample immersed in ethanol (Supplementary information 3). This may be related to the enhanced stability of the sample—increased signal—as well as the embedding media—decreased noise—during the scan acquisition. By imaging the entire intact ZF in the micro-CT, information such as 3D connectivity is retained and significant internal tissue damage resulting from sample preparation can be assessed. Since micro-CT is a nondestructive imaging technique, the same sample block can be retrieved and sectioned, and subsequent data can be correlated with histological (i.e., LM) and/or subcellular ultrastructural studies (i.e., EM) (Figure 3), as the sample was processed using a protocol compatible to all the mentioned imaging modalities. Sectioning for array tomography is the most time consuming step: it will take an experienced microtomist 6–8 hours to section through the entire GIS sagittal and collect the ribbons of sections on glass slides, following which the samples are conserved for future LM and EM imaging and analysis.

Micro-CT analysis allowed us to determine that the GIT of a 12 dpf ZF was approximately 2.5 mm long, from the oesophagus to the anus.

The liver, the largest of the digestive glands, plays a pivotal role in the maintenance of metabolic function and excretion [50]. Our micro-CT and array tomography reconstructions showed that the liver is a trilobe organ with a boomerang-like shape and lies ventrally and anterior to the swim bladder, surrounding the intestine (Figure 3). These observations are supported by previous histological observations [20]. In a 12 dpf larvae, we measured the liver to be approximately 25 μm (anterior-posterior diameter) and occupying an average volume of 5.10^6 μm^3 (approximately 2% of a 12 dpf ZF total body volume) within which 17% is occupied by blood vessels (Figure 4). Compared to a rat liver, a ZF liver volume is hence calculated to be 2 400 000 times smaller in volume and 2.2% less vascularised. From previous histological observations, the ZF liver is similar to that of other teleosts [51]: there are no portal triads nor hepatocytes arranged in plates but rather tubules of hepatocytes among portal veins, hepatic arteries, large biliary ducts, and sinusoids, which are distributed stochastically within the parenchyma [52]. Morphologically, the hepatocytes we analysed were polygonal, measured 17 μm in diameter (Figure 3), and formed tubules of small bile ducts, derived from the bile canaliculi, which was intrahepatic and had an average diameter of 2.2 μm. EM data showed vessels and sinusoids also lined with a monolayer of endothelial cells, exhibiting fenestrations varying from 80 to

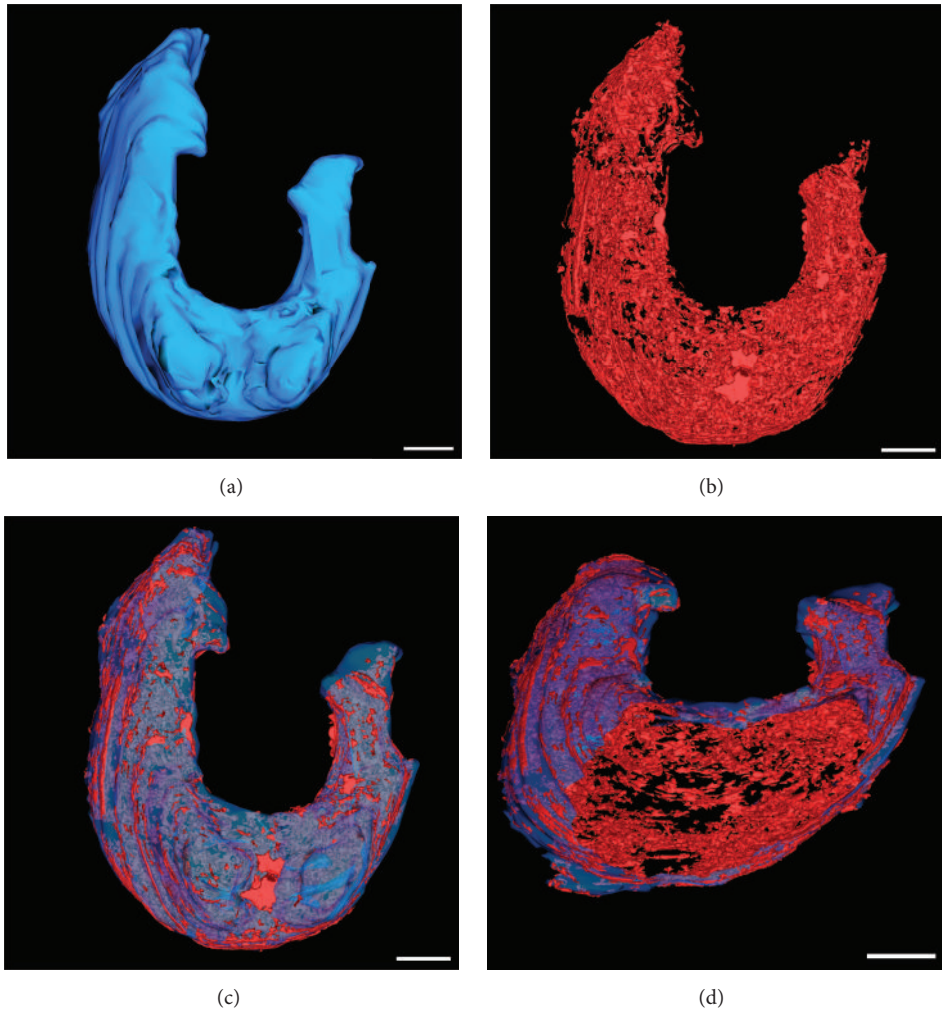

Figure 4: Zebrafish larvae (12 dpf) model of liver (blue) and its vasculature (red), generated by serial LM imaging of 416 consecutive sections of 500 nm. (a) Dorsal view of the liver. (b) Vasculature of the liver (17% of total volume) represented in (a). (c) Combined liver model and its vasculature. (d) Same as (c), viewed from a different angle and clipped opened to visualise the internal vasculature. Liver vasculature was modelled by thresholding the grey values corresponding to the vessels and sinusoids from individual LM images. For full animation, see Supplementary information. Scale bar = 50 μm.

230 nm in diameter, which means they can be up to double the size of the liver endothelial cells fenestrations reported in rats [53]. Notable was the apparent absence of Kupffer cells throughout the hepatic sinusoids, a distinct point of difference to mammalian livers.

Like in other animals who possess a pancreas, the ZF pancreas is a dichotomic organ and the site of glucohomeostasis [21, 54]. In the ZF, the pancreas is diffused and located around the liver and intestine: the exocrine part is mainly composed of pancreatic ducts and acinar cells. From our correlative imaging studies, the acinar cells were polyhedral, measured 8–10 μm in diameter, and were populated with relatively large zymogen granules (2–2.5 μm, compared to 500–800 nm found in rats) which discharge their contents into the pancreatic ducts to form the pancreatic juice. A principal islet of Langerhans (or Brockman body), identified in the pancreas head and measuring 50 μm in diameter, along with scattered secondary islets forms the endocrine component. The islets composition is as found in mammals, whereby a rich network of blood vessels surrounds neuroendocrine cells, namely, alpha-cells, beta cells, and delta-cells, which, respectively, produce the hormones glucagon, insulin, and somatostatin. Gamma and polypeptide producing- (PP-) cells appear to be absent (Figure 3) [24]. From our light microscopy analysis, the vasculature within the principal islet occupied approximately 12% of the islet. Glucagon and insulin granules were smaller than those found in the rat, measuring, respectively, 182 nm and 160 nm compared to 172 nm and 207 nm.

The gastrointestinal tract (GIT) is a long 3-fold tube, starting from the oesophagus to the anus, with morphological similarities to mammals: a simple columnar epithelium with folds (no real villi) including enterocytes, goblet cells, and endocrine cells. On the apical side, microvilli are present and the enterocytes are joined by tight junctions, which we determined by TEM to be 200–400 nm across.

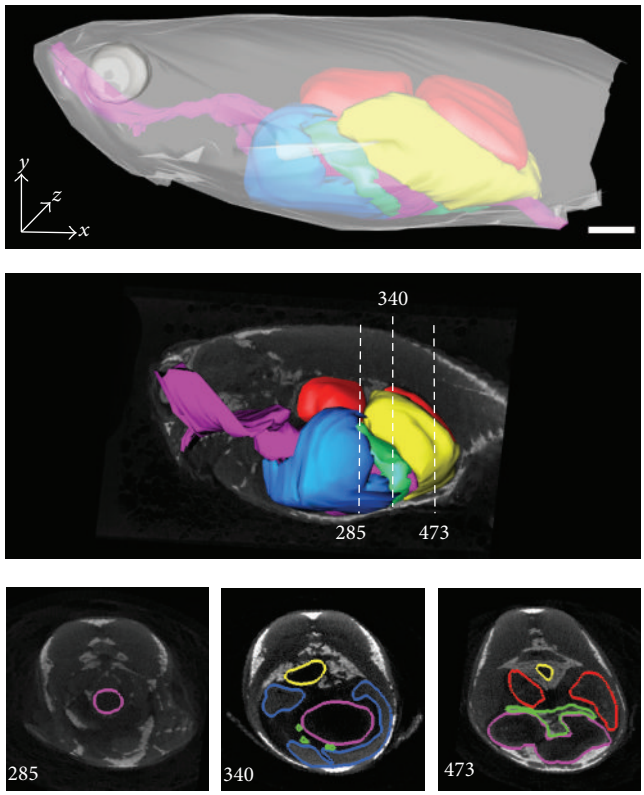

Figure 5: Adult zebrafish digestive system reconstruction by X-ray micro-CT, showing the GIT (pink), liver (blue), pancreas (green), swim bladder (yellow), and oocytes (red). Cross-sections are shown on the bottom line for different positions (here, positions 285, 340, and 473 are used as examples). Scale bar = 20 mm.

In the ZF, the oesophagus is short and muscular and is mainly composed of goblet cells and taste buds as it is the first site of enzymatic digestion. The intestine follows the oesophagus and histologists have divided it into 3 parts: the intestinal bulb, the midintestine, and the posterior intestine which can be differentiated by their shape, density of goblet cells, and the length of microvilli forming the brush border [18, 55]. Paneth cells, crypts, and organised lymphoid structures are absent across the entire intestine [55]. The intestinal bulb plays the role of food storage and fat absorption [24]. From our X-ray and LM observations, the "bulb" was dilated up to 80 μm wide; however, it could probably extend more following a large meal. Enterocytes were the main cell types and our analysis revealed that the enterocytes at the very beginning of the intestine have the longest microvilli (up to 7.5 μm long) after which they shorten to about 2-3 μm within the intestinal bulb (Figure 3). The midintestine is narrow and believed to be the site of protein absorption [19, 56]. Enterocytes in this area presented microvilli of similar length to those found in the intestinal bulb and were surrounded by more goblet cells than in the intestinal bulb. In the posterior intestine, goblet cells were still present but enterocytes presented short or few microvilli. In fact, most of the digestion process has already taken place and this part is mainly involved in osmoregulation [18].

Finally, we demonstrated that our whole-mount multimodal imaging approach could also be applicable to adult ZF (Figure 5). The versatile imaging workflow presented in this study validates the anatomical fine structures of the adult ZF GIS and compares the larvae ZF GIS systematically to the rodent experimental models (Table 1).

