Use of Micropatterned Cocultures to Detect Compounds That Cause Drug-Induced Liver Injury in Humans

Salman R. Khetani,* Chitra Kanchagar,† Okechukwu Ukairo,‡ Stacy Krzyzewski,† Amanda Moore,† Julianne Shi,† Simon Aoyama,† Michael Aleo,‡ and Yvonne Will§†

*Mechanical and Biomedical Engineering, Colorado State University, Fort Collins, Colorado; †Hepregen Corporation, Medford, Massachusetts; and §Drug Safety Research & Development, Pfizer R&D, Groton, Connecticut

†To whom correspondence should be addressed at Pfizer R&D, Compound Safety Prediction-WWMC, Cell Based Assays and Mitochondrial Biology, Eastern Point Rd, Groton, CT 06340. E-mail: yvonne.will@pfizer.com.

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Because drug-induced liver injury (DILI) remains a major reason for late-stage drug attrition, predictive assays are needed that can be deployed throughout the drug discovery process. Clinical DILI can be predicted with a sensitivity of ~50% and a false positive (FP) rate of ~5% using 24-h cultures of sandwich-cultured primary human hepatocytes and imaging of four cell injury endpoints (Xu et al., 2008). We hypothesized that long-term drug dosing in a functionally stable model of primary hepatocytes (micropatterned cocultures [MPCCs]) could provide for increased predictivity over short-term dosing paradigms. We used MPCCs with either primary human or rat hepatocytes to understand possible species differences along with standard endpoints (glutathione levels, ATP levels, albumin, and urea secretion) to test 45 drugs either known or not known to cause clinical DILI. Human MPCCs correctly detected 23 of 35 compounds known to cause DILI (65.7% sensitivity), with a FP rate of 10% for the 10 negative compounds tested. Rat MPCCs correctly detected 17 of 35 DILI compounds (48.6% sensitivity) and had a higher FP rate than human MPCCs (20 vs. 10%). For an additional 19 drugs with the most DILI concern, human MPCCs displayed a sensitivity of 100% when at least two hepatocyte donors were used for testing. Furthermore, MPCCs were able to detect relative clinical toxicities of structural drug analogs. In conclusion, MPCCs showed superiority over conventional short-term cultures for predictions of clinical DILI, and human MPCCs were more predictive for human liabilities than their rat counterparts.

Key Words: human hepatocytes; rat hepatocytes stromal cells; cocultures; micropatterning; drug-induced liver injury.

Drug-induced liver injury (DILI) is a leading cause of acute liver failures and transplants in humans that can lead to restrictions on use, black box warnings of marketed drugs following severe reactions, and the prelaunch and postmarket attrition of pharmaceuticals (Kaplowitz, 2005). In addition to its impact on the well-being of patients, the economic impact of DILI to patients, the health-care system, regulatory agencies, and pharmaceutical companies is significant (Rawlins, 2004). Thus, there is pressure to reduce the cost of development while increasing the likelihood of success. Preclinical animal studies are sometimes inadequate by themselves to evaluate human-relevant toxicity because of species-specific differences between human and animal hepatocellular functions, thereby necessitating supplementation of animal data with human-relevant assays (Olson et al., 2000; Shih et al., 1999). In addition, preclinical safety evaluation studies are conducted in young animals with limited genetic diversity (e.g., outbred or inbred rat strains) under controlled nutritional and housing conditions. However, it is known that patient-related risk factors include underlying disease, age, gender, comedinations, nutritional status, activation of the innate immune system, physical activity, and genetic predisposition (Ulrich, 2007). Species differences in drug metabolism, drug targets, and pathophysiology are factors that must be considered in the interpretation of preclinical findings and in assessing their relevance to humans. Therefore, compound prioritization and optimization in drug development is a critical task, and better predictive in vitro screening systems reflecting both animal and human in vivo toxicities are needed.

Several in vitro liver models are currently used in pharmaceutical practice: liver slices, microsomes, cell lines, and primary hepatocytes (Guillouzo and Guguen-Guillouzo, 2008; Lecluyse, 2001). Although liver slices retain in vivo cytoarchitecture, they have limited viability and are not amenable to high-throughput screening (HTS). Microsomes are used in HTS to identify enzymes involved in drug metabolism but lack the gene expression and cellular machinery required for toxicity testing. Although hepatocarcinoma-derived cell lines and immortalized hepatocytes can be reproducible and inexpensive, they display abnormal levels of liver-specific functions (Wilkening et al., 2003). Thus, of the aforementioned models, primary hepatocytes are considered to be the best
choice for toxicology applications because they are simple to use and their cytoarchitecture remains intact (Guillouzo and Guguen-Guillouzo, 2008); however, hepatic functions rapidly decline under conventional culture conditions, which makes them only suitable for short-term culture (Guillouzo and Guguen-Guillouzo, 2008; Khetani and Bhatia, 2008). Longer studies require the supplementation of culture medium with soluble factors that prolong culture survival but can influence hepatocyte differentiation and/or the use of extracellular matrix (ECM) sandwich or 3D configurations that maintain hepatocyte morphology and function to a greater extent than simple monolayer cultures on collagen (Guillouzo and Guguen-Guillouzo, 2008; Lecluyse, 2001). In contrast to hepatocytes in monolayer configuration, sandwiched cells re-establish polarity, express functional transporters in culture, and constitute a useful tool to predict hepatobiliary transport in vivo (Bi et al., 2006). However, sandwich cultures and 3D spheroids on ECM gels (composed primarily of laminin, collagen IV, heparan sulfate proteoglycan, and entactin) have been shown to display a sharp decline in liver functions and are only useful for acute dosing studies, 3 to 24 h (Khetani and Bhatia, 2008).

