3D Primary Human Hepatocytes (PHH) Spheroids Demonstrate Increased Sensitivity to Drug-induced Liver Injury in Comparison to 2D PHH Monolayer Culture

Application Note

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Introduction

Despite the use of *in silico*, *in vitro*, and preclinical animal models for liver safety assessment during drug discovery and development, Drug-induced Liver Injury (DILI) remains a leading cause of drug attrition, post-marketing withdrawals, and restrictive usage warnings[1]. Therefore, researchers are continuously exploring novel liver models that can faithfully recapitulate the physiology and biology of the human liver[2].

Primary human hepatocytes (PHHs) are considered the "gold standard" of liver functions as they contain all the critical components for drug metabolism, transportation, and disposition[3]. Although hepatocytes are often the *in vivo* targets of DILI, PHHs are not suitable for hepatotoxicity tests under conventional 2D monolayer culture conditions, due to the rapid loss of their hepatic phenotypes, functions, and cell viability. Recent advances have shown that 3D culture of PHHs as spheroids can significantly prolong the cell viability and extend hepatic function *in vitro*[4,5]. In addition, gene and protein expression studies indicate the 3D PHH spheroids closely mimic the native liver[6]. Together with the flexibility and adaptability to high throughput screening, the PHH spheroid model appears to be an appealing, physiologically relevant tool for *in vitro* hepatotoxicity studies.

In the present study, a 100-compound screen was performed (using DILI and control compounds selected based on recent publications[7,8]) to examine the response of 2D monolayer vs. 3D spheroid hepatocytes. Specifically, a two-week hepatotoxicity assay with three repeated doses of testing compounds was carried out with 3D PHH spheroids made from cryopreserved PHHs in Corning® 96-well spheroid microplates. For comparison, 2D monolayer cultures from the same PHHs were used in a single-dose, short-term cytotoxicity assay. Bioluminescent ATP assays were performed and 8-point dose response curves were generated. IC_{50} values for each compound were calculated. Using clinical C_{max} values as drug exposure references and the margin of safety (MOS) approach, quantitative analysis of assay specificity and sensitivity were performed. Results indicated that 3D PHH spheroids are 2 to 3 times more sensitive to DILI compound treatment than 2D PHH cultures.

Materials and Methods

Compounds

DILI or control compounds were selected based on published reports to represent each of the 5 different DILI severity categories (Table 1). All the chemicals used in this study, unless otherwise stated, were purchased from Sigma (list of compounds is available on request). Stock solutions for each compound were prepared, and 8-point serial dilutions of 2X working solutions were prepared in serum-free medium from compound stock solutions (in DMSO or medium). For 2D PHH monolayer cultures or 3D PHH spheroid cultures in 96-well microplates, 200 µL medium was used for each well. A bioluminescent ATP assay (CellTiter-Glo® 3D, Promega) was used to measure cell viability according to the manufacturer's procedures with modifications.

3D PHH Spheroid Culture and Compound Dosing

3D spheroid-qualified PHHs (Corning Cat. No. 454552) were thawed using high-viability recovery medium to ensure post-thaw viability at greater than 75%. William's E medium supplemented with insulin, transferrin, selenium, and 0.1 µM dexamethasone and antibiotics was used for both 3D PHH spheroid culture and 2D PHH monolayer culture. For plating, 10% fetal bovine serum (FBS) was also added to the supplemented William's E medium. PHHs were seeded at 1,000 cells/well in 100 µL medium using Corning 96-well spheroid microplates (Corning Cat. No. 4515 or 4520) to generate PHH spheroids for all the hepatotoxicity assays described in this study. After spheroid formation, serum-free medium was used for DILI and control compound hepatotoxicity assays.

Table 1. Selection of DILI and Control Compounds

DILI Severity Category	No. of Compounds
Severe clinical DILI	17
High clinical DILI concern	22
Low clinical DILI concern	24
Enzyme elevations in clinic	17
No DILI	20

For 3D PHH spheroid culture, compound dosing was performed after spheroid formation in serum-free medium by carefully removing 100 µL/well old media, then adding 100 µL of serial dilution of a test compound. 3D PHH spheroid cultures were treated with 3 repeated dosings on days 8, 12, and 15 (Figure 1). Bioluminescent ATP assays were performed after 24 hours of the last dosing.