4. Conclusion

The sample preparation protocol presented here offered not only great versatility—compatible with X-ray, light, and electron microscopy—but also optimum ultrastructural preservation of ZF larvae as well as improved sample contrast and conductivity, required for high-resolution X-ray and EM imaging. The workflow can be easily adapted to incorporate *in vivo* fluorescence or fluorescently labelled structures by adding a fluorescent live imaging step prior to sample processing. The concept of Correlative Light and Electron Microscopy (CLEM) can then be applied to complement dynamic functional information with high-resolution ultrastructural details, on the same sample. The use of tracers or fiducial markers such as laser etching techniques [57] or carbon-coating of pattern on glass slides [58, 59] can facilitate the relocalisation of the area, cell,or subcellular structure of interest between the macro- and nanometre scales imaging. This workflow presents a real advantage in the fields of research aiming at exploring drug transport and xenobiotic metabolism within the digestive glands (e.g., liver and pancreas) or studying and modifying malignant cell behaviour via novel anticancer therapeutic approaches (e.g., colon, pancreas, and liver cancer) (Figure 6). In fact, the low cost, small size, and relative speed at which drugs can be tested in ZF have already made it a popular model for the aforementioned studies. By following the entire workflow, gross differences between experimental and control fish can be rapidly determined by X-ray micro-CT, while detailed analysis of the drug treatment upon pathology can be subsequently evaluated, on the same sample. While all imaging modalities might not be available within a certain research laboratory or institute, this workflow can be adapted to only incorporate the imaging instrumentation available. Since the sample has been prepared to accommodate all imaging techniques aforementioned, one can skip imaging modalities which are unavailable without affecting the next. Also, depending on the study, one can also limit the imaging techniques to only include the ones needed to answer the biological question.

Our comparative microscopy maps and concomitant delivery of 3D imaging models of the ZF digestive system provide a comprehensive overview of the ZF GIS and establish the much needed foundation as well as a limit of confidence in the use of ZF for future research in gastrodigestive related illnesses.

Competing Interests

The authors declare that they have no competing interests.

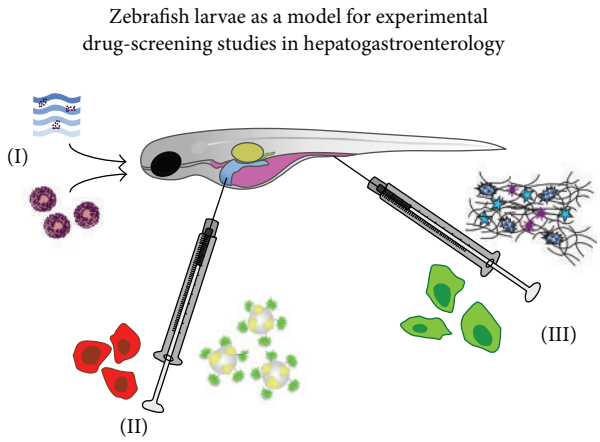

Figure 6: Illustration showing the different routes of administration possible in zebrafish larvae to study the uptake, transport, metabolism, and efficacy of therapeutic drug- and/or cell-based approaches. (I) Indirect administration of complexes dissolved in water or administered directly via the oral route, mixed with food pellets. (II-III) Local and targeted microinjection of fluorescent macromolecular complexes at the site of interest or the use of microcapillary needles to deposit genetically modified cells within the digestive glands (II) or intestines (III). Note that those three administering routes are typically employed in routine preclinical screening studies in rodent models and human studies as well underpinning the relevance of the zebrafish model to investigate the pharmacology, toxicology, and effectiveness of new therapeutic interventions. Taking advantage of the optical translucent properties of the larvae, subsequent whole-mount live-cell imaging allows systematic monitoring of the treatment regimes using fluorescent navigation. The results can be combined with correlated electron microscopy techniques as depicted under Figures 1–4. Colour legend for the zebrafish: swim bladder (yellow); stomach and intestines (purple); liver (blue); pancreas (green).

Authors' Contributions

Delfine Cheng and Gerald J. Shami contributed equally.

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Research Article

Oxidative Stress and Liver Morphology in Experimental Cyclosporine A-Induced Hepatotoxicity

Agnieszka Korolczuk,[1] Kinga Caban,[1] Magdalena Amarowicz,[1] Grażyna Czechowska,[2] and Joanna Irla-Miduch[1]

[1]*Department of Clinical Pathomorphology, Medical University, Jaczewskiego 8, 20-950 Lublin, Poland*
[2]*Department of Gastroenterology with Endoscopic Unit, Medical University, Jaczewskiego 8, 20-950 Lublin, Poland*

Correspondence should be addressed to Agnieszka Korolczuk; agnieszka.korolczuk@wp.pl

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Cyclosporine A is an immunosuppressive drug used after organ's transplantation. The adverse effects on such organs as kidney or liver may limit its use. Oxidative stress is proposed as one of the mechanisms of organs injury. The study was designed to elucidate CsA-induced changes in liver function, morphology, oxidative stress parameters, and mitochondria in rat's hepatocytes. Male Wistar rats were used: group A (control) receiving physiological saline, group B cyclosporine A in a dose of 15 mg/kg/day subcutaneously, and group C the CsA-vehicle (olive oil). On the 28th day rats were anesthetized. The following biochemical changes were observed in CsA-treated animals: increased levels of ALT, AST, and bilirubin in the serum, statistically significant changes in oxidative stress parameters, and lipid peroxidation products in the liver supernatants: MDA+4HAE, GSH, GSSG, caspase 3 activity, and ADP/ATP, NAD^+/NADH, and $NADP^+$/NADPH ratios. Microscopy of the liver revealed congestion, sinusoidal dilatation, and focal hepatocytes necrosis with mononuclear cell infiltration. Electron microscope revealed marked mitochondrial damage. Biochemical studies indicated that CsA treatment impairs liver function and triggers oxidative stress and redox imbalance in rats hepatocytes. Changes of oxidative stress markers parallel with mitochondrial damage suggest that these mechanisms play a crucial role in the course of CsA hepatotoxicity.

1. Introduction

Cyclosporine A (CsA) belongs to calcineurin inhibitors used in patients after kidney, liver, heart, lung, and heart-lung transplants for graft-versus-host disease (GVHD) prophylaxis [1, 2].

Moreover, CsA is used to treat the majority of autoimmune diseases [3], in dermatology to treat psoriasis, autoimmune dermatitis, or chronic idiopathic urticaria [4, 5].

The major adverse side effect of CsA is acute and chronic nephrotoxicity.

CsA can cause metabolic and electrolyte disorders, that is, weight gain, hyperglycaemia, hyperlipidaemia, hypercalcaemia, and hypomagnesaemia [6].

Experimental studies and clinical observations reveal that CsA can lead to drug-induced liver injury (DILI). In CsA-induced liver injury, functional and morphological changes are observed. The functional changes include elevated serum levels of liver transaminases and alkaline phosphatase, cholestasis, hyperbilirubinemia, increased production of bile salts, and impaired secretion of lipids [7–9].

The morphological changes observed in experimental animals receiving CsA include impaired trabecular structure, hepatic sinus congestion and widening, activation of the Kupffer cells, passive congestion and oedema of portal tracts, mild mononuclear cell infiltrations within portal tracts, and degenerative changes in the hepatocytes including their focal necrosis [10–12].

The mechanisms of CsA-induced liver injury involve the development of hypermetabolic state in the liver [13] and inhibition of ATP-dependent transport of bilirubin and bile salts through the hepatocyte canalicular membranes as well as of bile secretion [14, 15]. The use of antioxidants in experimental animals exposed to CsA reduces liver functional and

morphological damage [11, 12, 16, 17], which suggests the involvement of oxidative stress as one of the mechanisms of hepatotoxicity.

The aim of the present study was to evaluate the function and morphology of the liver in animals receiving a cumulative dose of CsA. We focused on the correlation between changes in the selected oxidative stress parameters and morphological and ultrastructural changes in hepatocytes.

2. Material and Methods

Adult male Wistar rats weighing 250–300 g were housed in a temperature-controlled environment with an alternating cycle of 12 h light and dark. They were on a low-sodium diet and had free access to water. The experimental protocols were conducted according to the guidelines of Institutional Animal Ethics Committee (IAEC) of the Medical University, Lublin.

The animals were divided into three groups (A, B, and C) (with 8 animals in each group):

A: control, NaCl 1 mL/kg/day, subcutaneously.

B: vehicle, olive oil 1 mL/kg/day, subcutaneously.

C: CsA, 15 mg/kg/day in olive oil, subcutaneously.

CsA, NaCl, and olive oil doses and way of administration were established according to previous studies [10, 16]. Animals were weighed daily while receiving treatment for 28 days. On the 29th day of an experiment all animals were anesthetized with pentobarbitone (Morbital, Biowet, Poland) and blood samples and liver specimens from the left and right lobe were obtained for biochemical, histological, and ultrastructural analyses.

2.1. Measurement of Liver Function. Serum levels of AST, ALT, and bilirubin were measured using the commercially available diagnostic Cormay kits (Cormay Diagnostics SA, Poland).

2.2. Biochemical Studies. The liver samples were homogenised in 20 mM phosphate buffer (pH 7.4), 0.5 g tissue in 2 mL. The homogenisation was made in cold-water bath (4°C) at 4000 rpm using a Teflon pestle homogeniser (Glas-Col, USA) for 3 min. The homogenate was centrifuged at 15 000 rpm for 20 min and the obtained supernatant was used for further biochemical studies. All spectrophotometric methods were performed using a microtiter plate reader (PowerWaveXS, BioTek, USA).

GSH, GSSG, and GSH/GSSG. Reduced (GSH) and oxidized (GSSG) glutathione determination was conducted using commercial kit BIOXYTECH GSH/GSSG-412 (*Oxis*Research, USA). The principle of the procedure is based on simultaneous determination of GSH and GSSG in two separate tubs. To determine GSSG, 1-methyl-2-vinylpyridinium trifluoromethane-sulfonate (M2VP) was used at a level that rapidly and completely scavenges GSH but does not interfere with the glutathione reductases that in turn reverse GSSG to GSH in the next step of procedure. Subsequently the procedure is common for both parameters. GSH in separate tubs (created or native) is extracted and reacts with Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid) forming a colour product with the maximum of absorbance at 412 nm. The concentrations of GSH and GSSG were rewritten from the separate calibration curves.

Lipid Peroxidation Products. The commercial kit BIOXYTECH LPO-586 (*Oxis*Research, USA) was used to measure malondialdehyde (MDA) and 4-hydroxyalkenals (4HAE) as indicators of lipid peroxidation. The method is based on the reactions between MDA and 4HAE with N-methyl-2-phenylindole at temperature of 45°C for 60 minutes, in which a stable chromophore with maximal absorbance at 586 nm is yielded. The procedure was conducted according to the manufacturer's description and the concentration of MDA+4HAE in tested samples was calculated from the formula of calibration curve $y = 0.0896x - 0.008$. The results were expressed in nmol/g liver tissue.

NAD^+/NADH and $NADP^+$/NADPH Ratios. The $NAD(P)^+$ and NAD(P)H levels were measured using BioChain NAD^+/NADH assay kits according to the manufacturer's instructions (BioChain, Hayward, CA). The principles of assay kits are based on a glucose dehydrogenase cycling reaction, in which the tetrazolium dye (MTT) is reduced by NAD(P)H in the presence of phenazine methosulfate (PMS). The intensity of the reduced product colour, measured at 565 nm, is proportionate to the NAD^+ concentration in the sample. The standards attached to the kits were used to prepare the calibration curves needed to calculate $NAD(P)^+$/NAD(P)H ratios.

