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# Perfused human hepatocyte microtissues identify reactive metabolite-forming and mitochondria-perturbing hepatotoxins

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ABSTRACT

Hepatotoxins cause liver damage via many mechanisms but the formation of reactive metabolites and/or damage to liver mitochondria are commonly implicated. We assess 3D human primary hepatocyte microtissues as a platform for hepatotoxicity studies with reactive metabolite-forming and mitochondria-perturbing compounds. We show that microtissues formed from cryopreserved human hepatocytes had bile canaliculi, transcribed mRNA from genes associated with xenobiotic metabolism and expressed functional cytochrome P450 enzymes. Hierarchical clustering was used to distinguish dose-dependent hepatotoxicity elicited by clozapine, fialuridine and acetaminophen (APAP) from control cultures and less liver-damaging compounds, olanzapine and entecavir. The regio-isomer of acetaminophen, *N*-acetyl-meta-aminophenol (AMAP) clustered with the hepatotoxic compounds. The principal metabolites of APAP were formed and dose-dependent changes in metabolite profile similar to those seen in patient overdose was observed. The toxicological profile of APAP was indistinguishable from that of AMAP, confirming AMAP as a human hepatotoxin. Tissue oxygen consumption rate was significantly decreased within 2 h of exposure to APAP or AMAP, concomitant with glutathione depletion. These data highlight the potential utility of perfused metabolically functional human liver microtissues in drug development and mechanistic toxicology.

## 1. Introduction

Hepatotoxicity is a major cause of lead compound attrition in drug development (Watkins, 2011) and drug-related clinical morbidity (Chalhoub et al., 2014) and there is still a need for better predictive model's for mechanistic studies (Peters, 2005). Hepatotoxins can cause liver damage via many mechanisms but the formation of reactive metabolites (Dahal et al., 2013), and/or damage to liver mitochondria (Labbe et al., 2008) are commonly implicated. While a fully-representative whole organ model featuring multiple cell types remains an ultimate goal, a hepatocyte-only model that can be used to reliably predict and study these major hepatotoxicity mechanisms would greatly aid drug development. Requirements for such a model include maintained hepatic phenotype, physiological metabolic pathways, and mitochondrial functionality. Transformed cell lines have undergone major phenotypic changes compared to their tissues of origin (Alexopoulos et al., 2010; Geiger et al., 2012), therefore healthy primary human cells are more suited to constructing improved models. Isolated human hepatocytes have become a crucial part of safe drug development (Soars et al., 2007) despite their tendency to dedifferentiate in culture (Elaut et al., 2006). Improvements in primary human hepatocyte cryopreservation techniques (Hewitt, 2010) have made high quality human cells more easily available and many improved model systems that offer more physiological relevance are being developed (Godoy et al., 2013; Peck and Wang, 2013; Rowe et al., 2013; Kostrzewski et al., 2017). Three dimensional (3D) human hepatic microtissues formed in a purpose-designed liver cell culture system (Sivaraman et al., 2005; Domansky et al., 2010; Clark et al., 2014) maintain hepatocyte functions for extended periods (Sarkar et al., 2015; Vivares et al., 2015). These attributes, and the model's amenity to direct measurement of the tissues' oxygen consumption (Domansky et al., 2010) suggest these tissues would have utility in toxicology studies where metabolism or mitochondrial dysfunction is implicated. We assessed the model's phenotypic stability, relevance of metabolic function to human liver, and investigate responses to known reactive metabolite-forming or mitochondria-disrupting hepatotoxins. The well-characterised

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**Table 1**
Clinical Cmax drug concentrations for model compounds. 30 × or 100 × the indicated concentrations were used in toxicological studies.

| Compound | Cmax | Reference | In vitro exposures | Exposure times | Endpoint |
|---|---|---|---|---|---|
| Clozapine | 3.8 ng/mL | Dahl et al. (1994) | 30 × Cmax, 100 × Cmax | 48 h, 96 h, 144 h | Albumin, Urea, LDH, WST-1, Total Protein. |
| Olanzapine | 11.7 ng/mL | Lucas et al. (1998) | 30 × Cmax, 100 × Cmax | 48 h, 96 h, 144 h | Albumin, Urea, LDH, WST-1, Total Protein. |
| Fialuridine | 370 ng/mL[a] | O'Brien et al. (2006) | 30 × Cmax, 100 × Cmax | 48 h, 96 h, 144 h | Albumin, Urea, LDH, WST-1, Total Protein. |
| Entecavir | 8.2 ng/mL | Matthews (2006) | 30 × Cmax, 100 × Cmax | 48 h, 96 h, 144 h | Albumin, Urea, LDH, WST-1, Total Protein. |
| Acetaminophen | 0.019 mg/mL | O'Brien et al. (2006) | 30 × Cmax, 100 × Cmax | 48 h, 96 h, 144 h | Albumin, Urea, LDH, WST-1, Total Protein. |
| | | | 1 mM, 13 mM | 1 h, 3 h, 6 h, 24 h | Metabolite quantification |
| | | | 30 × Cmax | 48 h | SOD1 |
| | | | 100 × Cmax | 2 h, 4 h, 6 h, | Oxygen consumption |
| | | | 100 × Cmax | 6 h | Albumin, LDH, WST-1, GSH, Total Protein, |
| *N*-acetyl-meta-aminophenol | 0.019 mg/mL | Hadi et al. (2013) | 30 × Cmax, 100 × Cmax | 48 h, 96 h, 144 h | Albumin, Urea, LDH, WST-1, Total Protein. |
| | | | 30 × Cmax | 48 h | SOD1 |
| | | | 100 × Cmax | 2 h, 4 h, 6 h, | Oxygen consumption |
| | | | 100 × Cmax | 6 h | Albumin, LDH, WST-1, GSH, Total Protein, |

[a] A nominal 1 μM Cmax was used for the withdrawn from trial drug, Fialuridine.

physiological and toxicological responses to acetaminophen (APAP) are compared to its analogue *N*-acetyl-meta-aminophenol (AMAP). We confirm and extend the findings of others (Hadi et al., 2013; van Swelm et al., 2014) that AMAP, once considered a less toxic analogue of APAP (Rashed and Nelson, 1989), is equally as hepatotoxic as APAP to human liver tissues.