We have previously developed a liver model with precise microscale architecture and optimal stromal interactions (micropatterned cocultures [MPCCs]) that displays phenotypic stability for several weeks in vitro as assessed by major liver-specific functions and gene expression (Bhatia et al., 1999; Khetani and Bhatia, 2008). In MPCCs, primary hepatocytes are patterned onto ECM domains of optimized dimensions and then surrounded by 3T3-J2 murine embryonic fibroblasts. Phase I and II enzymes and transporters are expressed at high levels in MPCCs for several weeks (Khetani and Bhatia, 2008), which has led to improved detection of drug metabolites over suspension cultures and enzyme fractions (Wang et al., 2010). Here, we explore whether repeated drug incubations using the MPCC model could improve the sensitivity of in vitro DILI detection, without increasing the false positive (FP) rate reported in in vitro models could improve the sensitivity of DILI detection. We have previously developed a liver model with pre-patterned surfaces, and enzyme fractions to display a sharp decline in liver functions and are only useful for acute dosing studies, 3 to 24 h (Khetani and Bhatia, 2008).

We have previously developed a liver model with precise microscale architecture and optimal stromal interactions (micropatterned cocultures [MPCCs]) that displays phenotypic stability for several weeks in vitro as assessed by major liver-specific functions and gene expression (Bhatia et al., 1999; Khetani and Bhatia, 2008). In MPCCs, primary hepatocytes are patterned onto ECM domains of optimized dimensions and then surrounded by 3T3-J2 murine embryonic fibroblasts. Phase I and II enzymes and transporters are expressed at high levels in MPCCs for several weeks (Khetani and Bhatia, 2008), which has led to improved detection of drug metabolites over suspension cultures and enzyme fractions (Wang et al., 2010). Here, we explore whether repeated drug incubations using the MPCC model could improve the sensitivity of in vitro DILI detection, without increasing the false positive (FP) rate reported in human sandwich cultures (Xu et al., 2008). Furthermore, we evaluated the suitability of secreted cell biomarkers, albumin and urea, as nondestructive and sensitive endpoints for DILI hazard identification in comparison with traditional cell lysate-based endpoints, ATP and glutathione (GSH) levels. Given the ready availability of primary rat hepatocytes as opposed to the limited availability, at times variable quality and high expense of primary human hepatocytes, we also explored here the extent (sensitivity, specificity) to which rat hepatocytes in the MPCC model could predict human-relevant DILI, potentially as a first step prior to use of human cultures in drug development.

**MATERIALS AND METHODS**

**Materials**

Plateable cryopreserved primary human hepatocytes were purchased from Celsis In Vitro Technologies (Baltimore, MD) and Life Technologies (Carlsbad, CA). Plateable cryopreserved primary rat (Sprague Dawley) hepatocytes were purchased from Life Technologies. The cryopreserved human hepatocytes used in this study were from different donors: Hu4165 from Life Technologies (a 57-year-old Caucasian female who died of anoxia and was previously on clonidine and sympathoid), YRT from Celsis (a 37-year-old Caucasian male who died of a cerebrovascular arrest (stroke) and had no known drug use), VEP from Celsis (a 56-year-old African American diabetic male on insulin therapy who died of cerebrovascular arrest and previously had kidney disease and hypertension), and JNB from Celsis (a 19-year-old Caucasian female with a history of lupus whose cause of death was listed as intracranial hemorrhage secondary to stroke). CellTiter-Glo (ATP levels) and GSH-Glo (GSH levels) were purchased from Promega (Madison, WI). All compounds were purchased from either Sigma (St Louis, MO) or Toronto Research Chemicals (North York, Ontario, Canada) and were of the highest purity available. Hepregen Corporation (Medford, MA) provided MPCCs (also known as HepatoPac), Hepregen proprietary hepatocyte culture medium (HCM), and the stromal cells (3T3-J2 murine embryonic fibroblasts). HCM can be purchased from Hepregen Corporation.

**Methods**

**Preparation of MPCCs with primary hepatocytes.** Cryopreserved human and rat hepatocytes were thawed at 37°C for 90–120 s followed by dilution with 50mL of warm HCM. The cell suspension was spun at 50 x g for 5 min. The supernatant was discarded, cells were resuspended in HCM, and viability was assessed using trypan blue exclusion (typically 80–95%). Liver-derived nonparenchymal cells, as judged by their size (~10 μm in diameter) and morphology (nonpolygonal), were consistently found to be less than 1% in these preparations. To create MPCCs in 96-well plates, we first produced a hepatocyte pattern by seeding hepatocytes on collagen-patterned substrates that mediate selective cell adhesion. The cells were washed with medium 4–6 h later to remove unattached cells (leaving ~5000 attached hepatocytes on 13 collagen-coated islands within each well of a 96-well plate) and incubated in HCM. Stromal fibroblasts were seeded 12–18 h later to create cocultures. Culture medium was replaced every 2 days (~65 μl per well) for 7–8 days prior to incubation with compounds. The MPCC model (HepatoPac) is patented and can be purchased from Hepregen Corporation.

**Hepatic health/functionality assays.** ATP and GSH levels in the cell lysates were measured using the CellTiter-Glo and GSH-Glo luminiscence kits from Promega per manufacturer’s instructions, respectively. Urea concentration was assayed using a colorimetric endpoint assay kit using diacetylmonoxime with acid and heat (Stanbio Labs, Boerne, TX). Albumin content was measured using an enzyme-linked immunosorbent assay (MP Biomedicals, Santa Ana, CA) with horseradish peroxidase detection and 3,5,5′-tetramethylbenzidine (Fitzgerald Industries, Acton, MA) as a substrate (Khetani and Bhatia 2008). All assays were multiplexed in the same set of wells, except for GSH-Glo, which required a separate set of cultures due to incompatibility with the CellTiter-Glo assay.

