3D Organotypic Cultures of Human HepaRG Cells: A Tool for In Vitro Toxicity Studies

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Drug-induced human hepatotoxicity is difficult to predict using the current in vitro systems. In this study, long-term 3D organotypic cultures of the human hepatoma HepaRG cell line were prepared using a high-throughput hanging drop method. The organotypic cultures were maintained for 3 weeks and assessed for (1) liver specific functions, including phase I enzyme and transporter activities, (2) expression of liver-specific proteins, and (3) responses to three drugs (acetaminophen, troglitazone, and rosiglitazone). Our results show that the organotypic cultures maintain high liver-specific functionality during 3 weeks of culture. The immunohistochemistry analyses illustrate that the organotypic cultures express liver-specific markers such as albumin, CYP3A4, CYP2E1, and MRP-2 throughout the cultivation period. Accordingly, the production rates of albumin and glucose, as well as CYP2E1 activity, were significantly higher in the 3D versus the 2D cultures. Toxicity studies show that the organotypic cultures are more sensitive to acetaminophen- and rosiglitazone-induced toxicity but less sensitive to troglitazone-induced toxicity than the 2D cultures. Furthermore, the EC50 value (2.7mM) for acetaminophen on the 3D cultures was similar to in vivo toxicity. In summary, the results from our study suggest that the 3D organotypic HepaRG culture is a promising in vitro tool for more accurate assessment of acute and also possibly for chronic drug-induced hepatotoxicity.

Key Words: 3D cultivation; organotypic; HepaRG; hepatotoxicity.

Drug-induced hepatotoxicity, which is difficult to predict and often occurs during the clinical phases of drug development or postmarketing, poses a serious human health concern and accounts for approximately 30–50% of acute liver failure cases (Tuschl et al., 2008). The use of animals in toxicology studies is highly controversial due to ethical and scientific reasons (e.g., interspecies differences in drug metabolism and toxicity). Currently, primary human hepatocytes (PHHs) are considered the gold standard to study drug metabolism and toxicity in vitro (LeCuyse, 2001). However, several disadvantages are associated with the use of PHHs, including their limited availability, varying disease etiology and therapy of donors, limited viability, and early dedifferentiation to progenitor-like cells lacking relevant gene expression (Aninat et al., 2006; Godoy et al., 2009). Although PHHs, due to their inherent interindividual variability, allow characterization of the susceptibility to metabolism-mediated drug toxicity, the genetic polymorphisms become necessary to risk assessment mainly if they alter the pharmacokinetic outcomes (Lipscomb et al., 2003). On the other hand, a stable biotransformation competence is a prerequisite for reliable and predictive in vitro toxicity assessment (Coecke et al., 2006). Therefore, there is an urgent need for alternative in vitro human hepatic models, especially those that retain their phenotypic characteristics for longer time periods and can be used to assess drug-induced hepatotoxic effects in humans more accurately.

Conventional 2D hepatocyte cultures do not represent the complex 3D structure of the liver in vivo (Lin et al., 2008), which is imperative for normal cell physiology and function (Griffith and Swartz, 2006; Lin et al., 2008; van Zijl and Mikulits, 2010). As such, physiological and functional hepatic models will have a tremendous impact on the accuracy of prediction. Studies have shown that the human HepaRG cell line (Gripón et al., 2002) is a useful cellular model to investigate drug-induced hepatotoxicity. The 2D monolayer cultures of HepaRG cells have been well characterized, and results indicate that they show phenotypic characteristics that are similar to adult PHHs, including phase I, II, and III enzymes, are sensitive toward prototypical inducers, and can be cultivated for several weeks with stable phenotype (Guillouzo et al., 2007; Josse et al., 2008; Kanebratt
and Andersson, 2008). However, these cells have not been well characterized for their ability to mimic long-term drug-induced toxicity and for phenotype alterations in 3D cultures.

Currently, several 3D in vitro human hepatic models are in use (i.e., isolated whole and perfused liver slices, and perfusion bioreactors). However, the existing systems are technically challenging, labor intensive, and not suitable for high-throughput applications. In addition, perfused whole or liver slices have relatively short viability (Guillouzo, 1998). Perfused whole human livers are viable only for 2–3 h, whereas human liver slices are viable for up to 3–5 days (Van de Bovenkamp et al., 2007) and therefore cannot be used for chronic toxicity studies. Even though the hollow fiber bioreactor systems allow good viability, perfused human livers are viable only for 2–3 h, whereas human liver slices are viable for up to 3–5 days (Van de Bovenkamp et al., 2007) and therefore cannot be used for chronic toxicity studies.