2D Monolayer Culture and Compound Dosing

2D monolayer cultures were prepared with Corning® BioCoat™ Collagen I 96-well microplates (Corning Cat. No. 354407), and PHHs were seeded at 60,000 cells/well. A single lot of hepatocytes was used for both 2D monolayer culture and 3D PHH spheroid cultures. High-viability recovery medium was used for thawing. For 2D culture, after thawing, cells were plated in William's E medium with 10% FBS for approximately 4 to 5 hours, after which medium was changed to remove dead cells. Compound dosing started 24 hours post-plating as described above. Bioluminescent ATP assays were performed 24 hours after dosing.

Results

3D Spheroid-qualified Primary Human Hepatocytes for 2D PHH Monolayer and 3D PHH Spheroid Toxicity Tests

As shown in Figure 1, a short-term toxicity assay was applied to 2D PHH monolayer cultures, due to the limited cell viability of PHHs under these conditions. In contrast, 3D PHH spheroids were subjected to a two-week hepatotoxicity assay with three repeated dosings of DILI or control compound.

Amiodarone is a known DILI compound that causes severe clinical DILI. Representative images of PHH spheroids are shown (Figure 2) with repeated dosing for 3D hepatotoxicity assay. Morphological changes of PHH spheroids indicate the loss of spheroid integrity and cell death at higher drug concentrations. IC_{50} values were calculated at 26.4 µM for PHH spheroids and at 209.1 µM for 2D PHH monolayer cultures. These results show that 3D PHH spheroids have greater sensitivity to amiodarone-induced hepatotoxicity than 2D PHH monocultures.

Liver Toxicity Screening with 3D PHH Spheroids and 2D PHH Monolayer Cultures

Using the 2D and 3D hepatotoxicity assay protocols described above, we have tested 100 DILI (severe clinical DILI, high clinical DILI concern, and low clinical DILI concern) and control compounds (enzyme elevation in clinic and no DILI). C_{max} is the maximum concentration of a drug measured in a patient's plasma. When IC_{50} values can be determined from *in vitro* assays, we took the margin of safety (MOS) approach where the ratio of IC_{50}/C_{max} is used to predict the risk of a test compound in a clinical setting. A summary of the results comparing the IC_{50}/C_{max} ratios from PHH spheroids and 2D PHH monolayer cultures for each tested compound in this screen are in Figure 3. A threshold of 50-fold IC_{50}/C_{max} ratio (MOS 50) is used to predict tested compounds as either positive or negative for causing hepatotoxicity.

3D PHH Spheroids Show Increased Sensitivity to DILI Compound Treatment

Based on the known status of each tested compound within the five DILI categories and MOS threshold, the resulting numbers of True Positive (TP), True Negative (TN), False Positive (FP), or False Negative (FN) predictions for each DILI category are summarized for both 2D and 3D hepatotoxicity studies. Sensitivity = TP/(TP+FN); Specificity = TN/(FP+TN), as shown in Figure 4.

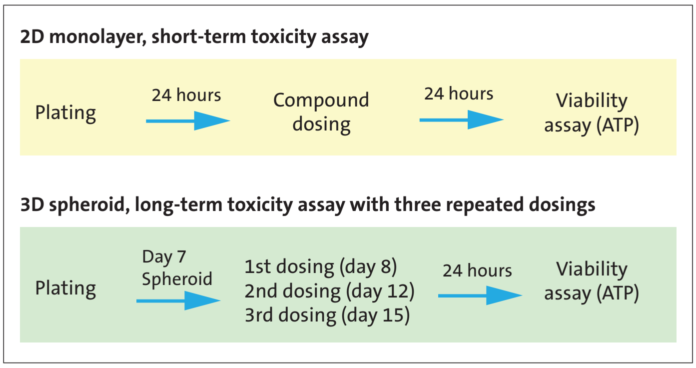

Figure 1. Dosing regimen for 2D PHH monolayer and 3D PHH spheroids.