**Compound selection and dosing.** To test for reproducibility of the selected hepatic health assays and MPCCs in a 96-well format, human cultures (JNB donor) were dosed twice over 5 days with six prototypical compounds (up to 100°Cmax for troglitazone, diclofenac, tolmetin, ibuprofen, and aspirin and up to 30°Cmax for tetracycline) in triplicate wells in two different sets of MPCC plates. All data were normalized to vehicle only controls. Next, to assess the sensitivity and specificity of MPCCs (Hu4165 donor), 10 compounds known to cause clinical DILI (DILI positive, true positives [TPs]), 10 compounds not known to cause clinical DILI (DILI negative, true negative [TN]), and 25 compounds not identified correctly as DILI positive in short-term sandwich cultures of primary human hepatocytes (false negative [FN] compounds) were selected from a previous study (Xu et al., 2008). Compounds selected spanned a variety of exposure (Cmax) ranges and a several drug classes in order to ensure that a diverse set of compounds was analyzed in this study. An additional 19 compounds classified as having most DILI concern were selected from the Liver Toxicity Knowledge Base (LTKB) organized by the Food and Drug Administration (FDA) (Chen et al., 2011) and tested on human
MPCCs (YRT and VEP donors). Compounds were dissolved in 100% dimethyl sulfoxide (DMSO), and the final concentration of DMSO in culture medium was kept at less than 0.5% vol/vol.

MPCCs were first allowed 7 days to stabilize as cocultures. Then, four doses (1, 30, 60, and 100*Cmax, total human plasma concentration) of each drug were applied to the cultures on days 7, 9, 12, and 14 days of culture (four repeat drug administrations in total), followed by assessment of liver cell health (ATP and GSH content) and functions (albumin and urea synthesis) either on day 12 (two repeat dosing over 5 days) or on day 16 (four repeat dosing over 9 days). ATP was not conducted on rat hepatocytes due to limited availability of cryopreserved vials for the donor used in this study. Results for each compound and assay were obtained for MPCCs using primary human or rat hepatocytes and stromal-only control plates in order to calculate the hepatocyte-only responses in MPCCs. GSH and ATP signals in MPCCs were ~1.5- to 2-fold and 8- to 10-fold higher than in stromal controls, respectively. GSH and ATP signals in fibroblast-only control cultures were subtracted from those of MPCCs to obtain calculated behavior of hepatocytes. Stromal controls did not secrete albumin or urea (data not shown) as these are liver-specific functions of hepatocytes.

**Data analysis.** Microsoft Excel (MS-Excel) was used for data analysis, whereas GraphPad Prism (San Diego, CA) was used for plotting data. For each assay, data were normalized to vehicle controls, and mean and standard deviation were calculated (three technical replicates) for each of the drug doses administered. TC50 (concentration that decreased the response by 50%) values for each assay were interpolated using linear curve fitting between the dose at which the assay signal was greater than 50% of control values and the dose at which the assay signal was less than or equal to 50% of control values. Sensitivity was defined as the fraction of correctly predicted positives to all positives in the clinic ([TP]/[TP + FN]), and specificity was defined as the fraction of correctly predicted negatives to all negatives in the clinic ([TN]/[TN + FP]). Each positive or toxic and negative or nontoxic call was confirmed in at least two independent experiments from the same donor. The initial assay validation data (Supplementary figs. 1–4) were generated on 5 different days.

**RESULTS**

**System Reproducibility**

The reproducibility of hepatic health-functional assays in 96-well format MPCCs was first determined via dosing with six prototypical compounds (trogloxetine, diclofenac, tolmetin, tetracycline, ibuprofen, and aspirin) in 3–5 separate sets (days) of human cultures. This was done using the JNB hepatocytes. The luminescent assays (GSH-Glo and CellTiter-Glo) in cell lysates had average coefficient of variation (CV) values between 7 and 11%, thereby showing reproducible micropatterning of cocultured cells within wells of the same plate and across different plates (Supplementary figs. 1 and 2). The supernatant-based assays (albumin ELISA and colorimetric urea) had higher average CV values of 17 to 24% (Supplementary figs. 3 and 4) due to yet undetermined causes. Nonetheless, the variability of the system was within ranges typical for primary cell-based assays and sufficient for subsequent toxicity analyses.

**Compound Selection and Criteria for Toxic/Nontoxic Calls**

A total of 45 compounds were tested using Hu4165 (at 1, 30, 60, and 100*Cmax doses) to determine the sensitivity and specificity of MPCCs. This hepatocyte lot and the compounds were chosen as a direct comparison to the Xu et al. (2008) hepatocyte imaging assay technology (HIAT) data. Ten of these compounds were reported previously as DILI positive (TP), meaning that human liver toxicity was observed for these drugs in conventional sandwich cultures at a concentration below 100*Cmax total (maximum plasma concentration) reported for humans (Xu et al., 2008). Ten drugs were void of such characteristic and were considered safe in humans (TN). These compounds were negative in the conventional sandwich cultures at test concentration of 100*Cmax (Xu et al., 2008). The third category of DILI compounds were those that could not be identified utilizing sandwich cultures (FN) according to the results published by Xu et al. (2008) but are known to cause clinical DILI.

In our study, a compound was considered positive in the assay if the TC50 of at least one of the multiplexed assays was less than 100*Cmax (total) for that particular compound. A compound was considered negative if the TC50 for all assays utilized was greater than 100*Cmax of a particular drug. Table 1 summarizes the compound list, Cmax values, the DILI categorization, and the interpretive calls made in human and rat MPCCs after 9 days of dosing (four repeat drug administrations in total) using multiple assay endpoints. Calls made after 5 days of dosing (two repeat drug administrations in total) are not shown because the sensitivity was lower compared with 9 days of dosing and specificity was within 10% between the two time points. Where applicable, the DILI severity score from the LTKB of the FDA is shown next to the compound names in Table 1. Compounds for which we detected different results in the rat and human MPCCs are highlighted for emphasis. Cmax values for compounds ranged widely as previously mentioned from 0.005µM for buspirone to 793.9µM for hydroxyurea.

