Functional Proliferating Human Hepatocytes: In Vitro Hepatocyte Model for Drug Metabolism, Excretion, and Toxicity

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ABSTRACT

To develop a functional alternative hepatocyte model for primary human hepatocytes (PHHs) with proliferative property, essential drug metabolic, and transporter functions, proliferating human hepatocytes (ProliHHs) expanded from PHHs were fully characterized in vitro. Herein, ProliHHs generated from multiple PHHs donors could be expanded more than 200-fold within four passages and maintained their metabolic or transporter capacities partially. Furthermore, ProliHHs were able to regain the mature hepatic property after three-dimensional (3D) culture. Particularly, the downregulated mRNA expression and function of three major cytochrome P450 (P450) enzymes in ProliHHs-P could be recovered by 3D culture. The metabolic variabilities across different PHHs donors could be inherited to their matured ProliHHs (ProliHHs-M). The intrinsinc clearances of seven major P450 enzymes in ProliHHs-M correlated well (r = 0.87) with those in PHHs. Also, bile canaliculi structures could be observed in sandwich-cultured ProliHHs (SC-ProliHHs), and the biliary excretion index of four probe compounds [cholyl-lys-dichlorofluorescein, 5-(and-6)-carboxy-2’’, 7’’-dichlorofluorescein diacetate (CDF), deuterium-labeled sodium taurocholate acid, and rosuvastatin] in SC-ProliHHs (>10%) were close to sandwich-cultured PHHs. More importantly, both ProliHHs-P and ProliHHs-M could be used to evaluate hepatotoxicity. Therefore, these findings demonstrated that the 3D and sandwich culture system could be used to recover the metabolic and transporter functions in ProliHHs for clearance prediction and cholestasis risk assessment, respectively. Together, ProliHHs could be a promising substitute for PHHs in drug metabolism, transport, and hepatotoxicity screening.

SIGNIFICANCE STATEMENT

This report describes the study of drug metabolic capacities, efflux transporter functions, and toxicity assessments of proliferating human hepatocytes (ProliHHs). The metabolic variabilities in different primary human hepatocyte donors could be inherited by their matured ProliHHs derivatives. Also, ProliHHs could form canicalicular networks in sandwich culture and display biliary excretion capacities. More importantly, both the proliferative and maturation statuses of ProliHHs could be used to evaluate hepatotoxicity. Together, ProliHHs were feasible to support drug candidate screening in hepatic metabolism, disposition, and toxicity.

Introduction

Assessments of drug metabolism, transport, and hepatotoxicity are essential parts of early drug discovery (Godoy et al., 2013; Zhang et al., 2016). Primary human hepatocytes (PHHs) have been widely recognized as the gold standard for predicting drug clearance and hepatotoxicity (Gómez-Lechón et al., 2003, 2004; Hewitt et al., 2007). However, the loss of character, including proliferative properties and metabolic and transported functions of PHHs after in vitro culture, limits their applications (Gómez-Lechón et al., 2003; O’Brien et al., 2006). Moreover, it is time consuming and costly to determine the variabilities of drug-metabolizing enzymes (DMEs) and transporters from different PHHs batches (Donato et al., 2013). Therefore, the establishment of a functional substitutable hepatocyte model is an important issue.

Significant efforts have been made in the past decade. In addition to PHHs, embryonic stem cell–derived, induced pluripotent stem cell–derived, or epigenetic reprogramming–induced hepatocyte technology was developed to solve the lack of donors. However, a better differentiation protocol was still missing to generate physiologically relevant hepatocytes (Brolén et al., 2010; Si-Tayeb et al., 2010; Ogawa et al., 2013; Huang et al., 2014). Also, there are several investigations

