3D spheroid culture as a tool for studying drug metabolism

Introduction

The primary human hepatocyte (PHH) three-dimensional (3D) culture model developed by Thermo Fisher Scientific holds promise for disease modeling and functional studies. As the liver is the principal site of metabolism for most drugs, primary hepatocytes have become the most popular *in vitro* tool to evaluate hepatic drug metabolism. However, the efficiency of 3D hepatic spheroids for assessing drug metabolism is relatively unknown. One major difference between 3D hepatic culture and conventional two-dimensional (2D) culture is the number of cells per well. A single hepatic spheroid typically consists of 1,000 to 3,000 cells, whereas there are between 50,000 (96-well plate) and 400,000 (24-well plate) cells per well in a conventional 2D culture. Hence, it is expected that metabolites synthesized in 3D culture will be present at lower concentrations relative to those found in 2D culture. However, we have observed that relative gene expression levels for certain **cytochrome P450 (CYP) enzymes** are higher in 3D hepatic spheroids than in their 2D counterparts. As CYP proteins are critical phase I enzymes for drug metabolism, we measured CYP activities for 6 different enzymes with distinct substrates via high-resolution mass spectrometry, directly comparing the activities of 2D and 3D cultures. Our data demonstrate the utility of Gibco™ 3D hepatic spheroid models for studying drug metabolism.

Materials and methods

Spheroid culture

Hepatic spheroids were generated using **Gibco™ cryopreserved spheroid–qualified human hepatocytes (Cat. No. HMCPSQ)** following the user guide [1]. Each well contained 3,000 PHHs. The spheroids formed within 5 days of cell seeding. Starting on day 5, half of the plating medium was changed every 48–72 hours. On day 9 of spheroid culture, 2D hepatocytes were seeded in a collagen-coated 96-well plate at 50,000 PHHs per well following published guidelines [2].

Metabolic assay

On day 10, the media of both 2D and 3D cultures were replaced with an incubation medium containing **Gibco™ Williams' E Medium** and **Primary Hepatocyte Maintenance Supplements**. Six compounds of interest were added to both 2D and 3D PHH cultures in serum-free Williams' E Medium. The compounds were selected such that several CYP enzymes that are important for hepatic drug metabolism could be interrogated. Table 1 lists the identity of each compound, the CYP enzymes primarily responsible for their metabolism, the metabolites analyzed, and the drug concentrations tested. Of the six compounds used, all except tolbutamide are known as high-turnover compounds with fast intrinsic clearance rates. The incubation time (Table 2) for each compound for both 2D and 3D studies was previously determined to be within the linear range of the assay. The medium was collected without disturbing the cells and stored at –80°C for later analysis.

Analysis of metabolites

Cell culture samples collected from both 2D and 3D hepatic cultures were analyzed for metabolite formation using the **Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer** (Table 3). This allowed for accurate, high-resolution mass measurement as well as fast scanning for metabolites (quantitative and/or qualitative) in complex matrices.

Liquid chromatography–mass spectrometry (LC-MS)

The **Thermo Scientific™ Vanquish™ Flex Binary UHPLC system** was used for liquid chromatography (LC) analysis. LC separation was performed on a **Thermo Scientific™ Hypersil™ BDS C18** column using mobile phases A (H_2O/0.1% formic acid) and B (ACN/0.1% formic acid) at a flow rate of 400 µL/min with gradient (5% to 90% in 15 min). The media collected from both 2D and 3D samples containing the respective metabolites were directly injected into the Vanquish system for LC-MS analysis on the Q Exactive Plus mass spectrometer. High-resolution full scan and HCD MS2 data were collected in a data-dependent fashion with polarity switching. The metabolites were readily identified and quantified using full scan mass with resolution 35,000. The various specifications of the mass spectrometer are provided in Table 3.

Table 1. Cytochrome P450 enzymes assayed and compounds analyzed in the metabolic assays.

CYP enzyme assayed	Compound used	Metabolite analyzed	Molecular weight of metabolite (m/z)
CYP2D6	Dextromethorphan	Dextrorphan	258.1852
CYP3A4	Midazolam	1-Hydroxymidazolam	342.0804
CYP1A2	Phenacetin	Acetaminophen	152.0706
CYP2B6	Bupropion	Hydroxybupropion	256.1099
CYP3A4	Testosterone	6β-Hydroxytestosterone	305.2111
CYP2C9	Tolbutamide	4-Hydroxytolbutamide	287.1060

Table 2. Concentration of compounds used and incubation time for 2D and 3D cultures.