**DILI Positive Compounds**

TC50 values for multiple assay parameters for the 10 HIAT-positive compounds used in this study are displayed graphically in Figure 1A for human MPCCs. Human MPCCs correctly detected all 10 compounds as toxic based on the TC50 criteria described above. Albumin secretion was reduced by at least 50% in all 10 compounds, urea secretion was reduced in 9 of 10 compounds (except mebendazole), ATP (data not shown) was reduced in 9 of 10 compounds (except phenacetin), and GSH was reduced in 7 of 10 compounds (except mebendazole, phenacetin, and flurbiprofen). Furthermore, 7 of 10 compounds (benzbromarone, clozapine, trazodone, diclofenac, quinine, mefanamic acid, and phenylbutazone) caused at least 50% reduction in all four parameters tested, whereas 3 compounds caused reduction in two parameters (phenacetin—albumin and urea, mebendazole—albumin and ATP, flurbiprofen—albumin and urea). Based on results above, albumin and urea were necessary and sufficient to identify all 10 compounds as toxic in the assay. Although GSH is a useful mechanistic marker to detect potential depletion via reactive metabolites, its depletion does not always result in overt toxicity (Watanabe et al., 2003). On the other hand, downregulation of ATP levels has been shown to indicate overt toxicity (Lawal and Ellis, 2010),
whereas albumin and urea are critical functions of the liver (Khetani and Bhatia, 2008).

Rat MPCCs (Fig. 1B) detected 8 of 10 HIAT-positive compounds as toxic using three assays (GSH, albumin, and urea), with mebendazole and phenacetin being classified as FNs. Urea secretion and GSH were reduced at least 50% in all eight compounds correctly detected as toxic, whereas albumin secretion was reduced in seven compounds. Additionally, seven

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound name</th>
<th>DILI category</th>
<th>Cmax (µM)</th>
<th>Clinical DILI</th>
<th>Sandwich culture (Xu et al., 2008; Hu4165)</th>
<th>Hu-MPCC DILI</th>
<th>Rat MPCC DILI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Benzbromarone (–2)</td>
<td>P1</td>
<td>4.361</td>
<td>Positive</td>
<td>Positive Positive Positive Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Clozapine (2)</td>
<td>P2</td>
<td>0.951</td>
<td>Positive</td>
<td>Positive Positive Positive Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Diclofenac (7)</td>
<td>P2</td>
<td>8.023</td>
<td>Positive</td>
<td>Positive Positive Positive Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Flurbiprofen (3)</td>
<td>P2</td>
<td>57.356</td>
<td>Positive</td>
<td>Positive Positive Positive Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Mefenamic acid (N/A)</td>
<td>P2</td>
<td>26.959</td>
<td>Positive</td>
<td>Positive Positive Positive Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Mebendazole (3)</td>
<td>P2</td>
<td>0.126</td>
<td>Positive</td>
<td>Positive Positive Positive Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Phenacetin (N/A)</td>
<td>P2</td>
<td>13.401</td>
<td>Positive</td>
<td>Positive Positive Positive Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Phenylbutazone (N/A)</td>
<td>P2</td>
<td>486.772</td>
<td>Positive</td>
<td>Positive Positive Positive Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Quinine (N/A)</td>
<td>P2</td>
<td>9.254</td>
<td>Positive</td>
<td>Positive Positive Positive Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Trazodone HCl (N/A)</td>
<td>P2</td>
<td>5.065</td>
<td>Positive</td>
<td>Positive Positive Positive Positive</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TPs in HIAT

11 Aspirin (N/A) O2 5.526 Negative Negative Negative Negative Negative
12 Buspirone (3) N1 0.005 Negative Negative Negative Negative Negative
13 Dexamethasone (3) N1 0.224 Negative Negative Negative Negative Negative
14 Dextromethorphan HBr (N/A) N1 0.028 Negative Negative Negative Negative Negative
15 Fluoxetine (3) N2 0.049 Negative Negative Negative Negative Negative
16 Lidocaine (N/A) N1 36.298 Negative Negative Positive Positive Positive
17 Miconazole (N/A) N1 0.024 Negative Negative Negative Negative Negative
18 Prednisone (N/A) N2 0.068 Negative Negative Negative Negative Negative
19 Propranolol (3) N1 0.201 Negative Negative Negative Negative Negative
20 Warfarin (5) N2 4.868 Negative Negative Negative Negative Positive

TNs in HIAT

21 Acetazolamide (N/A) P2 135.142 Positive Negative Positive Positive Positive
22 Betahistine 2HCl (6) P2 0.004 Positive Negative Negative Negative Negative
23 Captopril (7) P2 4.284 Positive Negative Negative Negative Negative
24 Chloramphenicol palmitate (N/A) P2 19.991 Positive Negative Negative Negative Negative
25 Ciprofloxacin HCl (7) P2 11.476 Positive Negative Positive Positive Positive
26 Clonidine citrate (N/A) P2 0.022 Positive Negative Negative Negative Negative
27 Clonipramine (N/A) P2 0.191 Positive Negative Positive Positive Positive
28 Cyclophosphamide (5) P2 265.359 Positive Negative Positive Positive Positive
29 Cyproterone acetate (N/A) O1 0.656 Positive Negative Positive Positive Positive
30 Danazol (8) P1 0.074 Positive Negative Negative Negative Negative
31 Dapsone (N/A) P1 6.007 Positive Positive Positive Positive Positive
32 Epron (N/A) P2 0.022 Positive Negative Negative Negative Negative
33 Hydroxyurea (8) P2 793.925 Positive Negative Positive Positive Positive
34 Imipramine HCl (3) P2 0.087 Positive Negative Positive Positive Positive
35 Isosulfan (8) P1 76.609 Positive Positive Positive Positive Positive
36 Maleic acid (N/A) O1 1.000 Positive Positive Positive Positive Positive
37 Methimazole (8) P2 1.868 Positive Positive Positive Positive Positive
38 Nifedipine (3) P2 0.271 Positive Negative Negative Negative Negative
39 Norgestrel (N/A) P2 0.009 Positive Negative Negative Negative Negative
40 Norotiptyline HCl (8) P2 0.122 Positive Negative Positive Positive
41 Phenolamine mesylate (No DILI concern) P2 0.086 Positive Negative Negative Negative
42 Piroxicam (3) P2 5.135 Positive Negative Negative Negative Positive
43 Progesterone (N/A) P2 0.193 Positive Negative Negative Negative Negative
44 Pyrazinamide (3) P2 407.174 Positive Positive Positive Positive Positive
45 Tamoxifen (6) P2 0.162 Positive Negative Positive Positive Positive