ABBREVIATIONS: AAFE, absolute average fold error; BEI, biliary excretion index; BSEP, bile salt export pump; CDF, 5-(and-6)-carboxy-2’’, 7’’-dichlorofluorescein diacetate; CL, cholesteryl-lys-fluorescein; CLint, intrinsic clearance; 2D, two-dimensional; 3D, three-dimensional; DILI, drug-induced liver injury; DME, drug-metabolizing enzyme; DMEM, Dulbecco’s modified Eagle’s medium; d8-TCA, deuterium-labeled sodium taurocholate acid; HBSS, Hank’s balanced salt solution; HIM, hepatic maturation medium; MRP2, multidrug-resistance-associated protein 2; P450, cytochrome P450; PHH, primary human hepatocyte; ProliHHs, proliferating human hepatocytes; ProliHHs-M, maturation status of ProliHHs; ProliHHs-P, proliferative status of ProliHHs; PS, penicillin-streptomycin; qPCR, quantitative polymerase chain reaction; SC-PHHS, sandwich-cultured PHHs; SC-ProliHHs, sandwich-cultured ProliHHs; SC-ProliHHs-M, sandwich-cultured ProliHHs-M; SC-ProliHHs-P, sandwich-cultured ProliHHs-P; T90, median toxic concentration; UHH, Upcyte human hepatocyte; WME, Williams’ medium E.
about reprogramming PHHs into hepatic progenitor cells to obtain cell proliferation features and maintain physiologically relevance, but these proliferative cells lose mature hepatic features quickly and have not been widely used in drug toxicity screening (Kim et al., 2018; Fu et al., 2019). Moreover, multiple reports on applications of HepaRG cells and Upcyte human hepatocytes (UHHS) in metabolic and toxicological evaluation were published (Andersson et al., 2012; Levy et al., 2015; Ramachandran et al., 2015). However, to the best of our knowledge, different clones, passages, and differentiation protocols in HepaRG cells may lead to poor repeatability (Petrov et al., 2018); the transcriptional levels of major cytochrome P450 (P450) enzymes in UHHS are still to be improved at donor-specific levels, and the efflux transporter functions for biliary excretion were not reported (Levy et al., 2015; Schaefer et al., 2016). Furthermore, genetic manipulation by overexpressing human papillomavirus genes (E6 and E7) or other immortal genes is not the best way to obtain expanded hepatocytes with genomic instability (Liang and Zhang, 2013; Ma et al., 2014).

Recently, the development of dedifferentiation strategy, a breakthrough in developing an in vitro surrogate hepatocyte model for PHHs, made it feasible for PHHs to expand as proliferating human hepatocytes (ProliHHs), which can be matured in a three-dimensional (3D) culture environment (Zhang et al., 2018). Unlike other dedifferentiation strategies for PHHs, ProliHHs are biphenotypic, partially maintaining mature hepatocyte features and gaining expression of progenitor-associated genes. Compared with human pluripotent stem cell–derived hepatocytes and human induced hepatocytes, ProliHHs show promising potential for in vitro drug safety assessment because of expression of transporter and activity of CYP2B6 (Zhang et al., 2018). However, it is still unclear whether ProliHHs can feasibly predict drug hepatic clearance, biliary excretion, and hepatotoxicity in vitro.

In this study, we generated ProliHHs from different PHHs donors. Three major DMEs’ expression and function in ProliHHs were systematically characterized, and the relevance of seven major DMEs was then investigated between the maturation status of ProliHHs (ProliHHs-M) and PHHs. After sandwich culture, cell polarization and efflux transporter function were identified in ProliHHs. Also, cytotoxicity induced by selected compounds was tested in ProliHHs. Together, these results suggested that ProliHHs can be an alternative hepatocyte model for PHHs in drug metabolism, transport, and hepatotoxicity screening.

Materials and Methods

Chemicals and Reagents. Phenacetin, buprophen, testosterone, chlorozoxazon, diclofenac acid, S-mephenytoin, dextromethorphan, dexamethasone, N-acetylcysteine, human [Leu15]-gastrin I, penicillin-streptomycin (100×), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, chlortetracycline, ibuprofen, lithocholic acid, chenodeoxycholic acid, chlorpromazine, cyclosporin A, rifampin, troglitazone, amiodarone, imipramine, isoniazid, valproic acid, rosuvastatin, A83-01, Wnt3a, and 7. 3-[4-(3-chloro-phenox)-phenyl]-propanoic acid. 