The 3D hepatocyte cultures show liver-like structures and functionality for several weeks and showed a dose-dependent effect to acetaminophen exposure (Leite et al., 2012).

The objective of this study was to obtain, maintain, and characterize long-term 3D HepaRG organotypic cultures using a scaffold-free, high-throughput hanging drop system (Drewitz et al., 2011). This method allows medium change and precise control over cell number and hence the spheroid size. In order to determine whether the 3D HepaRG organotypic cultures may be a more appropriate model to study drug toxicity, 2D and 3D HepaRG cultures were maintained in parallel for 3 weeks and compared in terms of (1) liver-specific functions, including albumin and urea production, phase I enzyme, and transporter activities; (2) expression of liver-specific proteins; and (3) acute toxicity of three drugs, namely acetaminophen, troglitazone, and rosiglitazone.

Our results show that the 3D organotypic HepaRG cultures maintain high liver-specific functionality during 3 weeks of culture and exhibit higher sensitivity for acetaminophen and rosiglitazone toxicity. The data suggest that the 3D organotypic HepaRG cultures provide a suitable in vitro tool for the assessment of drug-induced hepatotoxicity.

MATERIALS AND METHODS

Cell culture. The differentiated human HepaRG cells (Biopredic International, France) were maintained according to the supplier’s recommendations with minor modifications. Cells were thawed and seeded using Williams E medium; GlutaMAX (Invitrogen Life Technologies, Sweden) supplemented with the thaw, seed, and general purpose additive; and ADD670 (Biopredic International). On culture day 3, 50% medium was exchanged with Williams E medium, GlutaMAX supplemented with the serum-free induction additive, ADD650 (Biopredic International). Cultures were maintained in a sterile environment at 37°C, 95% air, and 5% CO₂.

3D HepaRG organotypic cultures. The HepaRG cells (2000 cells per drop) were seeded into the GravityPLUS plates (InSphero AG, Zurich, Switzerland). The spheroids were transferred to the GravityTRAP plates on culture day 4 and maintained until the end of the experimental period. The culture volumes were 40 and 70 µl in the GravityPLUS and GravityTRAP plates, respectively.

2D HepaRG monolayer cultures. The HepaRG monolayer cultures were cultivated on type 1 rat tail collagen (5 µg/cm²; BD Biosciences)-coated cell culture plates. Cells were seeded (72,000 or 480,000 cells per well in 96- or 24-well plates, respectively) to form confluent monolayers as recommended by the manufacturer. The culture volume was 100 or 300 µl in 96 or 24-well cell culture plates, respectively.

Clinical chemistry. Albumin, urea, pyruvate, lactate, and glucose concentrations were measured in the pooled media from 60 wells of 2D or 3D cultures. Specific consumption and production rates for albumin and metabolites were calculated by subtracting the respective blank medium (medium incubated without cells) and normalizing to the initial cell number (2000 cells per sphere in 3D and 72,000 cells per well in 2D).

Albumin. The albumin concentration in cultures was quantified using high-performance liquid chromatography (HPLC), according to a previously described method (Clark et al., 2007).

Glucose, lactate, and pyruvate. An HPLC method was used to determine the glucose, lactate, and pyruvate concentrations in supernatants as previously described (Strigan et al., 2011).

CYP2E1 enzyme activity. The CYP2E1 enzyme activity assay was performed on days 5, 10, 15, and 21 for both 2D and 3D cultures. The CYP2E1 activity assay was assessed by measuring the conversion of chlorozoxazone to OH-chlorozoxazone. Briefly, the 2D and 3D cultures were incubated (replicates of 3 or 17, respectively) with chlorozoxazone (300 µmol/L) in medium for 1 h, after which the pooled media was collected and stored at −20°C. For analyses, the frozen samples were thawed at room temperature and diluted (1:2) with 0.1% formic acid in ultrapure water. OH-chlorozoxazone was quantified by HPLC tandem mass spectrometry. The system consisted of an AB Sciex (Framingham) API3200 triple quadrupole mass spectrometer interfaced with an Agilent (Santa Clara) 1200SL HPLC. Chromatography was performed at 70°C with 20 µl injection volume using a Zorbax Eclipse XDB C18 column (50 mm × 4.6 mm, 1.8 µm particle size), at a flow rate of 1.5 mL/min. The column eluent was split to an electrospray ionization interface, operating at 650°C in multiple reaction monitoring (MRM) mode in negative polarity. The MRM reaction was 183.9 → 119.9. The mobile phase was 0.01% formic acid in ultrapure water (A) and acetonitrile (B). The proportion of the mobile phase B was increased linearly from 10 to 50% in 1.2 min and then the column was flushed with 95% mobile phase B and then allowed to re-equilibrate to the initial conditions. The total run time was 3.0 min. Retention time was 1.02 min for OH-chlorozoxazone.