FNs in HIAT

Notes. Shaded compound names indicate differences in calls between one or more culture models. Next to compounds names in parentheses are the DILI severity scores from the LTKB of the FDA (Chen et al. 2011), with N/A indicating not applicable/no information in the database, negative number indicating that drug was withdrawn from the market, and higher positive numbers indicating a greater DILI concern. DILI categorization: P1, DILI type 1, dose dependent (toxic); P2, DILI type 2, idiosyncratic (toxic); N1, not known to cause liver injury (nontoxic); N2, sporadic cases (< 10) of liver injury reported but generally considered safe drug to use by doctors (nontoxic); O1, hepatotoxic in animals untested in humans (toxic); O2, elevated liver enzymes observed in humans but does not lead to frank liver toxicity (nontoxic). HIAT, Hepatocyte Imaging Assay Technology using high content imaging on extracellular sandwich cultures of primary human hepatocytes.
compounds (benzbromarone, clozapine, trazodone, diclofenac, quinine, mefanamic acid, and flurbiprofen) caused at least 50% reduction in the three parameters tested for rat, whereas one compound caused reduction in two of the three parameters (phenylbutazone—urea and GSH).

**DILI Negative Compounds**

The 10 HIAT-negative compounds used in this study are displayed in Table 1 including their Cmax values, which ranged from 0.005 µM for buspirone to 36.3 µM for lidocaine. Nine of the 10 compounds in human MPCCs and 8 of the 10 compounds in rat MPCCs were correctly detected as negative (i.e., none of the assay signals dipped to 50% or lower) after 9 days of exposure. Lidocaine was detected as a FP in both human and rat MPCCs, whereas warfarin was detected a FP only in rat MPCCs.

**DILI FN Compounds**

Twenty-five compounds classified to cause human DILI, but previously not detected as positive in short-term cultures (Xu et al., 2008), were also tested in the MPCC model. These HIAT-FN compounds used are displayed in Table 1, including their Cmax values, which ranged from 0.004 µM for betahistine to 793.9 µM for hydroxyurea. Using human MPCCs, 13 of the 25 compounds were correctly detected as toxic after 9 days of dosing and based on the TC50 criteria described above. TC50 values for multiple assay parameters for these 13 compounds are displayed graphically in Figure 2A for human MPCCs. Urea

![Diagram](attachment:image.png)

**FIG. 1.** TC50 values (expressed in µM) of HIAT true positive compounds picked up correctly in human MPCCs (A) with comparison to rat MPCCs (B). Arrows indicate that TC50 could not be interpolated in dose range tested (1 to 100*Cmax). In rat MPCCs, mebendazole and phenacetin were detected as nontoxics (FNs). Data were generated using the Hu4165 hepatocyte donor lot.
FIG. 2. TC50 values (expressed in µM) of HLIAT-FN compounds picked up correctly in human MPCC (A) with comparison to rat MPCC (B). Arrows indicate that TC50 could not be interpolated in dose range tested (1 to 100*Cmax). In rat MPCC, pyrazinamide, isoniazid, dapsone, maleic acid, and imipramine were detected as nontoxics (FNs). Data were generated using the Hu4165 hepatocyte donor lot.
secretion was reduced by at least 50% in all 13 compounds, ATP (data not shown) was reduced in 12 compounds (except maleic acid), GSH was reduced in 11 compounds (except maleic acid and acetazolamide), and albumin secretion was reduced in 9 compounds (except cyproterone acetate, maleic acid, acetazolamide, isoniazid). Furthermore, 9 of 13 compounds (tamoxifen, imipramine, nortriptyline, clomipramine, dapsone, ciprofloxacin, cyclophosphamide, pyrazinamide, and hydroxyurea) caused at least 50% reduction in all four parameters tested, 2 compounds caused reduction in three parameters tested (isoniazid and cyproterone acetate—urea, GSH, ATP), 1 compound caused reduction in two parameters tested (acetazolamide—urea, ATP), and 1 compound caused reduction in one parameter tested (maleic acid—urea). Based on the results above, urea secretion was necessary and sufficient to call all 13 compounds as toxic. The 12 compounds that human MPCCs were unable to detect correctly as positive were betahistine, captopril, chloramphenicol, clomiphene, danazol, estrone, methimazole, nifedipine, norgestrel, phenolamine, piroxicam, and progesterone.

Rat MPCCs (Fig. 2B) identified 9 of the 25 HIAT-FN compounds correctly. Urea secretion was reduced at least 50% in all these nine compounds; albumin secretion was reduced in eight compounds (except nortriptyline), whereas GSH was reduced in two compounds, tamoxifen and cyclophosphamide. Additionally, two compounds (tamoxifen and cyclophosphamide) caused at least 50% reduction in the three parameters tested, six compounds caused reduction in two of the three parameters (piroxicam, clomipramine, cyproterone, ciprofloxacin, acetazolamide, hydroxyurea—albumin and urea), and one compound caused reduction in one parameter (nortriptyline—urea). The 16 compounds that rat MPCCs were unable to detect correctly as positive or toxic were betahistine, captopril, chloramphenicol, clomiphene, danazol, dapsone, estrone, imipramine, isoniazid, maleic acid, methimazole, nifedipine, norgestrel, phenolamine, piroxicam, and pyrazinamide.