Three major DMEs (CYP1A2, CYP2C9, and CYP3A4) in ProliHHs were measured in a P450 metabolic assay or sandwich culture.

The Sandalwood Culture of ProliHHs. For sandwich culture, trypsinized ProliHHs (3 × 10^5 cells/well) from proliferative status were seeded onto collagen I–coated 24-well plates. ProliHHs were incubated overnight, and HIM was renewed. Attached ProliHHs were overlaid with 0.25 mg/ml Matrigel in HIM and incubated under normoxia condition (5% CO2 incubator). A 50% HIM was then exchanged daily to allow further maturation for about 7–10 days. These ProliHHs-M were further used in P450 metabolic assay or sandwich culture.

The Derivation and Proliferation of ProliHHs from PHHs. Cryopreserved PHHs were firstly seeded at required density (5 × 10^5 cells/well) in a six-well plate, coated with collagen I and cultured under hypoxia conditions (5% O2, 5% CO2, 3%H2). PHHs were maintained in feeding medium with 5 μM forskolin, 1 μM dexamethasone, 20 ng/ml oncostatin M under normoxia condition (5% CO2 incubator). A 50% HIM was then exchanged daily to allow further maturation for about 7–10 days. These PHHs were incubated in 1 ml medium with probe substrates listed in Supplemental Table 3. The supernatants (100 μl/C2) were collected from 24-well plate at day 1. For ProliHHs-M, supernatants were collected from six-well plate when cells were attached to the plate at day 1. For ProliHHs-M, supernatants were collected from 24-well plate at day 0 (trypsinized from ProliHHs-M, day 2, and day 8. Human albumin ELISA Quantitation Set was purchased from Bethyl Laboratory (Montgomery, TX) and SYBR Green Master Mix (Yeasen) on Applied Biosystems 7500. Primers are listed in Supplemental Table 2.

Measurement of Human Albumin Levels. For ProliHHs-P and PHHs, supernatants were collected from six-well plate when cells were attached to the plate at day 1. For ProliHHs-M, supernatants were collected from 24-well plate at day 0 (trypsinized from ProliHHs-M, day 2, and day 8. Human albumin ELISA Quantitation Set was purchased from Bethyl Laboratory (Montgomery, TX) and SYBR Green Master Mix (Yeasen) on Applied Biosystems 7500. Primers are listed in Supplemental Table 2.

P450 Metabolic Capacity Determination. Clearance of probe compounds was determined at designated time points (day 7, passage 4 in ProliHHs-P and day 7 in ProliHHs-M). For measurement of activities of three major DMEs (CYP1A2, CYP2B6, and CYP3A4), ProliHHs-P (5 × 10^5 cells/well) and PHHs (1 × 10^5 cells/well) were seeded in a 24-well plate. For the measurement of seven metabolic activities of P450 enzymes, ProliHHs-M were seeded at 2.5 × 10^5 cells per well and cultured in a 24-well Ultra-Low Attachment plate in HIM for 7 days. ProliHHs and PHHs were incubated in 1 ml medium with probe substrates listed in Supplemental Table 3. The supernatants (100 μl) were collected from PHHs,
ProliHHs-P, and ProliHHs-M at 0, 1, 2, and 3 hours after administration of substrate. The metabolic assay was stopped by adding 300 μl ice-cold acetonitrile. The probe compounds were analyzed by LCMS-8030 (Shimadzu, Kyoto, Japan). Drug clearance was determined through parent compound disappearance rate as follows:

\[
\text{Elimination rate constant (k) = } (- \text{gradient})
\]

\[
\text{Half life (t½)(minutes) = } \frac{0.693}{k}
\]

\[
V(\mu l/million cells) = \frac{\text{Incubation volume(µl)}}{\text{Number of cells in incubation } \times 10^{6}}
\]

\[
\text{Intrinsic Clearance (CL}_{\text{intrinsic}}\text{(µl/min per million cells) = } \frac{0.693 \times V}{t_{1/2}}
\]