ADP/ATP Ratio. The tissue supernatant ADP/ATP ratio was calculated on the basis of fluorescence (luminescence) intensity measurement (BioVision kit, USA) using a fluorescence microplate reader Victor3V (Perkin Elmer, Finland).

2.3. Morphologic Studies. Liver samples were fixed in 10% buffered formalin and embedded in paraffin. After dewaxing 4 μm sections were stained with haematoxylin and eosin (H+E). All slides were evaluated under light microscopy (Olympus BX45) by one pathologist who assessed liver morphology. A minimum of 20 cortical fields was examined in each biopsy (magnification ×100).

2.4. Electron Microscopy. Tissue sections for electron microscopic examinations were fixed in 4% formaldehyde and 1% glutaraldehyde and processed to epoxy resin. Ultrathin samples were stained with uranyl acetate and lead citrate and examined with a Jeol-JEM 1011 electron microscope.

2.5. Statistical Analysis. Results were presented as mean ± SEM. Data were analysed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test and Student's t-test using a statistical software package (STATISTICA v.8.0 StatSoft, Poland). p value < 0,05 was considered statistically significant.

Table 1: Serum levels of AST, ALT, and bilirubin in all experimental groups.

	NaCl (A)	CsA (B)	Olive oil (C)
AST [U/L]	25,09 ± 3,01	62,4 ± 5,36*	25,11 ± 3,36
ALT [U/L]	7,76 ± 0,98	28,35 ± 5,12*	7,95 ± 1,26
BIL [mg/dL]	0,42 ± 0,038	1,05 ± 0,09*	0,46 ± 0,05

Values are mean ± SEM. *A versus B and B versus C and $p < 0,05$.

3. Results

3.1. Liver Function. CsA administration resulted in decreased liver function measured by serum levels of AST, ALT, and bilirubin when compared with the control group (Table 1). The results show statistically significant differences in all examined parameters in animals of group B comparing with animals of control group ($p < 0,05$): AST was 62,4 in group B versus 25,09 in group A, ALT 28,35 (group B) versus 7,76 (group A), and bilirubin 1,06 (group B) versus 0,42 (group A).

3.2. Microscopic Changes. No prominent morphologic changes were seen in the liver of group receiving olive oil or control animals (Figures 1(a) and 1(b)). Liver samples collected from CsA-treated animals (group B) showed several morphological changes. Lobular architecture of the liver was preserved. Passive hyperaemia within central veins with dilatation of hepatic sinusoids was observed (Figure 1(c)). Hepatocytes presented vacuolar degeneration of their cytoplasm (Figure 1(d)) and focal micro- and macrovesicular steatosis (Figure 1(e)). Foci of liver cell necrosis accompanied by mononuclear inflammatory infiltrates were noticed with the presence of single cells with pyknotic nuclei and strongly eosinophilic cytoplasm (Figure 1(f)).

3.3. Ultrastructural Changes. Electron microscope examination did not reveal pathologic changes within the hepatocytes of group receiving olive oil or control animals (Figure 2(a)). In animals treated with CsA, vacuolization of hepatocytes seen under a light microscope corresponded to dilatation of endoplasmic reticulum with the formation of different sized vacuoles seen in electron microscope (Figure 2(b)). Ultrastructural examination of these cells revealed also marked swelling of mitochondria with the presence of giant mitochondria in some cells (Figure 2(c)). In most of these organelles, the disruption and loss of the inner membrane and the cristae were observed (Figure 2(c)). Moreover, the formation of autolysosomes containing mitochondrial material was observed. Mitochondrial damage was observed approximately in 50 to 60% of hepatocytes of animals receiving CsA. Some of nuclei were shrunken with some of them performing karyorrhexis (Figures 2(b) and 2(c)). Single apoptotic cells with markedly condensed chromatin and shrunken cytoplasm were present (Figure 2(d)).

3.4. LPH, GSH, and GSSG. Treatment of animals with CsA alone produced a significant increase in hepatic LPH (3,13 nmol/g in controls to 12,68 nmol/g in CsA animals) and GSSG levels (0,38 nmol/g in controls to 1,33 nmol/g in CsA animals) as well as a significant decrease in hepatic GSH levels (6,65 nmol/g in controls to 3,3 nmol/g in CsA animals) compared with controls. The results showed statistically significant differences in all examined parameters in animals of group B comparing with animals of control group ($p < 0,05$), Table 2.

3.5. $NADP^+$/NADPH, NAD^+/NADH, and ADP/ATP Ratios. CsA induced a significant increase in $NADP^+$/NADPH ratio (from 0,72 in controls to 3,48 in CsA animals) and a decrease in NAD^+/NADH ratio (from 5,83 in controls to 1,78 in CsA animals), Table 2. The results showed statistically significant differences in all examined parameters in animals of group B comparing with animals of control group ($p < 0,05$). The mean ADP/ATP ratio was significantly higher in CsA-treated animals (4,95) compared with controls (0,8).

3.6. Caspase 3 Activity. Caspase 3 activity in the liver was significantly increased ($p < 0,05$) in animals receiving CsA when compared with controls (Table 2). It increased from 99,93% in controls to 232,22% in CsA group.

4. Discussion

In the present study assessing the effects of CsA on liver injury in animals, changes in liver function parameters, microscopic and ultrastructural lesions in the hepatocytes, and changes in oxidative stress parameters were analysed.

The essential adverse effect of CsA is nephrotoxicity, yet there are also reports describing damage to other organs or systems, such as the heart, central nervous system, and testicles [6, 18–21]. Moreover, clinical and experimental studies have demonstrated impaired liver function and morphology [10–12, 22, 23].

Our findings revealed that administration of CsA increased levels of AST, ALT, and bilirubin and these findings are consistent with the results of experimental studies of other authors, which show that elevated levels of these parameters evidence functional liver damage [8, 23–27].

Drug-induced liver injury can develop in the form of acute drug-induced hepatitis or cholestatic hepatitis [28, 29]. Toxic effects of drugs can cause degenerative changes in the hepatocytes, including their necrosis (paracetamol, bendazac, CsA, carbon tetrachloride, and ethionine), steatosis (tetracycline and ticlopidine), or cholestasis (methapyrilene and naphthyl isothiocyanate) [30]. Three major mechanisms of drug-induced liver injury have been implicated: (1) direct cell injury, (2) inhibition of mitochondrial beta-oxidation and the mitochondrial respiratory chain, and (3) immunologic reactions [31]. The symptoms of toxic action of drugs observed in histopathological examination include hepatocyte degeneration or necrosis, inflammatory infiltrates in the portal tracts, Kupffer cells, or stellate cell activation [32–34]. The mechanisms explaining drug-induced liver injury include mitochondrial damage and oxidative stress [29, 35–39].

Table 2: Hepatic levels of GSH, GSSG, LPH, $NADP^+$/NADPH, NAD^+/NADH, ADP/ATP, and caspase 3 in all experimental groups.

	NaCl (A)	CsA (B)	Olive oil (C)
GSH [nmol/g]	6,65 ± 0,64	3,3 ± 0,48*	6,37 ± 0,74
GSSG [nmol/g]	0,38 ± 0,07	1,33 ± 0,23*	0,4 ± 0,09
MDA+4HAE [nmol/g]	3,13 ± 0,94	12,68 ± 1,26*	3,41 ± 0,56
NAD^+/NADH	5,83 ± 0,81	1,78 ± 0,55*	5,54 ± 1,16
$NADP^+$/NADPH	0,72 ± 0,14	3,48 ± 0,49*	0,74 ± 0,14
ADP/ATP	0,8 ± 0,24	4,95 ± 0,64*	0,8 ± 0,24
Caspase 3 [%]	99,93 ± 18,94	232,22 ± 21,44*	103,13 ± 18,94

Values are mean ± SEM. *A versus B and B versus C and $p < 0,05$.

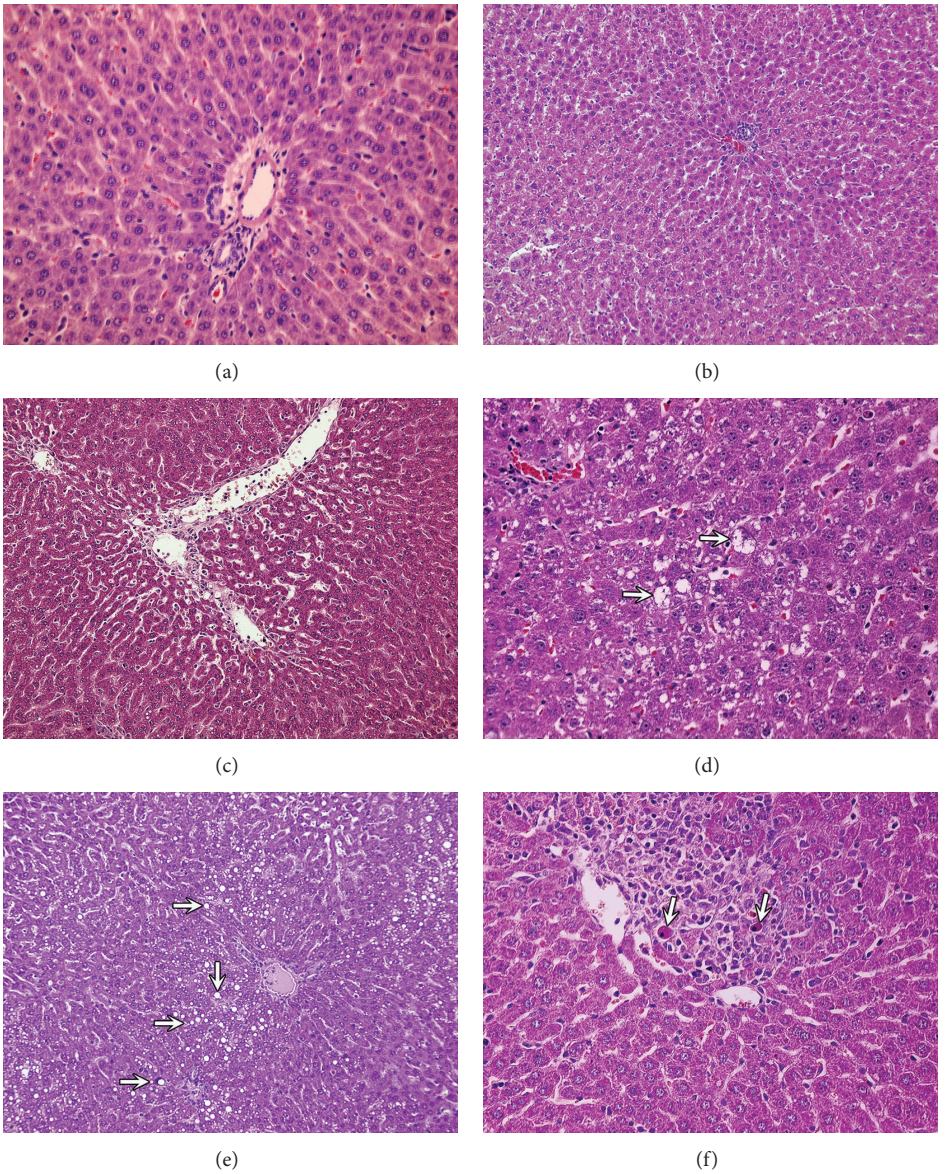
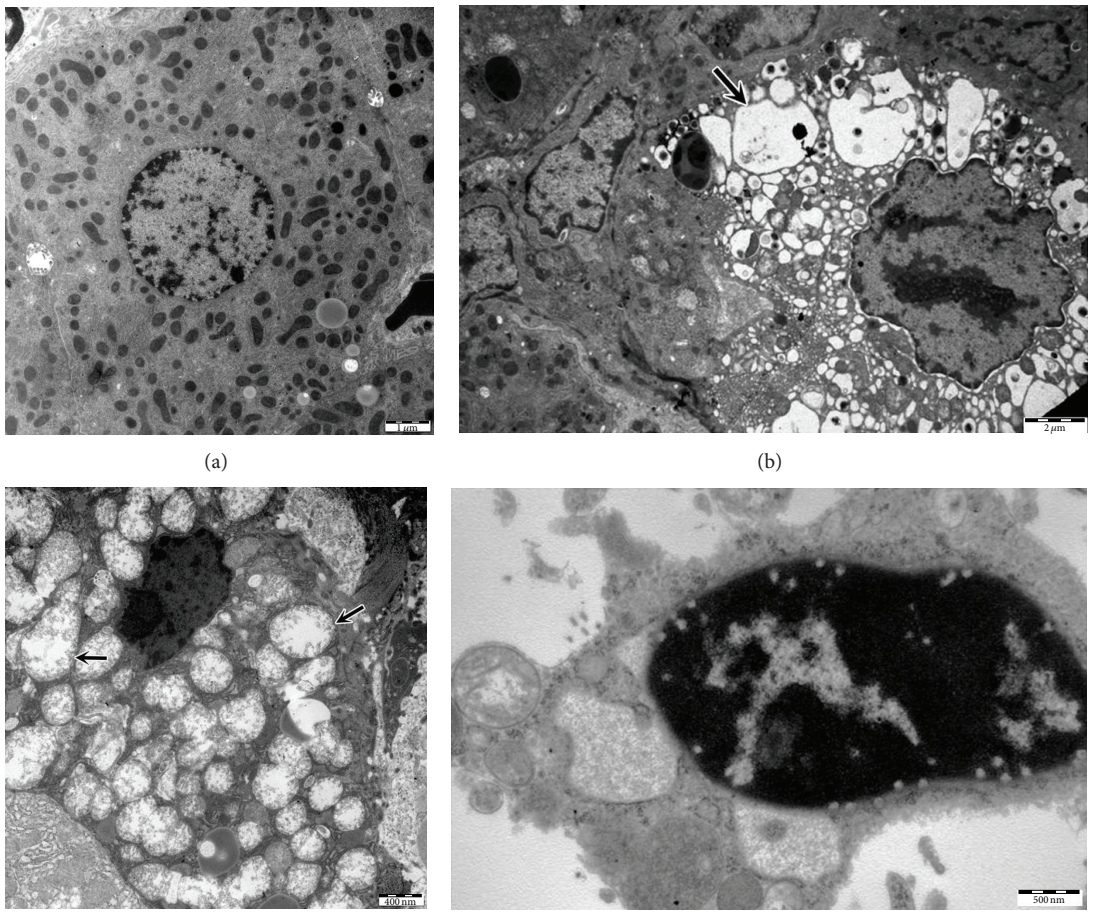