Sensitivity and Specificity at TC50

We next examined the sensitivity (defined as the fraction of correctly predicted positives to all positives/toxics in the clinic) and the specificity (defined as the fraction of correctly predicted negatives to all negatives/nontoxics in the clinic). As shown in Table 2, the sensitivities of human MPCCs and rat MPCCs were 65.7 and 48.6% after 9 days of dosing, respectively. The specificities of human MPCCs and rat MPCCs were 65.7 and 48.6% after 9 days of dosing, respectively. Of the compounds detected toxic in human MPCCs, except warfarin and piroxicam, which were detected as positive toxins in human and rat MPCCs only.

<table>
<thead>
<tr>
<th>Actual DILI</th>
<th>MPCC-predicted DILI (TC50)</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Human: 23</td>
<td>Human: 12</td>
<td></td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Rat: 17</td>
<td>Rat: 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Human: 1</td>
<td>Human: 9</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Rat: 2</td>
<td>Rat: 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Human: 24</td>
<td>Human: 21</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Rat: 19</td>
<td>Rat: 26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. Sensitivity: human, 65.7%; rat, 48.6%. Specificity: human, 90%; rat, 80%. Total of 45 compounds from the HIAT database (Xu et al. 2008) were tested, 35 DILI positive and 10 DILI negative in the clinic. Sensitivity and specificity after 9 days of dosing (four repeat drug administrations) are shown.

We selected 45 drugs from the HIAT database, 10 drugs known to cause clinical DILI (HIAT-positive), 10 drugs void of clinical DILI (HIAT-negative), and 25 drugs known to cause concern in the LTKB by the FDA (Chen et al., 2011). We found that administering two doses to MPCCs over 5 days was sufficient to detect the toxicities of compounds with the most DILI concern. Human MPCCs correctly detected 19 of 19 (100%) prototypical hepatotoxins when tested across at least two donors. Three of the 19 compounds (troglitazone, valproic acid, and dacarbazine) showed donor-dependent differences across the final toxic or nontoxic calls made. Lastly, the effects of structural analog compounds were assessed on human MPCCs (YRT and VEP hepatocyte lots). Figure 3 shows that the human MPCCs were able to distinguish the relatively nontoxic drugs (levofloxacin, zolpidem, and entacapone) from their toxic analogs (trobavoxacin, alpidem, and tolcapone) withdrawn from the market.

DISCUSSION

Species-specific differences in drug metabolism necessitate supplementation of animal data with human liver models (Olson et al., 2000). An ECM sandwich can positively impact liver functions (Guillouzo and Guguen-Guillouzo, 2008; Lecluyse, 2001); however, hepatocytes display a phenotypic decline after ~1 day (Khetani and Bhatia, 2008). Xu et al. (2008) showed using high-content imaging that short-term (24h) dosing of sandwich cultures is useful for DILI assessment. Although the low FP rate of ~5% has been corroborated even in cell lines (O’Brien et al., 2006; Tolosa et al., 2012), the sensitivity of HIAT is ~50%. We hypothesized that the sensitivity of short-term primary hepatocyte assay could be improved if a stable culture was subjected to repeat drug treatments. We selected MPCCs of primary cryopreserved hepatocytes and 3T3-J2 murine embryonic fibroblasts in a 96-well format, shown to stabilize key rat and human liver–specific functions for ~4 weeks (Bhatia et al., 1999; Khetani and Bhatia, 2008). We selected 45 drugs from the HIAT database, 10 drugs known to cause clinical DILI (HIAT-positive), 10 drugs void of clinical DILI (HIAT-negative), and 25 drugs known to cause
clinical DILI, but they were not previously identified as such in sandwich cultures (HIAT-FN) (Xu et al., 2008). We chose human and rat hepatocytes to determine whether repeat dosing could improve assay sensitivity regardless of species and the extent to which species affects assay performance. Assay parameters related to liver functions (albumin, urea) and mechanistically linked to DILI (GSH, ATP) were selected (Grattagliano et al., 2009). Albumin and urea rely on ATP levels affected by mitochondria, whereas drug-mediated GSH depletion can indicate potential formation of reactive metabolites or oxidative stress (Masubuchi et al., 2007). Albumin and urea were not secreted by 3T3 cells, and GSH and ATP levels were ~1.5- to 2-fold and ~8- to 10-fold higher in MPCCs compared with 3T3 controls, respectively. Drug dose escalation up to 100°Cmax was chosen according to Xu et al. (2008). Inter- and intraplate variability in assay parameters across triplicate wells in multiple sets of cultures were found to be robust (Supplementary figs. 1–4) to enable reproducibility in drug dose-response curves and subsequent toxic/nontoxic calls. Finally, our results showed that although two repeat drug dose administrations were sufficient to detect the toxicities of drugs with the most DILI concern (Table 3) as classified in the LTKB organized by the FDA (Chen et al., 2011), four administrations showed higher sensitivity across a broader drug set without specificity loss (data not shown). Longer term dosing beyond day 9 was not possible consistently across all donors due to a decline in liver functions in serum-free dosing medium, potentially due to lack of stromal proliferation/health because MPCCs otherwise survive to ~4 weeks in serum-supplemented medium (Khetani and Bhatia, 2008).

All 10 HIAT-positive compounds known to cause clinical DILI were detected correctly in human MPCCs using a TC50 cutoff. Albumin secretion was the most sensitive (10 of 10 compounds), followed by urea secretion and ATP levels (9 of 10) and GSH levels (7 of 10). Therefore, nondestructive measurement of albumin and urea in medium could suffice for an initial toxicity assessment, whereas parameters such as ATP and GSH can be subsequently used for probing mechanism. Rat MPCCs detected 8 of 10 HIAT-positive compounds as toxic, with mebendazole and phenacetin called as FNs. Human liver cells are known to be more sensitive to mebendazole toxicity than cells from rodents (Higa et al., 1992). Phenacetin can cause liver necrosis in Gunn rats (Calder et al., 1981); however, toxicity to Sprague Dawley rats used here is not known. The rank ordering of compounds and TC50 values (Fig. 1) in rat MPCCs were similar to those observed in human MPCCs, except for phenylbutazone, for which TC50 values were an order of magnitude greater in rat compared with that in human, the mechanism of which is not known.