**Hepatocyte Polarization and Biliary Excretion Capacity Measurement**

**Assay.** ProliHHs-P, ProliHHs-M, and PHHs were seeded at required density (3.5 × 10^5 cells/well) in 24-well plates. The formation of bile canaliculi networks in polarized hepatocytes were visualized by CDFDA (Levy et al., 2015). Generally, 5 mM stock CDFDA solution 1:500 was diluted in culture medium and incubated at 37°C for 30 minutes in the dark to detect the hepatocyte polarization. To measure the efflux transporter activities of ProliHHs and PHHs, different transporter substrates were selected: CLF [bile salt efflux pump (BSEP)], CDF [multidrug resistance-associated protein 2 (MRP2)], d8-TCA (BSEP), and rosuvastatin (MRP2). Biliary excretion index (BEI) values were determined over a 15-minute interval after preincubation with warm HBSS with or without Ca^2+ (Liu et al., 1999). For PHHs, the BEI study was performed on day 5. The fluorescence intensity of CDF and CLF in sandwich-cultured ProliHHs or PHHs were measured at excitation/emission wavelengths of 495/529 and 492/536 nm, respectively. According to our previous report protocol, the concentration of d8-TCA and rosuvastatin in sandwich-cultured ProliHHs or PHHs was analyzed by LCMS-8030 (Shimadzu) (Pan et al., 2012; Guo et al., 2014).

The BEI value was calculated as follows:

\[
\text{BEI} = \left( \frac{A_{\text{min}}, \text{C}3-\text{H}2-\text{H}1}{A_{\text{max}}, \text{C}3-\text{H}2-\text{H}1} \right) \times 100\%
\]

A was the concentration or amount of test compound accumulated in the absence \((A_{\text{min}}, \text{C}3-\text{H}2-\text{H}1)\) or presence \((A_{\text{max}}, \text{C}3-\text{H}2-\text{H}1)\) of Ca^2+, which was achieved through adding warm Ca^2+-free or standard HBSS buffer (Wu et al., 2016).

**In Vitro Cytotoxicity Assay.** ProliHHs-P and PHHs were seeded at required density (3 × 10^4 cells/well) in collagen I-coated 96-well plates. The medium containing test compounds were replaced in each well after cells attached and then incubated 24 hours. For ProliHHs-M, the expression levels of CYP1A2, CYP2B6, and CYP3A4 in ProliHHs-P and found that the expression levels of these genes were downregulated dramatically with serial passage compared with PHHs (Fig. 2A; Supplemental Fig. 3). Similarly, a decrease of metabolic capacity was also observed in ProliHHs-P, but weak metabolic activities were still maintained at passage 4 (Fig. 2B), whereas after the 3D maturation (ProliHHs-M), the expressions and functions of these induced enzymes recovered significantly. The expression levels of CYP1A2, 2B6, and 3A4 in ProliHHs-M were significantly higher than ProliHHs-P but still less than PHHs (Fig. 2A). The intrinsic clearances of selected P450 substrates also increased significantly (Fig. 2B). The results suggested that ProliHHs-M could achieve the P450 metabolic capacities of their donor PHHs to some extent, especially for CYP1A2 and 3A4 (Fig. 2C). Together, these findings indicated that ProliHHs were able to improve the gene expression and metabolic activities of three major DMEs after 3D culture.