Figure 1: Light microscope, H+E. (a) Control group. Liver architecture within normal limits. ×400. (b) Olive oil group. Mild passive hyperaemia within the central vein. ×200. (c)–(f) CsA group. (c) Passive hyperaemia within central veins with dilatation of hepatic sinusoids. ×200. (d) Vacuolar degeneration of the cytoplasm of hepatocytes (arrows). ×400. (e) Focal micro- and macrovesicular steatosis (arrows). ×200. (f) Focal liver cells necrosis accompanied by mononuclear inflammatory infiltrates. Single eosinophilic cells with signs of apoptosis present (arrows). ×400.



(a) (b)

(c) (d)

Figure 2: Electron microscope. (a) Control group. Hepatocyte with normal ultrastructural appearance. ×8000. (b–d) CsA group. (b) Hepatocyte with dilatation of endoplasmic reticulum with the formation of different sized vacuoles (arrow). ×8000. (c) Marked swelling of mitochondria with the presence of giant mitochondria (arrows) with disruption of mitochondrial cristae. Shrunken nucleus with condensed chromatin present. ×20000. (d) Apoptotic cell with markedly condensed chromatin and shrunken cytoplasm. ×60000.

In the present study, the light and electron microscopy findings demonstrated significant differences in liver morphology in rats receiving cyclosporine, as compared to the control group. Congestion and widening of hepatic sinuses, activation of Kupffer cells, passive congestion and swelling in the portal tracts, mild inflammatory mononuclear cell infiltrates within the portal tracts, and hepatocyte degenerative changes, vacuolar degeneration and steatosis, were present. Similar findings have also been described in other experimental studies [10–12, 24, 27, 40] demonstrating focal necrosis of hepatocytes and the presence of apoptotic cells [10–12, 40].

Drug toxicity-induced changes at the ultrastructural level predominantly regard the mitochondria [29, 36, 37]. Due to their characteristic structure and function different from those of other cell elements, the mitochondria are the key target of drug toxicity [29]. Oxidative stress mostly affects these cell structures. The evaluation of mitochondrial ultrastructure in correlation with the assessment of the selected oxidative stress parameters was an important element of our study.

The role of oxidative stress in chronic CsA treatment has been examined in several studies [41–47]. CsA induces intramitochondrial Ca^{++}, increases oxidative stress and ROS production, and inhibits mitochondrial glucose metabolism (the Krebs cycle and oxidative phosphorylation) and ATP production [6]. It is postulated that CsA is an uncoupler and inhibitor of the mitochondrial transport system. CsA-induced ROS generation is activated by NADPH oxidase, xanthine oxidase, cytochrome P450 CsA metabolism, or decreased intracellular antioxidant systems [48]. The ROS level in the kidney is dose-related [45, 49]. Increase in ROS results in lipid peroxidation and increases its products such as MDA. Moreover, CsA treatment reduces GSH, an important antioxidant [40, 41, 50–53]. Reduced glutathione (GSH) converts lipid peroxides to nontoxic products, thus maintaining the integrity of the mitochondria and cell membranes. GSH is converted by glutathione peroxidase to GSSG in the glutathione redox cycle. Regeneration of GSH by glutathione reductase uses NADPH. In present study, significant increases in MDA and decreases in GSH levels in animals treated with CsA were observed; our results are

consistent with those reported in other studies [12, 23, 24, 54, 55]. It may be speculated that marked decreases in GSH not only were produced by engagement in ROS reduction but could also result from impaired regeneration by glutathione reductase.

Lipid peroxidation products, MDA and mainly 4HAE, decrease the ADP- and NADH-dependent mitochondrial respiratory chain [56], which results in disturbances in membranous Na^+K^+ ATPase activity and decreases in the main mitochondrial product: ATP. To assess mitochondrial phosphorylation and redox state, ADP/ATP, NAD^+/NADH, and $NADP^+$/NADPH ratios were investigated. CsA-treated animals showed a significant increase in the ADP/ATP ratio when compared with controls. Impaired mitochondrial respiration could be one of the possible explanations. These findings correlate with decreases in NAD^+/NADH ratio. Decreased regeneration of NAD^+ and/or increased $NADH^+$ might result from respiratory chain impairment. Oxidative stress increases NAD^+ and ATP consumption and promotes PARP-1 (nuclear enzyme: poly, ADP-ribose, and polymerase-1) dependent cell death [57]. Decreased NAD^+/NADH ratios promote further mitochondrial damage and can lead to cell death.

Increases in $NADP^+$/NADPH ratio were noticed in animals treated with CsA. It could be associated with consumption of NADPH in antioxidative mechanisms. De Hornedo et al. [58] in their *in vitro* study have found that CsA causes dysfunction of enzymes responsible for NADPH production, such as mitochondrial dehydrogenases. NADPH is also a substrate for NADPH oxidase in ROS production. We cannot exclude that the increased $NADP^+$/NADPH ratio observed in this protocol is the result of NADPH oxidase stimulation and dysfunction of peroxisome dehydrogenases and in consequence a decrease in NADPH. Furthermore, it cannot be excluded that an increase in $NADP^+$/NADPH ratio could be associated with increased activity of glutathione reductase at the beginning of the experiment. Decreased GSH and increased GSSG levels seem to support this hypothesis.

Another observation of our study was a substantial increase in caspase 3 activity in the group receiving CsA, as compared to the control group. Substantially increased caspase 3 activity in the liver of rats given CsA compared to controls has also been demonstrated by Wolf et al. [59]. Moreover, similar changes have also been observed in kidneys of rats receiving cyclosporine [60]. In the case of apoptosis, released cytochrome c binds cytoplasmic scaffold (apaf-1) and procaspase 9, forming the apoptosome. The apoptosome activates procaspase 9. The above process required energy derived from ATP. When some mitochondria remain intact and their function consisting in ATP production is normal, activated procaspase 9 and other proapoptotic proteins activate caspase 3, which uncouples specific cell proteins and subsequently activates procaspases 6, 7, and 2, which uncouple some other proteins [61, 62]. The above changes induce programmed cell death, that is, apoptosis [63]. The initiation of apoptosis is thought to be triggered by increased permeability of the outer mitochondrial membrane and escape of cytochrome c from the mitochondrion to the cytosol.

Since mitochondria are one of the main physiologic sources of ROS, we have also focused on their structural appearance. Ultrastructural findings showed swelling of mitochondria in the hepatocytes that differed in size with rupture of their cristae, the inner and outer membrane present in some of them. Degradation of mitochondria and autolysosomes containing the mitochondrial material were also observed. Single apoptotic cells were present. Similar observations were described in previous studies [57, 64, 65]. Structural damage to mitochondria seems to result from their impaired function. De Hornedo et al. [58] have documented that CsA produces a depolarization of mitochondrial membranes that parallels with ROS production. CsA promotes a caspase-independent release of proapoptotic cytochrome c and Smac/Diablo from mitochondria [64]. Increased release of cytochrome c to the cytosol has also been described [65], suggesting that CsA opens the so-called mitochondrial permeability transition pores (MPTP) created at sites of contact of the inner and outer mitochondrial membranes.

Apoptosis is associated with condensation of the nucleus and cytoplasm as well as cytoplasm fragmentation without the loss of cell membrane integrity. Thus, it cannot be excluded that apoptosis was one of the mechanisms of necrosis of single hepatocytes observed by us. This hypothesis of mitochondrial degradation with release of proapoptotic cytochrome c could partially explain the presence of single apoptotic cells in our ultrastructural studies.

Oxidative stress as the possible mechanism of CsA hepatotoxicity was investigated by studies with the use of antioxidants [10, 11, 23, 25, 26, 62, 66–68]. Their findings have demonstrated hepatoprotective effects of melatonin [11, 16], L-arginine [24], taurine [23], quercetin, vitamin E [69], n-acetylcysteine [68], and S-adenosylmethionine [22].