MRP-2 transporter activity. A fluorescence-based assay was used to investigate MRP-2 transporter activity in 2D and 3D HepaRG cultures. The membrane permeable and nonfluorescent substrate 5-chloromethylfluorescein diacetate (CMFDA) was used as substrate. The CMFDA is converted by
intracellular esterases to a membrane-impermeable compound, which subsequently reacts with glutathione to form glutathione-methylfluorescein (GSMF). The GSMF is a substrate of the membrane transporter MRP-2 and is excreted out of the cell into the bile canaliculi. The 3D organotypic cultures were incubated on culture day 10 with 5µM CMFDA for 30 min. The dye solution was aspirated, and the cells were subsequently incubated with serum-free medium for 45 min. The cultures were then washed (2×) with PBS solution (pH 7.4). The fluorescence (excitation/emission wavelengths: 492/517 nm, respectively) in the 2D cultures was monitored using an Olympus IX 70 fluorescence microscope (Muenster, Germany). The fluorescence in the 3D cultures was assessed using a CLSM confocal microscope (LSM 510; Zeiss, Jena, Germany) equipped with an argon/neon laser. The FITC-WGA channel (excitation 488 nm, band pass filter 500–530 nm) was used for imaging.

Hematoxylin and eosin staining. The HepaRG spheroid cultures on day 20 were washed (2×) with cold PBS and incubated in 4% formaldehyde (overnight at 4°C). Fixed spheroids were pooled together, collected in a microcentrifuge tube and submitted to Histo-Center AB (Stockholm, Sweden) for further processing and standard hematoxylin and eosin (HE) staining procedures.

Immunohistochemistry (IHC) of HepaRG spheroid cultures. HepaRG spheroid cultures were fixed and collected as described previously on culture days 10 and 21 and sent to Histo-Center AB for further processing and immunohistochemical staining for CYP2E1 (the antibody was previously produced in our laboratory at Karolinska Institutet), MRP-2 (ab3373, Abcam, United Kingdom), CYP3A4 (PAP01, Cypex Limited, United Kingdom), albumin (A80-229F, Bethyl Laboratories, Inc.), and Ki67 (M7240, Dako, Sweden). The donkey DyLight anti-mouse 488 (ab98794, Abcam) and anti-rabbit 650 (A80-229F, Bethyl Laboratories, Inc.) secondary antibodies were used. The fluorescence was monitored using a confocal microscope (LSM 710; Zeiss, Jena, Germany).

Acute toxicity studies. Stock solution of acetaminophen was prepared in dimethylsulfoxide. The 2D and 3D cultures were exposed to various concentrations of acetaminophen (0.5, 1, 5, 10, 15, 20, 40, or 80 mmol/l), troglitazone, or rosiglitazone (1.5, 10, 50, 100, 200, 300, or 500 mmol/l) for 24 h on culture days 4 and 21. Corresponding untreated and vehicle controls were also tested. Triton X-100 (0.1%) was used as positive control. Cell viability was assessed using a MicroBeta2 LumI-JET ATP assay kit (Promega, Sweden) according to the manufacturer’s recommendations. The luminescent signal was measured using a MicroBeta2 LumiJET ATP assay kit (Promega, Sweden) according to the manufacturer’s recommendations. The 2D and 3D cultures were incubated on culture day 10 with 5µM CMFDA for 30 min. The dye solution was aspirated, and the cells were subsequently incubated with serum-free medium for 45 min. The cultures were then washed (2×) with PBS solution (pH 7.4). The fluorescence (excitation/emission wavelengths: 492/517 nm, respectively) in the 2D cultures was monitored using an Olympus IX 70 fluorescence microscope (Muenster, Germany). The fluorescence in the 3D cultures was assessed using a CLSM confocal microscope (LSM 510; Zeiss, Jena, Germany) equipped with an argon/neon laser. The FITC-WGA channel (excitation 488 nm, band pass filter 500–530 nm) was used for imaging.