**The Metabolic Activities of P450 Enzymes in ProliHHs.** ProliHHs were assessed by gene expressions and probe substrate clearances of selected major DMEs. We first analyzed the expression levels of CYP1A2, CYP2B6, and CYP3A4 in ProliHHs-P and found that the expression levels of these genes were downregulated dramatically with serial passage compared with PHHs (Fig. 2A; Supplemental Fig. 3). Similarly, a decrease of metabolic capacity was also observed in ProliHHs-P, but weak metabolic activities were still maintained at passage 4 (Fig. 2B), whereas after the 3D maturation (ProliHHs-M), the expressions and functions of these indicated enzymes recovered significantly. The expression levels of CYP1A2, 2B6, and 3A4 in ProliHHs-M were significantly higher than ProliHHs-P but still less than PHHs (Fig. 2A). The intrinsic clearances of selected P450 substrates also increased significantly (Fig. 2B). The results suggested that ProliHHs-M could achieve the P450 metabolic capacities of their donor PHHs to some extent, especially for CYP1A2 and 3A4 (Fig. 2C). Together, these findings indicated that ProliHHs were able to improve the gene expression and metabolic activities of three major DMEs after 3D culture.
organoids) (Zhang et al., 2018). However, 3D organoids are not suitable for quantitative transporter studies. Therefore, a sandwich-culture model was employed to evaluate if ProliHHs could form canalicular networks after sandwich culture.

The sandwich-culture strategy is illustrated in Fig. 4A. Typically, ProliHHs-P or ProliHHs-M were trypsinized and then sandwich-cultured in 24-well plates. SC-ProliHHs, cultured for 5 days, were used as a positive control. After sandwich culture for 7 days, cell morphology, bile canalicular structure, and cell polarization of ProliHHs were investigated. Sandwich-cultured ProliHHs-M (SC-ProliHHs-M) exhibited good hepatocyte-like morphology, including cubical shape, tight junctions, and bile canalicular-like structures, whereas the hepatocyte-like cell appearance in sandwich-cultured ProliHHs-P (SC-ProliHHs-P) was not as obvious as in SC-ProliHHs-M (Fig. 4B). Both SC-ProliHHs-P and SC-ProliHHs-M were able to form bile canalicular networks, evidenced by fluorescent dye (Fig. 4C).

Furthermore, the expression levels of essential uptake/efflux transporters were investigated for cells derived from both ProliHHs systems. First, real-time qPCR results showed the expression levels of these transporters were downregulated significantly in ProliHHs-P but significantly upregulated in ProliHHs-M (Fig. 5A). To examine efflux transporter functions, such as BSEP and MRP2, sandwich-cultured cells were incubated with their substrates (CLF and d8-TCA for BSEP, CDF and rosuvastatin for MRP2). Although the expression levels of these genes in ProliHHs-P were rarely detected than PHHs, all the tested compounds showed biliary excretion trends in ProliHHs-P (BEI values 10%). For SC-ProliHHs-M, the BEI values of tested compounds were increased or even comparable to PHHs (Fig. 5B). Together, these findings suggested that ProliHHs in sandwich-culture could maintain polarity, form bile canalicular networks, and display biliary excretion capacity.

Hepatotoxicity Assessment in ProliHHs. To further investigate whether ProliHHs can be used to evaluate drug-induced hepatotoxicity,
12 compounds were selected to perform 24-hour acute toxicity assessment. The TC\(_{50}\) values of three compounds (rifampicin, isoniazid, and valproic acid) in PHHs were from reported literature (Supplemental Table 4). Both ProliHHs-P and ProliHHs-M responded to these drugs in 24 hours and displayed good correlation with PHHs (Fig. 6, A and B). Compared with PHHs, the 2D culture system gave a much higher TC\(_{50}\) estimation for imipramine and tamoxifen approximately above 3-fold (Fig. 6A), whereas the TC\(_{50}\) value of troglitazone and imipramine in ProliHHs-M was much higher than PHHs (Fig. 6B). When TC\(_{50}\) values were compared with PHHs, the AAFE was 1.27 and 2.13 for ProliHHs-P and ProliHHs-M, respectively. Together, these results demonstrated that both ProliHHs-M and ProliHHs-P could be an alternative hepatocyte model for PHHs in drug cytotoxicity evaluation. Considering the cost and convenience, ProliHHs-P should be a better choice for early discovery drug screening phase.