Compound used	Concentration (µM)	Incubation time (hr)	
		2D	3D
Dextromethorphan	150	2	8
Midazolam	100	2	8
Phenacetin	200	2	8
Bupropion	500	2	8
Testosterone	400	2	8
Tolbutamide	500	4	16

Table 3. Specifications for Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer.

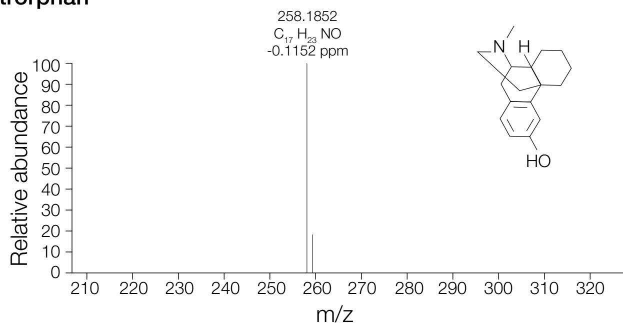
Category	Specification
Ion source	Thermo Scientific™ Heated Electrospray Ionization (HESI-II) Probe
Ionization mode	ESI positive/negative switching
Sheath gas flow rate	50 units N_2
Auxiliary gas flow rate	10 units N_2
Spray voltage	+3.2/–3.0 kV
Ion transfer tube temperature	300°C
S-lens radio-frequency level	50.0
Heater temperature	425°C

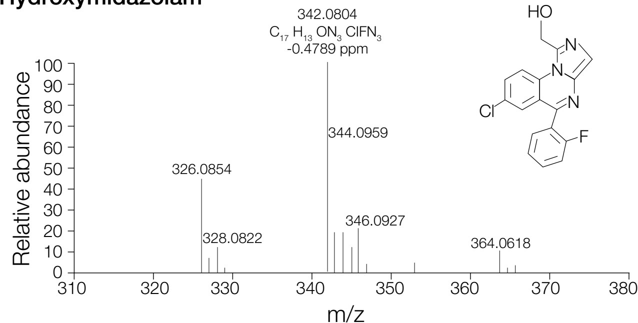
Conclusions

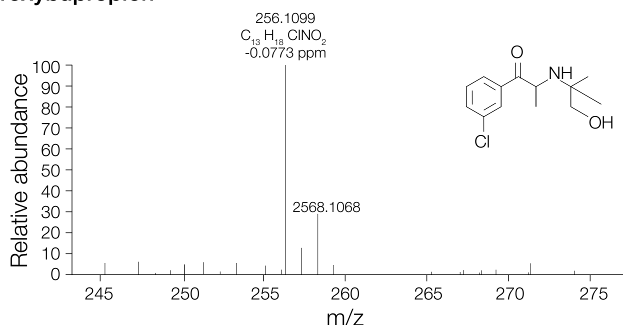
PHH 3D spheroids provide many benefits compared to 2D cultures, including histotypic and phenotypic longevity. However, production of 3D hepatocyte culture is achieved with a small number of cells per spheroid, raising concerns regarding a sufficient assay window for studying drug metabolism. The current study assessed differences in CYP450 enzyme activities in 2D and 3D PHH cultures.

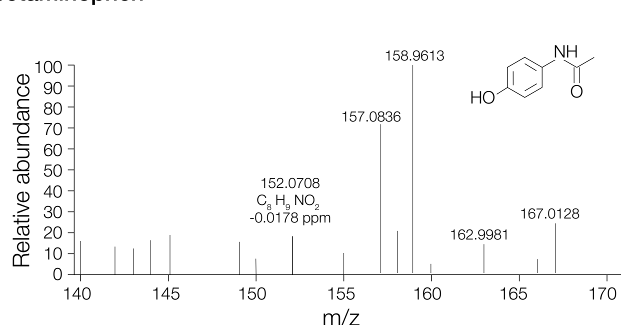
From the data presented, the following conclusions can be reached:

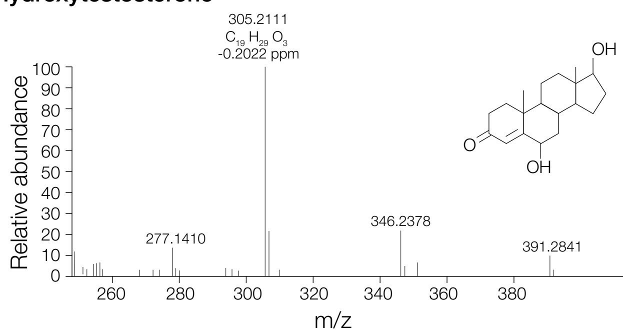
1. Metabolites produced by the 3D hepatic spheroids can be easily identified and quantified using a high-resolution mass spectrometer such as the Q Exactive Plus mass spectrometer (Figure 1).
2. 3D hepatic spheroid culture is suitable for studying drug metabolism. A sufficient assay window for metabolite formation was observed for the CYP450 enzymes studied (Figures 1 and 2).
3. In general, 3D hepatic cultures were more efficient in drug metabolism compared to 2D cultures. This finding is consistent with our previous observation that 3D hepatic spheroids express various CYP450 genes and albumin at higher levels than are found in 2D cultures [3]. Previously, we also found higher CYP3A4 activity in 3D cultures using a luminescence-based assay.
4. Based on the data generated, we conclude that 3D hepatic spHeroid culture is a suitable system for high-throughput drug screening and metabolic assays.











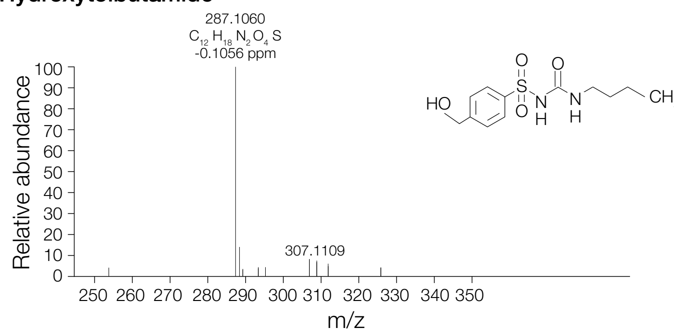


Figure 1. Identification of marker metabolites. The marker metabolites synthesized from the respective compounds were identified and quantified using high-resolution mass spectrometry (HRMS). HRMS readily identified the metabolites secreted from a single spheroid consisting of only 3,000 cells.