The specificity of human MPCCs based on 10 HIAT-negative compounds not known to cause clinical DILI (TN) was 90%, with lidocaine detected a FP after 9 days of dosing, which is in contrast to Xu et al. (2008) at 24 h of exposure. Some possible causes are that repeat dosing with lidocaine is detrimental

**FIG. 3.** Effect of structural analog compounds on human hepatocyte functions in MPCCs. Cultures were dosed at 50°Cmax for each compound twice over 5 days followed by assessment of liver functions. Alpidem Cmax, 0.2µM; zolpidem Cmax, 0.13µM; trovafloxacin Cmax, 4.08µM; levoflaxacin Cmax, 15.7µM; tolcapone Cmax, 16.5µM; entacapone Cmax, 3.9µM. Data are mean ± SD for separate experiments conducted with hepatocytes lots YRT and VEP. *p < 0.05 (Student’s t-test).
to hepatocytes, donor differences in CYP450s could lead to variable toxicity, and lidocaine inhibits CYP1A in cells over time leading to accumulation of toxic levels (Wei et al., 1995).

However, further experiments would be needed to verify one or more of these hypotheses. Repeat administration of warfarin also caused decrease in MPCC functions; however, human MPCCs experienced a ~30–40% decrease in urea secretion and GSH levels, whereas rat MPCCs experienced ~60–70% down-regulation of albumin synthesis, urea secretion, and GSH levels. The LTKB tags warfarin with some DILI concern in humans. Species-specific warfarin metabolism may underlie differential toxicities observed (Biagini et al., 2006).

Of the 25 HIAT-FN compounds, 13 were detected as positive/toxic in human MPCCs, whereas 9 were detected correctly in rat MPCCs. All detected compounds overlapped between species except for piroxicam, which was detected only in rat. Piroxicam has been found to be toxic to rat hepatocytes (Jurima-Romet et al., 1994), but in humans it causes borderline elevations of liver tests in ~15% of patients (Chen et al., 2011). Therefore, based on the 35 toxic drugs (HIAT-positive and HIAT-FN) chosen here, the sensitivities for human and rat MPCCs were 65.7 and 48.6%, respectively. For some undetected HIAT-FN compounds, toxicity differences among different strains of rats with variable drug metabolism competencies have been reported (Kovalenko et al., 2007; Masubuchi et al., 1999). Phentolamine is listed in the LTKB as having no DILI concern but reported to have DILI potential by Xu et al. (2008). It is evident that in order to build predictive models, compounds characterized for severity and incidence rate of DILI are needed. The growing LTKB should provide guidance in drug selection for continued validation of MPCCs and other liver models.

**Human MPCCs correctly detected all 19 additional drugs with the most DILI concern (per LTKB) when at least two donors were used (Table 3). Differences in TC50 values across the two donors were observed. Furthermore, troglitazone, valproic acid, and dacarbazine were not detected as toxic in one of the two donors. Further molecular profiling of the donors would be needed to elucidate mechanisms underlying donor differences. Therefore, multidonor human MPCCs coupled with repeat dosing are suited to screen out most, if not all, highly liver toxic compounds during prospective drug screening. For some TNs tested here, according to Xu et al. (2008), there is some DILI concern (i.e., buspirone, dexamethasone) listed in the LTKB. Additional donors would need to be screened using cellular stress markers (O’Brien et al., 2006; Xu et al., 2008; Tolosa et al., 2012), 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 coexisting genetic (i.e., P450 polymorphisms) and environmental (i.e., coadministered drugs) factors. The ability to prioritize structural analog compounds for further development represents a key utility of MPCCs and other in vitro systems. For instance,**

### TABLE 3

**Prototypical Hepatotoxins Tested in Human MPCCs From Two Donors (VEP and YRP From Celsis In Vitro Technologies) and Calls Made Based on TC50 Criteria (See Materials and Methods)**