**Discussion**

A variety of hepatocyte models, such as embryonic stem cell– or induced pluripotent stem cell–derived hepatocyte-like cells, human induced hepatocytes, HepaRG cells, and UHHs, have been developed to study drug metabolism, transport, and drug-induced liver injury (DILI) (McGill et al., 2011; Levy et al., 2015; Ni et al., 2016; Bell et al., 2017). However, these hepatocyte models for application are limited by the lack of physiologic relevance or donor variability (Brolén et al., 2010; Katsuda et al., 2012; Huang et al., 2014). It is vital to develop new promising cell models that address these issues. Recently, with the help of dedifferentiation strategy, PHHs could be expanded, display biphenotypic and stable genetic characteristics, and also could be converted back to mature “hepatocytes” after 3D culture (Zhang et al., 2018).

Compared with PHHs, the advantages of ProliHHs include low cost, ease of obtaining, diversified donor background, etc. However, it is not clear if it is feasible to evaluate drug metabolism, biliary excretion, and toxicity, especially from the transport aspect, using this novel cell model. Therefore, in this paper, for the first time, the drug metabolic capacities and efflux transporter functions of ProliHHs, as well as their potential in toxicity assessments, were fully characterized.

According to the previous study, ProliHHs could be expanded up to 10,000-fold at passage 8 from young PHH donor cells (Zhang et al., 2018). In this study, the total cell number of ProliHHs was enlarged more than 200-fold at passage 4, which is enough for the following studies, and no further proliferation test was performed (Supplemental Fig. 2).
These results are consistent with previous reporting that PHHs will lose their hepatic phenotype and metabolic capacity rapidly during in vitro culture (Gómez-Lechón et al., 2014), and the 3D scaffold culture system could preserve the P450 gene expression and hepatocyte-specific functions for PHHs (Schaefer et al., 2016; Heslop et al., 2017; Bell et al., 2018). These data indicated that ProliHHs from different donors could preserve the P450 gene expression and hepatocyte-specific functions for PHHs (Schaefer et al., 2016; Heslop et al., 2017; Bell et al., 2018). These data indicated that ProliHHs from different donors

Fig. 4. Bile canaliculi formation and polarization of ProliHHs. (A) The schematic overview showed the protocol for sandwich culture of PHHs, ProliHHs-P, and ProliHHs-M. FM, feeding medium. (B) Phase microscopy showed morphology of SC-ProliHHs-M similar to SC-PHHs. (C) CDFDA staining: CDFDA fluorescence intensity tends to converge to the junction of ProliHHs. The bile canaliculi structure is marked with a red arrow. ProliHHs were derived from donor MRW. Scale bars, 100 μm.

Fig. 5. Transporter expression and function of ProliHHs in sandwich culture system. (A) Real-time qPCR analyses of major transporter genes (NTCP, OATP1B1, BSEP, and MRP2) in ProliHHs at indicated stages, gene expressions are normalized to SC-PHHs. (B) Four different compounds were used to determine the BEI of SC-PHHs and SC-ProliHHs. ProliHHs were generated from donor MRW. *P < 0.05; **P < 0.01; ***P < 0.001.
gradually lost hepatic functions with serial passages and regained primary hepatocyte appearances and functions after 3D culture.

The loss of the phenotype of hepatocytes and the increase of the phenotype of liver progenitors were observed during the proliferating process of ProliHHs (Fig. 1, C–E; Supplemental Fig. 3). The expression of major DMEs and transporters also rapidly declined in ProliHHs-P (Fig. 2A; Fig. 5A), which was common for the dedifferentiation process (Kim et al., 2018; Zhang et al., 2018; Fu et al., 2019). The phenotypes of PHHs could be regained in ProliHHs-M after 3D culture, for instance, the improvement of metabolic capacity given the fact that ProliHHs-M were more flexible to predict intrinsic clearances of DMEs (Fig. 2, B and C; Fig. 3). The intrinsic clearances of selected P450 substrates in ProliHHs-M were able to predict the CLint ranking of their PHH donors to some extent (Fig. 2C). These results indicated that the metabolic variabilities in different donors (interindividual variability) could be inherited by their derived ProliHHs, respectively, and the donor specific functions could be rebuilt in a short term.