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RESULTS

Spheroid Formation and Structure

As illustrated in Figure 1, compact cell aggregates were formed on culture day 3. Initial cell seeding densities of 500, 2000, or 8000 cells produced spheroids with diameters of approximately 100, 200, or 450 µm on culture day 6, respectively. The 3D spheroids did not grow in size throughout the experimental period indicating constant cell number over time. The HE staining (Fig. 2) of 3D HepaRG spheroids at day 20 shows compact structure of the microtissue, consisting of viable cells with intact 4’,6-diamidino-2-phenylindole–stained nuclei and cytoplasm. Some lipid droplets were also observed.

IHC Analyses of the HepaRG Spheroid Cultures

We carried out IHC analyses for some key hepatic enzymes. As revealed from Figures 3a and 3b, the HepaRG organotypic cultures exhibited prominent CYP3A4 protein expression that appeared to be higher on day 10 than on day 21. Liver-specific albumin expression was significantly observed within hepatocytes throughout the 3D organotypic cultures on both culture days 10 and 21 (Figs. 3c and 3d), whereas only low expression of the Ki67 protein was observed in the spheroid cultures on both culture days, indicating minimal proliferation (Figs. 3d and 3e).

Functional Properties of the Spheroids

The spheroids were monitored for production of typical hepatic products such as albumin, urea, glucose, lactate, and pyruvate. The albumin production was consistently higher in 3D cultures, compared with 2D cultures throughout the experimental period (Fig. 4a). The urea production was generally higher in 3D cultures compared with 2D cultures (Fig. 4b) and mainly detected in 3D cultures after more than 2 weeks of cultivation with an extensive increase on days 19 and 21. A similar pattern was also seen for glucose production (Fig. 4c) mainly after 2 weeks of 3D culture. In contrast to the 3D cultures, glucose was consistently consumed in the 2D cultures throughout the experimental period. Lactate production was consistently higher in the 3D versus the 2D cultures (Fig. 4d), whereas pyruvate was produced mainly at the end of the cultivation period (Fig. 4e). In contrast, the 2D cultures consumed pyruvate from the culture medium during the whole cultivation.

CYP2E1 Protein Expression and Enzyme Activity

The CYP2E1 expression was observed throughout the 3D HepaRG spheroids as illustrated in Figures 5a and 5b. CYP2E1 activity was observed in both culture systems during 3 weeks of cultivation (Fig. 5c). The enzyme activity was consistently higher (approximately sevenfold) in the 3D versus the 2D cultures. It decreased in the 3D cultures on day 21.

MRP-2 Protein Expression and Activity

Figures 6a and 6b illustrate MRP-2 protein expression throughout the HepaRG spheroid cultures on days 10 and 21, respectively. Accordingly, the MRP-2 transporter was found to be functional in both the cultures. The accumulation of the fluorescent substrate in the bile-canaliculi was observed in both the 2D and 3D cultures on day 10 (Figs. 6c and 6d). The 2D cultures show distinct clusters of hepatocyte-like cells, where the bile-canaliculi are localized, whereas in case of 3D cultures the accumulated substrate was observed throughout the spheroid.

Acute Toxicity Studies

The acute toxicity (24 h exposure) of acetaminophen, troglitazone, and rosiglitazone was determined on culture days 5
Moreover, to check interlab reproducibility, we compared EC$_{50}$ values of acetaminophen and troglitazone (both 2D and 3D HepaRG cultures) assessed at Lab 1 (Karolinska Institute, Sweden) and Lab 2 (Saarland University, Germany). The results of these independent experiments are shown in Table 3.

Figures 7a and 7b illustrate representative concentration-response curves for 2D and 3D HepaRG cultures exposed to acetaminophen for 24 h. The EC$_{50}$ value was approximately 10- and 3-fold lower in the 3D versus the 2D cultures on culture days 5 and 22, respectively ($p < 0.001$). The EC$_{50}$ value increased for both cultures on day 22; however, the larger increase (approximately 10-fold) was observed in the 2D versus the 3D cultures. Figures 7c and 7f illustrate the concentration-response curves for rosiglitazone after 24h exposure. The 3D cultures were more sensitive to rosiglitazone-induced toxicity at both culture days ($p < 0.001$). The EC$_{50}$ value remained the same for the 2D cultures on the 2 test days, but a small increase (approximately 1.2-fold) was observed in the 3D cultures on day 22 compared with day 5.