## 2. Materials and methods

### 2.1. Hepatocyte culture

Cryopreserved human hepatocytes were purchased from multiple vendors. Supplementary information file 1 details the vendor, donor age and ethnicity for 9 lots were used in this study. These donors were selected due to their high functionality in the system and abundant availability of cryopreserved vials at the time of work. Cells were recovered according to the suppliers' instructions relevant to each lot. Post-thawing cell yield and viability was assessed by trypan blue exclusion and was > 85% for all lots. Single cell suspensions were seeded ($6 \times 10^5$ viable cells per compartment) into scaffolds housed in 12 compartment LiverChip™ bioreactors (CN Bio Innovations, UK) with downward flow (1 μL/s) in a total volume of 1.6 mL per compartment (Sarkar et al., 2015). After 8 h the flow controller automatically reversed the direction for the remainder of culture. Williams' E Medium containing primary hepatocyte thawing and plating supplements (Life Technologies, UK) was used for seeding, and maintenance supplements (Life Technologies, UK) was used from the day after seeding. All cultures were maintained in a standard humidified atmosphere at 37 °C with 5% $CO_2$ and had a first complete medium change the day after seeding cells and every subsequent 48 h. For clarity, supplementary information file 2 gives a detailed description of the LiverChip™ system used and the timeline for each figure in this manuscript.

### 2.2. Microscopy

The scaffolds/tissues were removed to a sterile conventional 12 well culture plate and washed with phosphate buffered saline (PBS). Bright-field images were taken using an inverted light microscope (Leica, UK). For confocal microscopy, tissues were fixed with 4% paraformaldehyde (Sigma, UK) for 10 min, washed with PBS, permeabilised with 0.1% Triton X 100 in PBS for 5 min and washed twice with PBS. Actin was labelled with 50 μg/mL phalloidin-TRITC (Sigma, UK) for 40 min, before washing twice in PBS. Tissues were counterstained with Hoechst stain (Invitrogen, UK) and mounted using AF1 mounting solution (Citifluor, UK). Images were obtained on an upright Zeiss LSM 510 confocal microscope.

### 2.3. CDFDA staining of bile canaliculi

Scaffolds were removed, washed with Hank's Balanced Salt Solution (HBSS) and incubated with 10 μM 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA), (Invitrogen, UK) in HBSS for 40 min at 37 °C. The accumulation of 5-(and-6)-carboxy-2′,7′-dichloro-fluorescein (CDF) in the canalicular domains between hepatocytes was observed using a Nikon Ti Eclipse fluorescence microscope with FITC filter. In some experiments MRP2 was inhibited by pre-incubation with 10 mM Probenecid (Invitrogen, UK) for 10 min.

### 2.4. Transcriptional analysis

Total RNA was extracted from freshly-thawed cells or from scaffolds on day 7 of culture using an RNA miniprep kit (Qiagen, UK). Reverse transcription and PCR was performed using Superarray reagents (Qiagen, UK). PCR was performed using a Via 7 (Applied Biosystems, UK) or Quantstudio 6 real time PCR system (Applied Biosystems, UK). The fold-change in each transcript represented on the Human Drug Metabolism Array plate was determined using a vendor supplied spreadsheet (supplementary data file 3).

### 2.5. Metabolite quantification

#### 2.5.1. Quantification of basal CYP activity

Conversion of Tacrine (5 μM) to 1-hydroxytacrine, Diclofenac (90 μM) to 4-hydroxydiclofenac, Bufuralol (10 μM) to hydroxybufuralol and Midazolam (5 μM) to 1-hydroxymidazolam was used to quantify the activities of CYP-1A2, -2C9, -2D6 and -3A4, respectively. A mixture of substrates was prepared at 1000-fold concentration in DMSO and added to medium immediately before incubation. Compounds were added to microtissues during a full medium change and incubated for 1 h under standard culture conditions, representing 2.25 complete medium circulations. Metabolites were quantified by Mass Spectrometry against quantitative standard curves by a contract research organisation (XenoGesis Ltd., Nottingham, UK).

#### 2.5.2. Quantification of APAP metabolism

For metabolite quantification, APAP was dissolved directly in cell culture medium to a final concentration of 1 mM or 13 mM. APAP metabolites formed after times were quantified in media samples using a quadrupole linear ion trap mass spectrometer (AB Sciex 4000 QTrap) coupled to a Dionex Ultimate 3000 HPLC system. 10 μL of each sample was separated with a Phenomenex Kinetex 2.6 u C18 100A 100 × 2.10 mm column. A gradient consisting of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) was used with a flow rate of 300 μL/min. The column oven and auto-sampler were maintained at 40 °C and 4 °C respectively. The mass spectrometer was