It is known that the mitochondrion is a major intracellular source of ROS. As major ROS generators, mitochondria are targets of high ROS exposure with consequences, such as oxidative damage to mitochondrial DNA (mtDNA) [63]. Considering high number of mitochondria in hepatocytes and main functions of mitochondria, such as energy production and cell death regulation [70, 71], it can be supposed that damage to these organelles is a consequence of elevated ROS. Due to oxidative stress, mitochondria become more susceptible to the action of drugs; mitochondrial damage is a relevant mechanism of toxicity of drugs, including CsA [10, 63, 72]. While elevated ROS associated mtDNA damage has been implicated in cell apoptosis, the precise mechanism whereby mtDNA damage mediates apoptotic signaling is incompletely understood [63]. The impaired liver function and impaired morphology of mitochondria observed by us at the ultrastructural level suggest that oxidative stress triggered the damage to these cytoplasmic organelles and that this mechanism plays an important role in CsA-induced liver injury. Further experimental and molecular studies including cell cultures studies and use of selected antioxidants are considered by us to support this hypothesis.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Study of Valproic Acid-Enhanced Hepatocyte Steatosis

Renin Chang,[1] Mei-Chia Chou,[2] Li-Ying Hung,[3] Mu-En Wang,[3] Meng-Chieh Hsu,[3] and Chih-Hsien Chiu[3]

[1]*Department of Emergency Medicine, Kaohsiung Veterans General Hospital, No. 386, Dazhong 1st Road, Zuoying District, Kaohsiung 813, Taiwan*
[2]*Department of Physical Medicine and Rehabilitation, Kaohsiung Veterans General Hospital, Pingtung Branch, No. 1, Anpin 1st Lane, Jhaosheng Road, Longtan Village, Neipu Township, Pingtung County 912, Taiwan*
[3]*Department of Animal Science and Technology, National Taiwan University, No. 50, Lane 155, Sec. 3, Keelung Road, Taipei 106, Taiwan*

Correspondence should be addressed to Chih-Hsien Chiu; chiuchihhsien@ntu.edu.tw

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Valproic acid (VPA) is one of the most widely used antiepilepsy drugs. However, several side effects, including weight gain and fatty liver, have been reported in patients following VPA treatment. In this study, we explored the molecular mechanisms of VPA-induced hepatic steatosis using FL83B cell line-based *in vitro* model. Using fluorescent lipid staining technique, we found that VPA enhanced oleic acid- (OLA-) induced lipid accumulation in a dose-dependent manner in hepatocytes; this may be due to upregulated lipid uptake, triacylglycerol (TAG) synthesis, and lipid droplet formation. Real-time PCR results showed that, following VPA treatment, the expression levels of genes encoding cluster of differentiation 36 (*Cd36*), low-density lipoprotein receptor-related protein 1 (*Lrp1*), diacylglycerol acyltransferase 2 (*Dgat2*), and perilipin 2 (*Plin2*) were increased, that of carnitine palmitoyltransferase I *a* (*Cpt1a*) was not affected, and those of acetyl-Co A carboxylase α (*Acca*) and fatty acid synthase (*Fasn*) were decreased. Furthermore, using immunofluorescence staining and flow cytometry analyses, we found that VPA also induced peroxisome proliferator-activated receptor γ (PPARγ) nuclear translocation and increased levels of cell-surface CD36. Based on these results, we propose that VPA may enhance OLA-induced hepatocyte steatosis through the upregulation of PPARγ- and CD36-dependent lipid uptake, TAG synthesis, and lipid droplet formation.

1. Introduction

Valproic acid (2-propylpentanoic acid, VPA), a chemically synthesized small compound, has been one of the most widely used antiepilepsy drugs over the past 40 years. It is also commonly prescribed for patients with bipolar disorder, neuropathic pain, migraine headache, and schizophrenia. However, clinical follow-up studies have reported that more than 40% of patients who received VPA also developed unexpected obesity and fatty liver disease [1–3]. Previous studies have proposed the involvement of downregulated mitochondrial β-oxidation, accumulation of VPA metabolites (4-ene-VPA and 2,4-diene-VPA), and carnitine deprivation in VPA-induced liver injury [4, 5]. Furthermore, animal studies have demonstrated that VPA alters hepatic triacylglycerol (TAG) and cholesterol biosynthesis, fatty acid catabolism, and lipid transport-related gene expression patterns [6, 7]. However, how VPA affects hepatic lipid metabolism to induce fatty liver remains largely unknown.

Hepatic steatosis, also known as fatty liver, may be caused by imbalanced lipid metabolism, including lipid uptake/secretion, *de novo* lipogenesis, and fatty acid oxidation [8]. According to previous studies, of the TAG stored in steatotic hepatocytes, 59% were formed from circulating free fatty acids, 26% from *de novo* lipogenesis, and 15% from dietary chylomicron remnants [9, 10]. These data emphasize the importance of lipid transport regulation on fatty liver development and provide critical clues regarding VPA-induced hepatic steatosis. Although it is believed that fatty acids are able to enter cells via passive diffusion, carrier-mediated fatty acid uptake has also been proposed previously

[11, 12]. Fatty acid translocase (FAT)/CD36 is a membrane-associated multifunctional receptor which has two transmembrane segments and an extracellular glycosylated loop. CD36 regulates lipid uptake in different cells via binding to different ligands including long-chain fatty acids [13], and native/oxidized lipoproteins [14–16]. Importantly, a recent study showed that increased hepatic CD36 expression is highly correlated with human insulin resistance and nonalcoholic steatohepatitis (NASH) development [17].

On the other hand, the nuclear transcription factor peroxisome proliferator-activated receptor γ (PPARγ), which is a pivotal regulator of lipid metabolism, may also play a critical role in VPA-induced fatty liver disease. PPARγ, along with the liver X receptor (LXR) and pregnane X receptor (PXR), was found to be an upstream regulator of CD36; the presence of PPARγ, LXR, and PXR binding sites in the *Cd36* promoter was previously identified [18]. In addition, PPARγ was also demonstrated to be a major mediator of oleic acid- (OLA-) induced hepatic lipogenesis and lipid droplet formation [19] and found to be highly expressed in high-fat diet-fed mice [20]. Based on these studies, we investigated the involvement of PPARγ- and CD36-regulated lipid metabolism in VPA-induced hepatic steatosis.

2. Materials and Methods

2.1. Chemicals. Valproic acid sodium salt, Nile Red, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT), F12K medium powder, Hoechst 33342, dimethyl sulfoxide (DMSO), and sodium oleate were purchased from Sigma-Aldrich. Fatty acid-free bovine serum albumin was purchased from Merck.

2.2. Cell Culture. FL83B cells were obtained from the Taiwan Bioresource Collection and Research Center (BCRC number: 60325) and cultured in F12K medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco) at 37°C in a humidified atmosphere with 5% CO_2. To make the stock medium containing 4 mM OLA, sodium oleate was conjugated to BSA at a molar ratio of 6 : 1 in F12K medium. For VPA stock preparation, VPA was dissolved in distilled water. All compounds were sterilized by filtration.

2.3. Intracellular Lipid Staining and Quantification. To determine intracellular lipid accumulation, a Nile Red and Hoechst 33342 double staining protocol was applied. Briefly, cells were seeded on either a 0.17 mm coverslip or a black 96-well plate (Nunc) and incubated overnight to allow adherence. After 24-hour OLA and VPA treatment, cells were washed with PBS and then fixed with 4% paraformaldehyde for 10 min. After fixing, cells were washed again with PBS and then stained with Nile Red (1 μg/mL) and Hoechst 33342 (2 μg/mL) in the dark for 30 min. For microscopy imaging, cells were washed with PBS and then mounted with ProLong Gold Antifade Mountant (Molecular Probes). Images were captured using an Olympus IX-70 inverted microscope. For quantitative analyses, stained cells were washed with PBS, and the Nile Red (excitation at 485 nm, emission at 535 nm) and Hoechst 33342 (excitation at 350 nm, emission at 461 nm) fluorescent signals were quantified using a Biotek H1 Hybrid Microplate Reader.

2.4. Cell Viability Assay. The effects of OLA and VPA on cell viability changes were analyzed by the MTT assay. Briefly, cells were seeded in 96-well plates (Nunc) and allowed to adhere overnight before treatment. After 24-hour treatment, the medium was replaced with fresh medium containing MTT (5 mg/mL dissolved in PBS for stock solution, diluted 1 : 10 with normal medium for assay). After a 1.5-hour incubation at 37°C, the MTT solution was removed, and then 200 μL of DMSO was added to each well to dissolve the insoluble formazan. Cell viability was determined by measuring OD values at 570 nm (reference at 690 nm) using a BioTek μQuant microplate spectrophotometer.

2.5. qPCR Analyses. For qPCR analyses, total RNA were extracted from cells using the TRIzol Reagent (Invitrogen) following the recommended protocol provided by the manufacturer. After total RNA extraction, first-strand cDNA was synthesized using the Tetro Reverse Transcription kit (Bioline) with a mixture of oligo $(dT)_{20}$ and random hexamer primers. cDNA samples were then quantified and diluted into the same concentrations for subsequent qPCR assays. To determine mRNA expression changes, the relative quantification ($\Delta\Delta C_T$) method was performed using the StepOne Real-Time PCR System with Fast SYBR Green Master Mix reagent (Applied Biosystems) and custom-designed gene-specific primer pairs (Table 1). In our study, all gene expression levels were normalized with that of 18S rRNA as the internal control.

2.6. Flow Cytometry. To investigate the effects of VPA on cell-surface CD36 expression levels, flow cytometry analyses were conducted. Treated cells on 10 cm dishes (Nunc) were harvested by trypsinization and centrifugation. Cells were washed in ice-cold PBS and resuspended in FACS buffer (2% FBS and 0.1% NaN_3 in PBS). Mouse IgA isotype control antibody (GTX35045, GeneTex) or anti-CD36 mouse monoclonal IgA antibody (sc-13572, Santa Cruz Biotechnology) was added and incubated on ice for 30 min. After primary antibody labeling, cells were washed once with FACS buffer and then incubated with DyLight 650-conjugated goat-anti-mouse IgA antibody (Abcam) in the dark for another 30 min on ice. After secondary antibody labeling, cells were washed again in FACS buffer, filtered with Cell Strainer Cap (BD Biosciences), and then analyzed on LSRFortessa cell analyzer (BD Biosciences) with BD FACSDiva software.

2.7. Western Blotting. For Western blotting, protein from cultured FL83B cells was extracted using RIPA buffer, followed by BCA protein (Pierce) quantification. Samples were then mixed with Laemmli buffer and boiled at 95°C for 10 min. Denatured protein samples were then separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad) using standard protocol. After transfer, membranes were incubated with specific antibodies at 4°C overnight, then washed with

Table 1: Sequences of primer pairs used in study.

Gene	Forward/reverse primer sequences (5′ to 3′)	Reference
18S rRNA	CGGACAGGATTGACAGATTG CAAATCGCTCCACCAACTAA	NT039649
Cpt1a	CTGAGCCATGAAGCCCTCAA CACACCCACCACCACGATAA	NM013495
Acca	GGAGCTAAACCAGCACTCCC GGCCAAACCATCCTGTAAGC	NM133360
Fasn	TAGAGCAGGACAAGCCCAAG GAGGCGTAGTAGACAGTGCAGAG	NM007988
Scd1	GGCCTGTACGGGATCATACTG CAGAGCGCTGGTCATGTAGTA	NM009127
Acsl1	AGCACCGTACACTGGAGGAA AGGAAAACCTCTGGTCCACTG	NM007981
Gpat	ATGAAACGCACACAAGGCAC CCCTTATGGACGTCTCGCTC	NM008149
Dgat1	TTGACCTCAGCCTTCTTC CATTGCCATAGTTCCCTTG	NM010046
Dgat2	GCTGGCATTTGACTGGAACA GCCACACGGCCCAGTTT	NM026384
Plin2	GAACAGTGGAGTAGATAATG TGAGAGCCTGGTGATAAG	NM007408
Cd36	TTACACATACAGAGTTCGTTATC TCCAACAGACAGTGAAGG	NM001159558
Lrp	GACCGACTGGCGAACAAAT CTGGGTGTTGGTCCTCTGTA	Degrace et al. [21]
Ldlr	CTGTGGGCTCCATAGGCTATCT GCGGTCCAGGGTCATCTTC	Lelliott et al. [22]
Mttp	ATTGAGCGGTCTGGATTTACAAC AGGTAGTGACAGATGTGGCTTTTG	Lelliott et al. [22]
Pparg	CGGGCTGAGGAGAAGTCACA GTCTGTCACACAGTCCTGTCA	NM001127330

TBST, and incubated with HRP conjugated secondary antibodies for 1 hour at room temperature. For signal developing, ECL select reagent (GE healthcare) and ChemiDoc™ Touch Imaging System (Bio-Rad) were used. Densitometry analyses were performed using Image J software (National Institutes of Health).