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound name</th>
<th>DILI label</th>
<th>Cmax (µM)</th>
<th>Clinical DILI</th>
<th>Hu-MPCC TC50 (VEP)</th>
<th>Hu-MPCC TC50 (YRT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amiodarone (8)</td>
<td>Boxed warning</td>
<td>0.806</td>
<td>Positive</td>
<td>15.9 (14.2 – 20.5)</td>
<td>11.6 (8.1 – 16)</td>
</tr>
<tr>
<td>2</td>
<td>Bicalutamide (8)</td>
<td>Warnings</td>
<td>1.970</td>
<td>Positive</td>
<td>22.3 (10.3 – 38.5)</td>
<td>48.4 (19.2 – 90.6)</td>
</tr>
<tr>
<td>3</td>
<td>Bosentan (7)</td>
<td>Boxed warning</td>
<td>1</td>
<td>Positive</td>
<td>11.7 (11.4 – 12)</td>
<td>23.7 (12.1 – 35.3)</td>
</tr>
<tr>
<td>4</td>
<td>Bromfenac (−2)</td>
<td>Withdrawn</td>
<td>2.34</td>
<td>Positive</td>
<td>40.6 (27.8 – 65.2)</td>
<td>29 (19.8 – 35.5)</td>
</tr>
<tr>
<td>5</td>
<td>Carbamazepine (7)</td>
<td>Warnings</td>
<td>15</td>
<td>Positive</td>
<td>46.3 (44.5 – 48.4)</td>
<td>49.3 (37.4 – 66.3)</td>
</tr>
<tr>
<td>6</td>
<td>Dacarbazine (6)</td>
<td>Boxed warning</td>
<td>43.91</td>
<td>Positive</td>
<td>Negative</td>
<td>70.4 (46.3 – 94.5)</td>
</tr>
<tr>
<td>7</td>
<td>Glafenine (−2)</td>
<td>Withdrawn</td>
<td>1.878</td>
<td>Positive</td>
<td>12 (albumin only)</td>
<td>91.4 (albumin only)</td>
</tr>
<tr>
<td>8</td>
<td>Ibafenic (−2)</td>
<td>Withdrawn</td>
<td>100</td>
<td>Positive</td>
<td>56.8 (35.4 – 74.3)</td>
<td>29.6 (9.5 – 66.9)</td>
</tr>
<tr>
<td>9</td>
<td>KETOCONAZOLE (8)</td>
<td>Boxed warning</td>
<td>7</td>
<td>Positive</td>
<td>6.5 (6.1 – 6.6)</td>
<td>6.3 (5.3 – 7.3)</td>
</tr>
<tr>
<td>10</td>
<td>Leflunomide (8)</td>
<td>Boxed warning</td>
<td>24.79</td>
<td>Positive</td>
<td>31.7 (23.6 – 39.4)</td>
<td>31.9 (29.9 – 33.8)</td>
</tr>
<tr>
<td>11</td>
<td>Nefazodone (8)</td>
<td>Boxed warning</td>
<td>0.858</td>
<td>Positive</td>
<td>24.6 (17.7 – 31.7)</td>
<td>39.25 (32.5 – 45.5)</td>
</tr>
<tr>
<td>12</td>
<td>Nevirapine (8)</td>
<td>Boxed warning</td>
<td>37.468</td>
<td>Positive</td>
<td>77.3 (48.3 – 92.8)</td>
<td>77.1 (64.1 – 90.1)</td>
</tr>
<tr>
<td>13</td>
<td>Rifampin (8)</td>
<td>Warnings</td>
<td>9</td>
<td>Positive</td>
<td>47.6 (10.3 – 85.1)</td>
<td>28.7 (15.4 – 39.3)</td>
</tr>
<tr>
<td>14</td>
<td>Ticlopidine (4)</td>
<td>Warnings</td>
<td>7.094</td>
<td>Positive</td>
<td>7.4 (6.5 – 8.2)</td>
<td>23.8 (9.3 – 32)</td>
</tr>
<tr>
<td>15</td>
<td>Tolcapone (8)</td>
<td>Boxed warning</td>
<td>16.5</td>
<td>Positive</td>
<td>6.5 (6.2 – 6.8)</td>
<td>6.5 (6.3 – 6.6)</td>
</tr>
<tr>
<td>16</td>
<td>Troglitazone (−2)</td>
<td>Withdrawn</td>
<td>6.387</td>
<td>Positive</td>
<td>6.4 (6.2 – 6.5)</td>
<td>Negative</td>
</tr>
<tr>
<td>17</td>
<td>Trovafoxacin (−2)</td>
<td>Withdrawn</td>
<td>4.078</td>
<td>Positive</td>
<td>23.7 (6.5 – 35.5)</td>
<td>35.7 (15.4 – 56.1)</td>
</tr>
<tr>
<td>18</td>
<td>Valproic acid (8)</td>
<td>Boxed warning</td>
<td>30.086</td>
<td>Positive</td>
<td>77.3 (48.3 – 92.8)</td>
<td>77.1 (64.1 – 90.1)</td>
</tr>
<tr>
<td>19</td>
<td>Zafrilukast (8)</td>
<td>Warnings</td>
<td>1.211</td>
<td>Positive</td>
<td>6.3 (6.1 – 6.5)</td>
<td>6.4 (6.1 – 6.8)</td>
</tr>
</tbody>
</table>

**Notes.** Shaded compound names indicate differences in calls between the two hepatocyte donors used. Next to compounds names in parentheses are the DILI severity scores from the LTKB of the FDA (Chen et al., 2011), with negative numbers indicating that the drug was withdrawn from the market and higher positive numbers indicating a greater DILI concern. For the human hepatocyte donors, average TC50 values are given for three assays tested (albumin, urea, GSH) and ranges of TC50 in parentheses.
we have shown that the in vitro effects of thiazolidinediones (i.e., troglitazone, rosiglitazone) on human MPCCs correlate with severity of clinical DILI (Khetani and Bhatia, 2008). Here, we show that the severe liver toxicity of trovafloxacin, alipidem, and tolcapone (all withdrawn from market) can be observed in human MPCCs over relatively safe structural analogs.

Compounds tested here may cause injury through toxic metabolites that deplete GSH and bind to macromolecules (i.e., acetylamidone, diclofenac), dissipation of mitochondrial membrane potential (i.e., troglitazone, nefazodone), accumulation within mitochondria and disruption of respiration (i.e., amiodarone), steatosis (i.e., amiodarone, valproic acid), and inhibition of bile salt export protein (i.e., clozapine, rifampin) (Grattagliano et al., 2009). Repeat dosing may subject cells to several instances of multiple forms of aforementioned mechanisms that get integrated in a functional hepatocyte leading to overt cellular stress. A long-term model such as MPCCs could also prove useful for elucidating underlying mechanism of cellular injury and to evaluate toxicity due to drug-drug interactions. For instance, we have shown that acetylamidone toxicity in MPCCs is sensitive to induction of CYP450 levels via phenobarbital or inhibition of phase II enzymes via probenecid, which is consistent with the role of toxic metabolites (Grattagliano et al., 2009; Khetani and Bhatia, 2008).

In summary, improvement in sensitivity of DILI predictions without loss of specificity can be obtained through repeat dosing in MPCCs. Rat MPCCs provide for potentially acceptable sensitivity (~50%) as an initial screen; however, the increase in FPs in rat needs verification with more compounds. Studies are ongoing to evaluate MPCC sensitivity via longer term dosing (months), to improve specificity via repeat dosing (months), and to evaluate toxicity due to drug-drug interactions. For instance, we have shown that acetaminophen toxicity in MPCCs is sensitive to induction of CYP450 levels via phenobarbital or inhibition of phase II enzymes via probenecid, which is consistent with the role of toxic metabolites (Grattagliano et al., 2009; Khetani and Bhatia, 2008).

Supplementary DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

REFERENCES