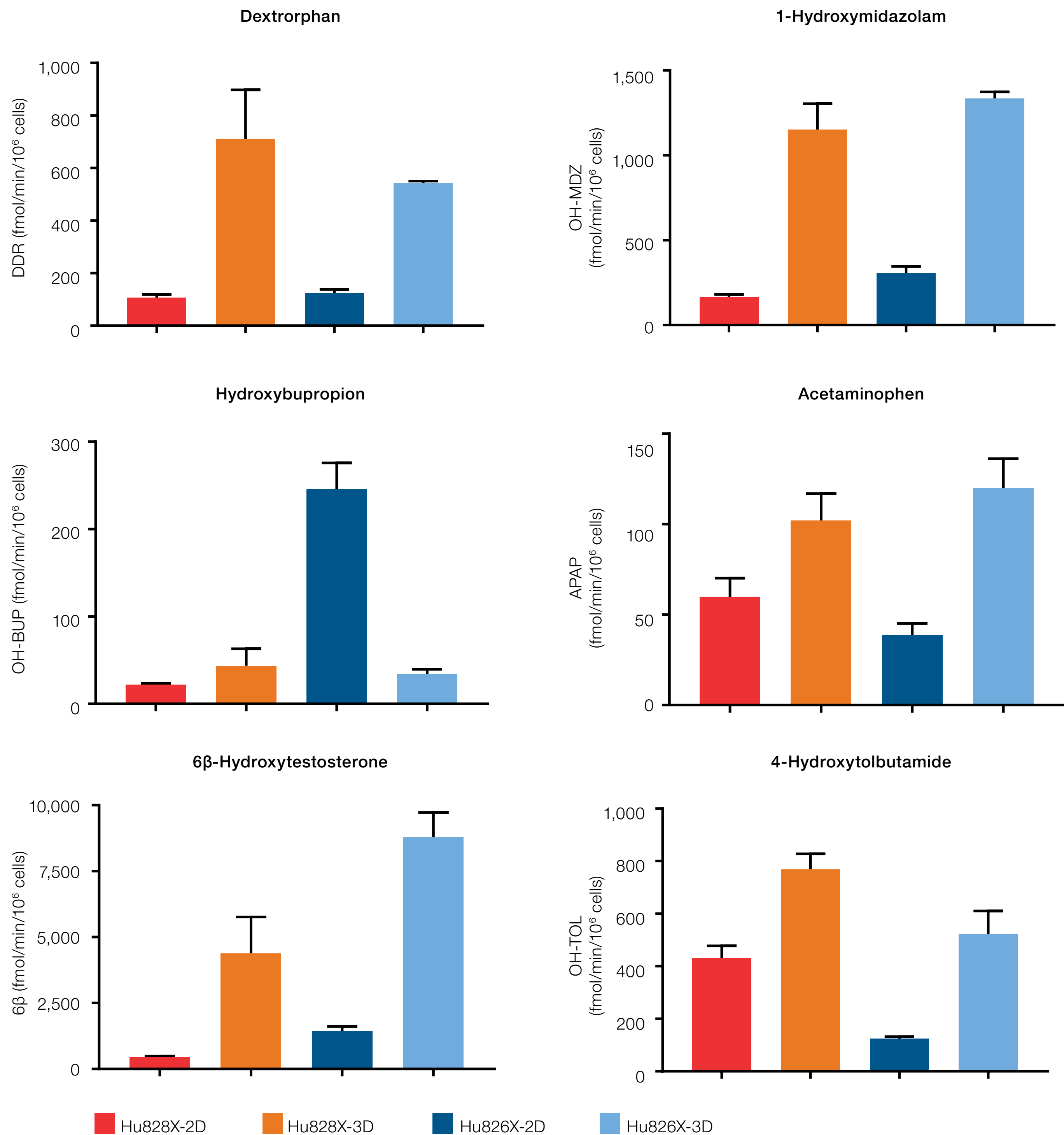


Figure 2. Comparison of metabolites between the 2D and 3D cultures. The metabolites quantified using HRMS were converted to mole amounts based on the standard curves of the respective metabolites. Two different lots of PHH, Hu828X and Hu826X (partially redacted), were used in this assay. Results were normalized to incubation time of individual substrates and number of cells per well in the 2D and 3D cultures. Data are mean ±space SD; n = 3.

References

1. Thermo Fisher Scientific. User Guide: Cryopreserved 3D-Spheroid Qualified Human Hepatocytes. Pub. No. MAN0018280.
2. Thermo Fisher Scientific. Thawing and Plating Cryopreserved Hepatocytes. Protocol available at thermofisher.com/us/en/home/references/protocols/drug-discovery/adme-tox-protocols/thawing-and-plating-hepatocytes-protocol.html.
3. Thermo Fisher Scientific. Primary Human Hepatocyte 3D Spheroids for Studying Hepatic Function and Drug Toxicity. Poster available at assets.thermofisher.com/TFS-Assets/BID/posters/human-hepatoctye-3d-spheroids-hepatic-function-drug-toxicity-poster.pdf.

Ordering information

Product	Cat. No.
Primary Hepatocyte Maintenance Supplements	CM4000
Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer	IQLAAEGAAPFALGMBDK
Vanquish Flex Binary UHPLC	IQLAAAGABHFAPUMBJC
CYP2D6 BACULOSOMES Plus Reagent, rHuman	P2283
CYP3A4 BACULOSOMES Plus Reagent, rHuman	P2377
CYP1A2 BACULOSOMES Plus Reagent, rHuman	P2792
CYP2B6 BACULOSOMES Plus Reagent, rHuman	P3028
CYP2C9 BACULOSOMES Plus Reagent, rHuman	P2378
Hypersil BDS C18 Columns	28105-052130
Gibco Spheroid-Qualified Human Hepatocytes, cryopreserved	HMCPSQ
Gibco Williams' E Medium	A1217601

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