2.8. Immunofluorescence Staining. In this study, an immunofluorescence staining technique was conducted to investigate the PPARγ nuclear translocation state. Briefly, cells were seeded, treated, and fixed as mentioned in the lipid staining section and then permeabilized with 0.25% Triton X-100 in PBS for 2 min at room temperature. After permeabilization, cells were washed in PBS, blocked with 1% BSA and 0.3 M glycine in PBS for 30 min at room temperature, and then stained with anti-PPARγ antibody (H-100, Santa Cruz Biotechnology) overnight at 4°C. On the next day, cells were washed three times with PBS for 5 min each to remove excessive primary antibodies. After the wash, Hoechst 33342- (2 μg/mL) and DyLight 488-conjugated goat-anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories) were added and incubated in the dark for 1 hour at RT. After fluorescent labeling, slides were washed, mounted, and imaged under an Olympus IX-70 microscope as described before.

2.9. Statistical Analysis. Statistical analyses were conducted using SigmaPlot software (Version 12.0, Systat Software). All values were expressed as means ± SEM (the number of biological replicates in each experiment is indicated in the figure legend). The fluorescence intensity and mRNA/protein expression data were analyzed by one-way and two-way ANOVA, followed by Duncan's multiple comparison. $P < 0.05$ was considered a significant difference.

3. Results

3.1. VPA Enhances Oleic Acid-Induced Lipid Accumulation and Causes Lipotoxicity in FL83B Cells. To address the effects of VPA on hepatic steatosis, we developed an *in vitro* hepatic steatosis model using the mouse FL83B cell line and a Nile Red lipid staining technique. FL83B is a noncancer hepatocyte cell line originated from fetal C57BL/6

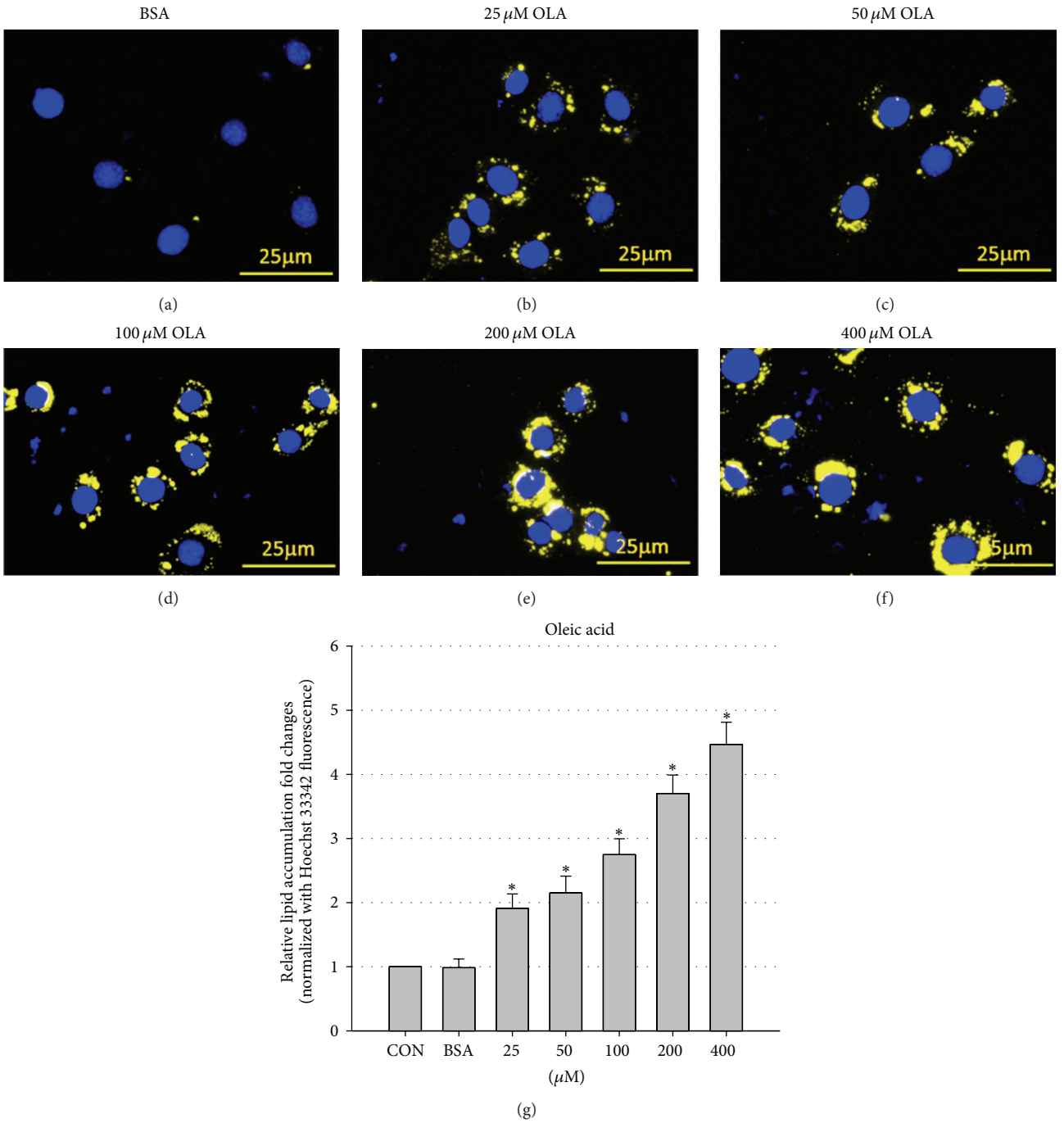

Figure 1: OLA induces lipid accumulation in FL83B cells in a dose-dependent manner. Microscopic images of FL83B cells treated with BSA only (a), or 25 μM (b), 50 μM (c), 100 μM (d), 200 μM (e), and 400 μM (f) BSA-conjugated OLA for 24 hours. Cells were fixed and stained with Hoechst 33342 (blue, nucleus) and Nile Red (yellow, intracellular lipid droplets). Quantification of intracellular lipid accumulation was performed using a fluorescent microplate reader (g). Values are means ± SEM of four independent experiments. $*$ above the bars refer to significant differences ($P < 0.05$). CON: control, treated with normal culture medium only.

mice [23], the most widely used strain for studies of fatty liver, obesity, and many other metabolic diseases. Using Nile Red and Hoechst 33342 double staining (Nile Red stains intracellular neutral lipids, while Hoechst 33342 stains the nucleus for cell number normalization), we found that 24-hour OLA treatment can induce lipid droplet formation and neutral lipid accumulation in a dose-dependent manner in FL83B cells (Figure 1). Next, we evaluated the steatosis-promoting effects of VPA using the same staining method. Our results showed that although treatment with VPA alone did not induce significant lipid accumulation in FL83B cells (Figure 2(a)), VPA enhanced 100 μM OLA-induced steatosis

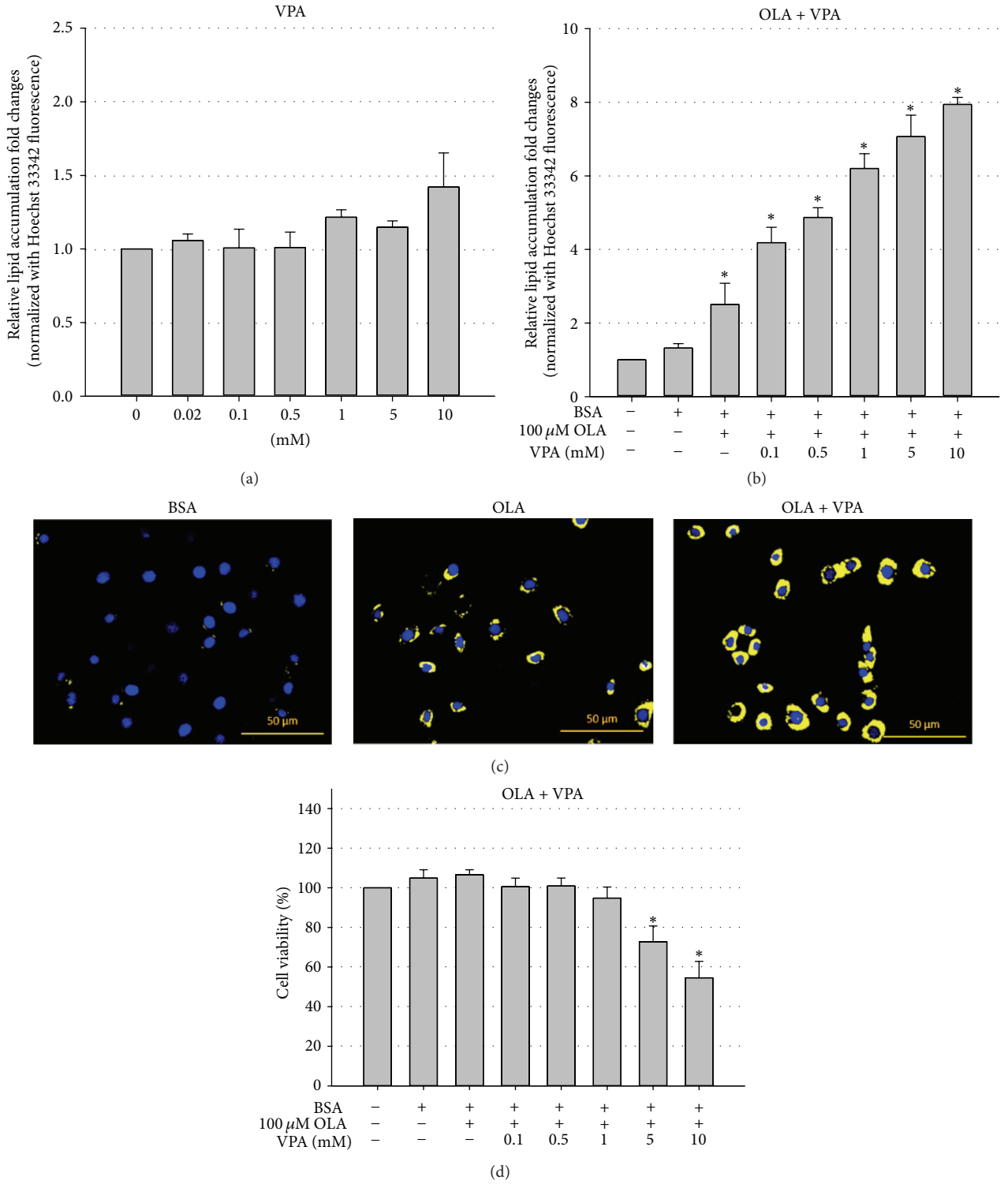

Figure 2: VPA enhances OLA-induced lipid accumulation in FL83B cells and induces cytotoxicity. Nile Red and Hoechst 33342 double staining showed that 24-hour VPA single treatment (a) did not significantly induce steatosis but enhanced OLA- (100 μM) induced intracellular neutral lipid accumulation (b) and lipid droplet formation (OLA = 100 μM, VPA = 1 mM) (c) in a dose-dependent manner in FL83B cells. MTT cell viability results also indicate high doses (5 and 10 mM) of VPA-induced lipotoxicity in FL83B cells (d). Values are means ± SEM of four independent experiments. $*$ above the bars refer to significant differences ($P < 0.05$).