It is worthwhile to note that although mRNA expression of P450 enzymes in ProliHHs-M is much less than that in PHHs, metabolic activity mediated by these enzymes in ProliHHs-M was close to that in PHHs. For example, CYP2B6 mRNA level in ProliHHs-P was about 1/300 as much as that in PHHs, whereas its CLint was about one-third in PHHs. For example, CYP2B6 mRNA level in ProliHHs-M was about one-third of PHHs (Supplemental Fig. 4). This fact might be attributed to dexamethasone (CYP2C19 inducer) added in HIM (Kanebratt and Andersson, 2008; Schaefer et al., 2016). Therefore, further manipulation of the culture system will be required to generate more functional ProliHHs-M that are capable of efficiently replacing PHHs for drug metabolic studies.

Sinusoidal and canalicular transport properties are critical for primary hepatocytes. The lack of uptake and efflux transporter functions limits various alternative hepatocyte models in the preclinical evaluation of DILI and cholestasis (Donato et al., 2013; Bell et al., 2016; Zhang et al., 2016). Previous studies reported that CDFDA has been commonly used to study the function of efflux transporters, e.g., MRP2 (Anthérieu et al., 2010; Levy et al., 2015). In this study, we found that ProliHHs could be polarized in sandwich culture, which was essential for the proper efflux of bile acids or toxic drugs, and both cells from proliferative and maturation status of ProliHHs could form canalicular networks in sandwich culture (Fig. 4, B and C). Furthermore, compounds that may lead to cholestasis were investigated for their potential DILI risk using SC-ProliHHs. To evaluate the BSEP efflux function, d8-TCA was selected to assess the efflux transporter functions in SC-ProliHHs (Fig. 5B). Three selected cholestatic drugs (rifampin, glibenclamide, and ketoconazole) were reported as the inhibitors of BSEP (Ni et al., 2016; Wu et al., 2016). In this study, all of them significantly inhibited d8-TCA biliary excretion in SC-ProliHHs-P (Supplemental Fig. 5). Of course, the sandwich-cultured cells derived from ProliHHs-M displayed higher BEI values than ProliHHs-P, which were close to the BEI values of PHHs (Fig. 5B). However, considering the timing and cost of 3D culture, SC-ProliHHs-P were a better choice for the early drug discovery stage. More comprehensive studies, including organic anion-transporting polypeptide/sodium-taurocholate cotransporting polypeptide uptake and multidrug resistance gene 1 (MDR1) (P-glycoprotein) efflux function, are required to fully characterized in SC-ProliHH in the future.

The evaluation of drug hepatotoxicity is essential for preclinical drug safety study (Donato et al., 2013; Sebastian et al., 2014; Zhang et al., 2016). In this study, to investigate whether ProliHHs can be used to evaluate drug-induced cytotoxicity, 12 compounds were selected to perform acute toxicity assessment. All tested compounds covered a broad toxicity dose range (TC50 ranging from 1 to 5000 μM) with varied liver toxic mechanisms, for example, metabolic activation (cheneodeoxycholic acid, ibuprofen, and isoniazid), cholestasis (lithocholic acid, chlorpromazine, and imipramine), reactive oxygen species (cyclosporin A, rifampin, amiodarone, and valproic acid), and multiple

![Graph](image-url)
mechanisms (trotiglazone and tamoxifen) (Wang et al., 2002; Kemp and Brouwer, 2004; Ni et al., 2016; Xie et al., 2019). In most cases, the TC50 values of the selected compounds in both ProliHHs-M and ProliHHs-P were within 3-fold accuracy with those in PHHS (Fig. 6, A and B). The higher TC50 values of trotiglazone in ProliHHs-M may be attributed to the low expression of uptake transporter organic-anion-transporting poly


Functional ProliHHs Derived from PHHs


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