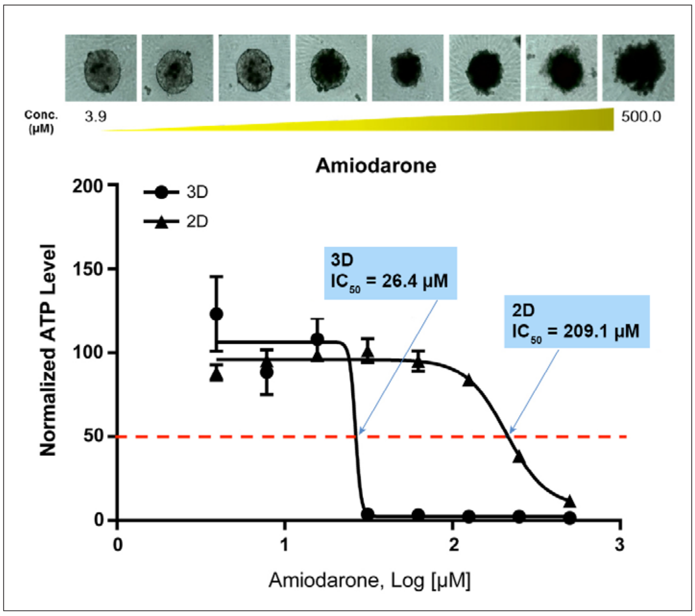

Figure 2. PHH spheroid dose response to the DILI compound amiodarone. Representative images of PHH spheroids after three repeated dosings of amiodarone. From the dose response curves generated with both 2D monolayer and 3D PHH spheroids, IC_{50} values were calculated using GraphPad Prism software.

To compare the performance of 2D PHH monolayer and 3D PHH spheroids in hepatotoxicity assays, assay sensitivity and specificity are calculated using MOS thresholds of 10, 25, and 50, respectively (Table 2). At MOS 10, 25, and 50, the sensitivity of 3D hepatotoxicity assays is 2.9-, 3.6-, and 2.6-fold higher than the 2D assays using the same lot of hepatocytes. Interestingly, co-culture liver spheroids of PHH and non-parenchymal liver cells have recently been shown to have 2- to 3-fold higher sensitivity in comparison to 2D PHH monolayer culture (Proctor, et al., 2017) in hepatotoxicity assays. In summary, our data and evidence in published literature reports clearly demonstrate that 3D PHH spheroids are a novel and powerful tool that can significantly improve the performance and sensitivity of *in vitro* hepatotoxicity assays.