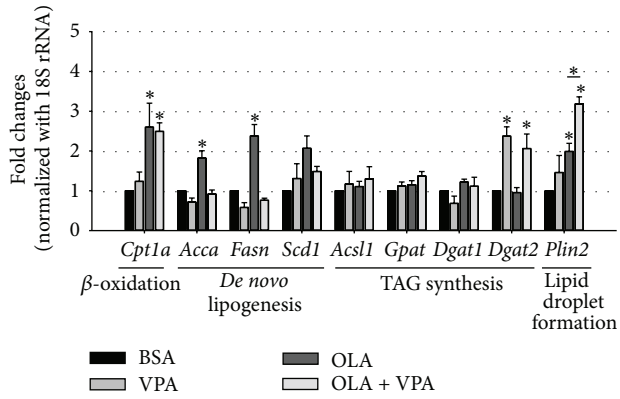

Figure 3: Regulation of expression of lipid metabolism-related genes by OLA and VPA. The mRNA levels of lipid catabolism/anabolism-related genes including *Cpt1*, *Acca*, *Fasn*, *Scd1*, *Acsl1*, *Gpat*, *Dgat1*, *Dgat2*, and *Plin2* in FL83B cells treated with BSA, 100 μM OLA, 1 mM VPA, or 100 μM OLA plus 1 mM VPA for 24 hours were analyzed using real-time PCR. Relative fold changes were calculated using Ct values obtained from three independent experiments and are shown as means ± SEM. $*$ above the bars refer to significant differences ($P < 0.05$).

in a dose-dependent manner (tested doses ranged from 0.1 to 10 mM) in FL83B cells (Figures 2(b) and 2(c)). More importantly, we demonstrated that high-dose (5 and 10 mM) VPA single (data not shown) or cotreatment also induces significant cytotoxicity (Figure 2(d)), which may contribute to NASH development.

3.2. Effects of VPA on the Expression of Lipid Metabolic Genes. In order to investigate the molecular mechanisms of VPA-enhanced hepatic steatosis, we assayed the expression patterns of lipid metabolic genes using real-time PCR. Our results suggest that VPA enhances hepatic steatosis possibly by increasing TAG synthesis and lipid droplet formation, but not by upregulating *de novo* lipogenesis or decreasing fatty acid oxidation. As shown in Figure 3, VPA cotreatment did not inhibit the OLA-induced expression of *Cpt1a*, which encodes the rate-limiting enzyme carnitine palmitoyltransferase I, controlling hepatic mitochondrial β-oxidation. Furthermore, we found that VPA did not increase but rather decreased the expression of *de novo* lipogenesis genes including *Acca* (acetyl-CoA carboxylase α, catalyzing the conversion of malonyl-CoA from acetyl-CoA, the rate-limiting step of fatty acid synthesis), *Fasn* (fatty acid synthase, synthesizing palmitic acid using acetyl-CoA and malonyl-CoA as substrates), and *Scd1* (stearoyl-CoA desaturase-1, the key enzyme involved in monounsaturated fatty acid production via introducing double bonds into palmitoyl-CoA and stearoyl-CoA).

However, when assaying TAG synthesis genes including *Acsl1* (acyl-CoA synthetase long-chain family member 1), *Gpat* (glycerol-3-phosphate acyltransferase), *Dgat1* (diacylglycerol acyltransferase 1), and *Dgat2* (diacylglycerol acyltransferase 2), we found significantly increased *Dgat2* expression following treatment with VPA alone and in combination with OLA (Figure 3). In addition, we found that VPA also enhanced the expression of *Plin2* (perilipin 2), which encodes a lipid droplet membrane-associated protein (Figure 3). These data suggest that VPA may enhance OLA-induced hepatic steatosis by increasing TAG synthesis and lipid droplet formation.

3.3. Effects of VPA on Lipid Transport Genes. In addition to lipid anabolic and catabolic genes, we also evaluated the regulation of lipid transport genes using real-time PCR. Among the four genes we assayed including *Cd36* (cluster of differentiation 36), *Lrp1* (low-density lipoprotein receptor-related protein 1), *Ldlr* (low-density lipoprotein receptor), and *Mttp* (microsomal triacylglycerol transfer protein), we found that VPA treatment profoundly increased the mRNA levels of *Cd36* and *Lrp*, which facilitate the import of long-chain fatty acids and VLDL (very low-density lipoproteins), respectively (Figure 4(a)). On the other hand, another VLDL uptake receptor gene, *Ldlr,* showed slightly decreased expression, and the lipid export protein gene *Mttp* remained unchanged under VPA treatment (Figure 4(a)). These data suggest the involvement of increased lipid uptake in VPA-enhanced hepatic steatosis, especially CD36-mediated fatty acid uptake, because its mRNA level was extraordinarily upregulated.

To further confirm the importance of CD36 in VPA-induced hepatic steatosis, we analyzed cell-surface CD36 levels using flow cytometry analyses. Consistent with the real-time PCR results, we found that cell-surface CD36 levels were significantly increased under VPA treatment (Figures 4(b) and 4(c)), indicating that VPA may increase lipid uptake in FL83B cells by elevating CD36 expression and translocation to the cell membrane.

3.4. VPA Increased OA Induced PPARγ Protein Expression and Nuclear Translocation. Finally, we also addressed the role of the nuclear transcription factor PPARγ in VPA-induced steatosis. Because PPARγ was previously reported to upregulate the expression of *Plin2*, *Cd36*, and many other steatotic genes in liver [18, 24], we evaluated the mRNA, protein expression levels, and protein nuclear translocation of PPARγ in FL83B cells under VPA treatment. As shown in Figure 5, we found that although the mRNA levels of *Pparg* were not altered by either VPA or OLA treatment (Figure 5(a)), VPA did increase OA induced PPARγ protein expression (Figure 5(b)) and nuclear translocation (Figure 5(c)). Using the immunofluorescence staining technique, we found that VPA on its own triggered and also enhanced OLA-induced PPARγ nuclear translocation. Our data suggests that PPARγ may be an upstream regulator via which VPA induces hepatic steatosis.

4. Discussion

VPA is currently one of the most widely used antiepilepsy drugs in the world. However, according to the clinical statistics, a high prevalence of fatty liver diseases induced by unknown mechanisms is observed in VPA-treated patients

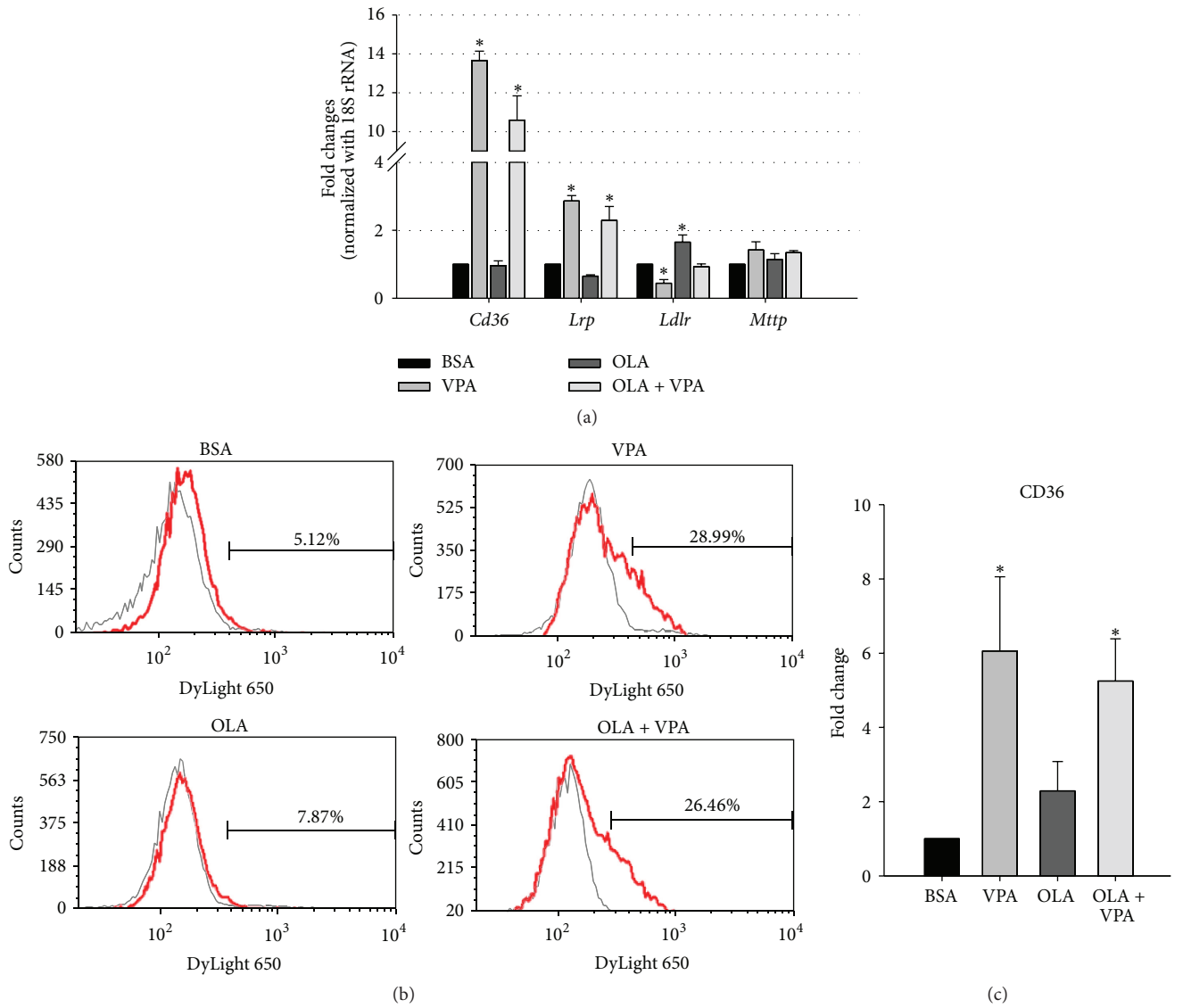

FIGURE 4: VPA increases the mRNA and cell-surface protein expression levels of fatty acid translocase CD36. The mRNA expression levels of lipid transport-related genes including *Cd36*, *Lrp*, *Ldlr*, and *Mttp* were analyzed using real-time PCR (a). Relative fold changes were calculated using Ct values obtained from three independent experiments and are shown as means ± SEM. ∗ above the bars refer to significant differences ($P < 0.05$). The Fl83B cell-surface CD36 expression levels were analyzed by flow cytometry using mouse anti-CD36 antibody and DyLight 650-conjugated secondary antibody, and representative histograms (b) and quantitative flow cytometry data (c) are shown. ∗ above the bars refer to significant differences ($P < 0.05$). For background fluorescent subtraction, mouse isotype IgG antibody was used (OLA = 100 μM, VPA = 1 mM, 24 hours).

[1–3]. Therefore, we developed a highly reproducible *in vitro* cell model to investigate the possible mechanisms of VPA-induced hepatic steatosis. In this study, we used FL83B, a noncancer hepatocyte cell line derived from fetal C57BL/6 mice, which presents similar morphology and physiological functions to those of normal hepatocytes [23]. Since FL83B is derived from C57BL/6 mice, the most commonly used strain in studies of obesity, fatty liver, and many other metabolic diseases, this model may easily be used to interpret *in vivo* findings.

To induce steatosis in FL83B cells, we treated the cells with BSA-conjugated OLA and analyzed the steatotic levels using Nile Red and Hoechst 33342 double staining. According to Greenspan et al., when excited by light of wavelength 450–500 nm, the lipophilic fluorescent dye Nile Red incorporated in neutral lipids emits strong yellow-gold fluorescence with wavelengths greater than 528 nm [25]. To further increase the accuracy of Nile Red lipid staining results, we also used Hoechst 33342 nuclear staining in our system for cell number normalization. Via this, we can readily image and quantify intracellular neutral lipid-rich droplets using fluorescent microscopy and a microplate reader, respectively. As shown in Figure 1, we confirmed that this staining method successfully detects OLA-induced lipid accumulation in FL83B cells.

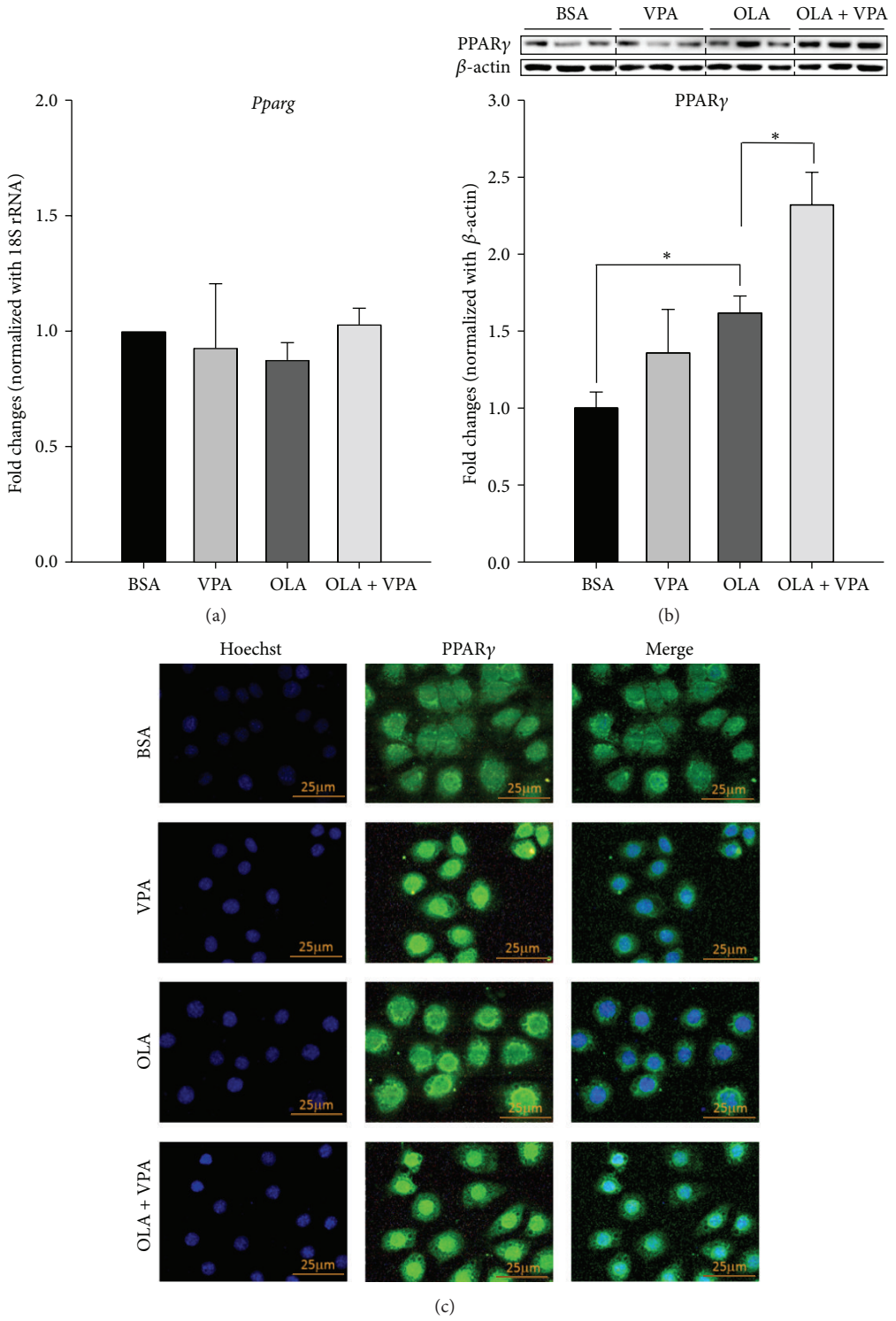

Figure 5: VPA enhances oleic acids increased PPARγ protein expression and nuclear translocation, but not the mRNA levels. Real-time PCR (a), Western blotting (b), and immunofluorescence staining (c) were conducted following 24-hour treatment with BSA, VPA (1 mM), OLA (100 μM), or OLA plus VPA. For real-time PCR, relative fold changes were calculated using Ct values obtained from three independent experiments and are shown as means ± SEM. $*$ above the bars refer to significant differences ($P < 0.05$). Densitometric analyses for Western blotting were conducted for sample sets obtained from three independent experiments, and results are shown as means ± SEM. $*$ above the bars refer to significant differences ($P < 0.05$). In the immunofluorescence staining images, nuclear and intracellular PPARγ proteins were stained by Hoechst 33342- (blue) and DyLight 488-conjugated antibody (green), respectively.

Next, we used this cell model to evaluate whether VPA has direct effects on hepatocyte steatosis. According to the results shown in Figure 2, we found that treatment with VPA alone at doses ranging from 0.02 to 10 mM did not significantly induce steatosis in FL83B cells (Figure 2). However, when cells were cotreated with 100 μM OLA, VPA significantly enhanced OLA-induced lipid accumulation in a dose-dependent manner (Figures 2(b) and 2(c)). More importantly, we found that VPA not only enhanced fatty acid-induced hepatocyte steatosis, but also caused cell death (Figure 2(c)). These results are consistent with previous *in vivo* and clinical findings: a study by Zhang et al. revealed that low-dose VPA treatment did not induce profound hepatic steatosis and liver injury in rats. However when rats were fed a high-fat diet for 8 weeks, VPA cotreatment significantly increased hepatic steatosis and liver cell death [26]. Interestingly, although VPA treatment *per se* was found to induce body weight gain and fatty liver, clinical data also revealed that VPA-induced fatty liver was more significant in patients with higher body weight [2].

Since VPA only enhanced OLA-induced lipid accumulation in FL83B cells but did not induce steatosis in the absence of OLA, we hypothesized that VPA may induce hepatic steatosis via increasing lipid uptake, neutral lipid synthesis, and lipid droplet formation, rather than by downregulating beta-oxidation or upregulating *de novo* lipogenesis in hepatocytes. Consistent with this hypothesis, we found that VPA significantly upregulates lipid uptake, TAG synthesis, and lipid droplet formation, but not the expression profiles of genes involved in *de novo* lipogenesis and beta-oxidation. Using real-time PCR, we found that VPA did not alter the mRNA levels of *Cpt1a*. Furthermore, VPA did not increase the mRNA expression of *Scd1* and even decreased the OLA-induced expression of *Acca* and *Fasn* (Figure 3). These data indicate that VPA may not directly induce lipid accumulation in hepatocytes via increasing *de novo* lipogenesis or downregulating beta-oxidation. However, when assaying the mRNA expression levels of *Acsl1*, *Gpat*, *Dgat1,* and *Dgat2*, which encode the key enzymes in TAG synthesis, we found that VPA significantly increased the level of *Dgat2*. Furthermore, we found that VPA also remarkably enhanced OLA-induced PLIN2 mRNA expression, which is important for OLA-induced lipid droplet formation [27]. Besides, it is worth noting here that although VPA treatment alone induced *Dgat2* expression, lipid accumulation only increased when cells were cotreated with OLA, indicating the importance of fatty acid uptake in VPA-induced hepatic steatosis (Figure 3).

Therefore, we next assayed expression changes in lipid uptake/export related genes including *Cd36*, *Lrp*, *Ldlr*, and *Mttp*. As shown in Figure 4(a), we found that although VPA treatment slightly decreased *Ldlr* expression, it significantly increased *Lrp* expression both alone and in combination with OLA treatment. The decrease in *Ldlr* expression here may be compensated by the increased *Lrp* expression, because these molecules were found to mediate the intake of similar lipoproteins into liver [28]. On the other hand, no changes were observed in the expression of the TAG export protein gene *Mttp* in all treatment groups. Interestingly, we found that VPA significantly increased the mRNA expression of CD36 more than 10-fold, both alone and in combination with OLA treatment. CD36 is a cell membrane-bound protein, which plays an important role in the cellular uptake of long-chain fatty acids, native lipoproteins, and oxidized LDL [15, 21, 29, 30]. Previous studies have shown decreased liver TAG content and increased plasma free fatty acid levels in CD36 knockout mice, indicating the significance of CD36 in the control of hepatic lipid transportation balance [31]. More importantly, using a flow cytometry assay, we found that VPA not only increased CD36 mRNA levels, but also enhanced cell-surface CD36 protein expression (Figures 4(b) and 4(c)).

Because the expression of CD36, DGAT2, and PLIN2 in liver is previously known to be regulated by PPARγ [18, 32, 33], a crucial nuclear transcription factor controlling cellular glucose and lipid metabolism, we also investigated whether PPARγ is involved in VPA-induced hepatic steatosis. Upon ligand binding and activation, PPARγ translocates to the nucleus, forms heterodimers with the retinoid X receptor (RXR), and binds to DNA peroxisome proliferator hormone response element (PPRE) regions to regulate downstream gene expression levels. PPARγ is highly expressed in adipocytes, acting as an important regulator of adipogenesis [34]. Although its expression level in liver is lower than in adipose tissue, previous studies have shown that PPARγ regulates hepatic lipid accumulation; high-fat diet-fed mice exhibited significant elevation of PPARγ expression [20, 24, 35, 36]. Our real-time PCR, Western blotting, and immunofluorescence staining results revealed that VPA enhanced OLA increased PPARγ protein expression and nuclear translocation, but not the mRNA levels (Figure 5). Importantly, these data also indicated that the regulating effects of VPA on PPARγ may rely on translation or protein degradation regulations, instead of transcriptional modulations.

5. Conclusions

In conclusion, our data indicate that VPA may enhance hepatic steatosis via increasing the protein expression and transcriptional activity of PPARγ, as well as by inducing the expression of downstream genes including *Cd36*, *Dgat2*, and *Plin2* to increase lipid uptake, TAG synthesis, and lipid droplet formation in hepatocytes.

Conflict of Interests

The authors declare that there is no conflict of interests in this study.

Authors' Contribution

Renin Chang and Mei-Chia Chou contributed equally to this paper.

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