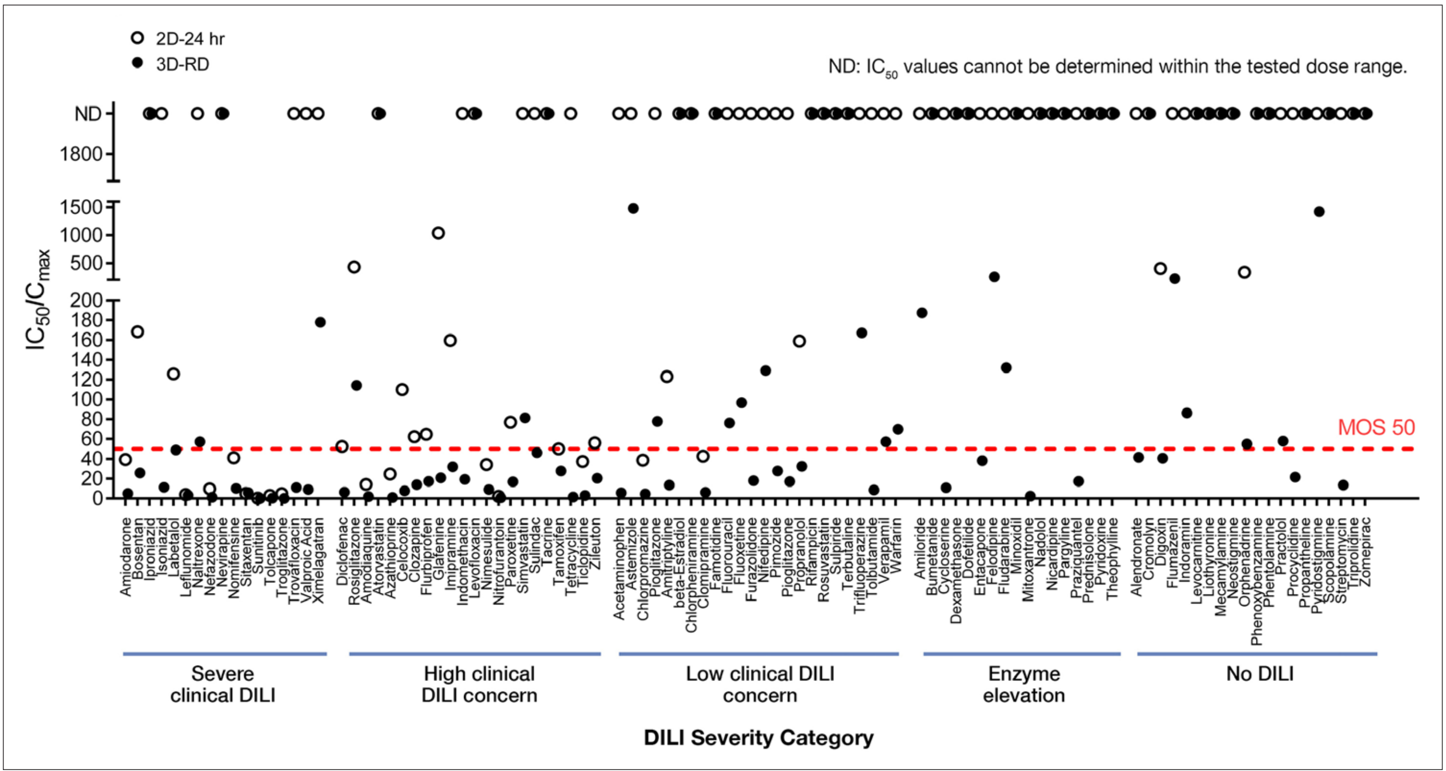

Figure 3. IC_{50}/C_{max} ratio for DILI and control compounds tested in 2D or 3D hepatotoxicity assays Hepatocytes from **the same lot of** 3D spheroid-qualified were used for both 3D liver spheroid (1,000 cells/spheroid) and 2D PHH monolayer cultures (60,000/well) with 96-well Ultra-Low Attachment surface spheroid microplates or 96-well Corning® BioCoat™ Collagen I microplates, respectively. IC_{50} values measured from 2D and 3D hepatotoxicity assays were corrected with clinical C_{max} (not shown) for 100 tested drugs that belong to five DILI severity categories. Margin of Safety ratio (IC_{50}/C_{max}) for each tested compound is plotted and the dotted red line shows the threshold of 50X MOS.

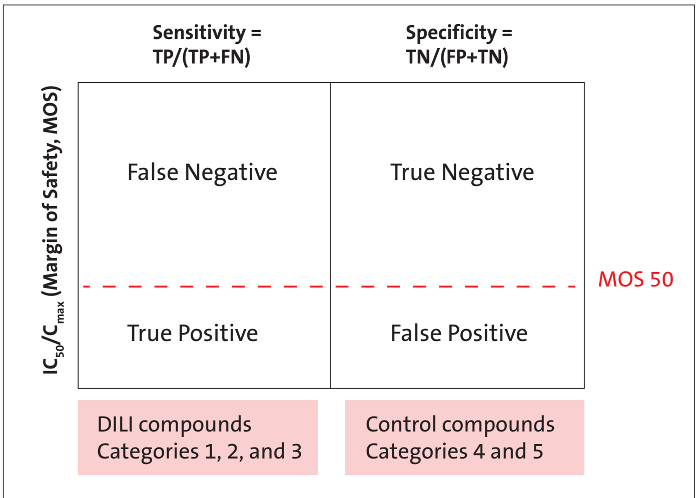

Figure 4. Assay performance assessment for *in vitro* hepatotoxicity. If the IC_{50}/C_{max} ratio for a tested compound is above MOS 50 threshold, it is considered negative for the hepatotoxicity assay. If there was no IC_{50} determined in the assay for a tested compound it is considered negative as well. If the IC_{50}/C_{max} ratio for a tested compound is below MOS 50 threshold it is considered positive.

Table 2. 3D PHH spheroids hepatotoxicity assay show 2-to 3-fold improvement in assay sensitivity vs. 2D PHH monolayer cultures.

MOS Threshold	10		25		50	
Assay	3D	2D	3D	2D	3D	2D
Sensitivity	32%	11%	51%	14%	62%	24%
Specificity	95%	100%	86%	100%	84%	100%

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