**DISCUSSION**

Currently, there is an urgent need for *in vitro* hepatic models that can predict drug-induced hepatotoxic effects in humans more accurately. The main objective of this study was to obtain, maintain, and characterize long-term 3D HepaRG spheroid cultures using the hanging drop method.

Depending on the initial cell number, formation of 3D HepaRG organotypic cultures takes 3–6 days in the GravityPLUS plates. In the hanging drop system, the formation of spheroids is induced by integrin-extracellular matrix binding followed by cadherin-cadherin interactions, resulting in strong cell-cell adhesion (Lin et al., 2006).

The spheroid size is critical due to the risk of necrotic cores caused by limited oxygen diffusion in spheroids with diameters larger than 200 µm (Lin et al., 2008). An initial cell number of 2000, which resulted in the reproducible production of spheroid sizes of about 200 µm, was found to be the best in our study. The HE staining showed a compact spheroid structure with high cell viability without a necrotic core. Spheroids of proliferating or expanding cells show rapid increase in spheroid diameter as shown in a recent study with HepG2 spheroids (Mueller et al., 2011a). HepaRG cultures do not proliferate at differentiated state (Antherieu et al., 2012). In our study, the sizes of the HepaRG spheroids were constant throughout the experimental...
period. Therefore, we assume a constant cell number in the HepaRG spheroids. The clinical chemistry data normalized to the initial seeded cell numbers show that these 3D cultures are highly active during 3 weeks of cultivation; albumin and urea production was consistently higher in the 3D versus the 2D cultures. Gene expression analyses have shown that hepatocytes maintained as spheroids express most genes involved in albumin secretion, ammonia removal, and drug metabolism at higher levels and for longer time periods compared with monolayer cultures that dedifferentiate rapidly (Sakai et al., 2010; Tamura et al., 1995). Interestingly, the spheroids produced increasing amounts of glucose in the late cultivation phase. In contrast, the 2D monolayer cultures constantly consume glucose. Sakai et al. (2010) have reported the expression of glucose-6-phosphatase in rat hepatocyte monolayer cultures, which was the only gene expressed at a similar level as the liver tissue and liver spheroids, suggesting only minimal sugar metabolism as dedifferentiation occurs more easily in monolayers. Concurrent net glucose and lactate production was also observed in PHHs cultivated in complex 3D bioreactors (Zeilinger et al., 2011). Parallel to glucose production, lactate and pyruvate secretion was also increased in the late cultivation phase of the spheroid cultures. The production of glucose via gluconeogenesis and simultaneous production of lactate via glycolysis may seem

**FIG. 3.** Protein expression in 3D HepaRG cultures. The expression of CYP3A4 (a and b), albumin (c and d), and Ki67 (e and f) in 3D HepaRG cultures on days 10 and 21. Scale bars represent 50 µm.
peculiar. However, this phenomenon has been reported and suggested due to tight compartmentalization of glycolysis and gluconeogenesis (Jones et al., 2002; Phillips et al., 2002). This observation is also supported by our results hinting that lactate produced via glycolysis is secreted and not directly used as gluconeogenic precursor. Amino acids and glycerol are other gluconeogenic precursors. Further studies on the metabolome and 13C metabolic flux analyses can help identify active metabolic pathways and the degree of exchange reactions of glucose, lactate, or other metabolites between intra- and extracellular compartments.

The HE staining on HepaRG spheroids (day 20) clearly illustrates viable cells with intact nuclei and cytoplasm throughout the spheroids. However, the presence of some fat droplets was observed within the hepatocytes which may be due to aging although a minimal amount of intrahepatic lipid storage may be hepatoprotective (Gibb and Anderson, 2008). 2D HepaRG cultures constitute hepatocytes and cholangiocytes in approximately 1:1 ratio (Cerec et al., 2007). In contrast, Leite et al. (2012) showed the presence of cholangiocytes presumably at the core of the spheroids. However, in our system, very few cholangiocytes (Supplementary fig. S1) were observed in the 3D HepaRG spheroids indicating that most cells within the spheroids are hepatocytes that acquire normal cytoskeleton structure and maintain intercellular organization and liver-specific functions. It has been suggested that in hepatocyte spheroids the stable maintenance of cell-cell interaction and communication may activate the expression of liver-enriched transcription factors and therefore a highly differentiated hepatic phenotype (Sakai et al., 2010).

The IHC analyses revealed that the spheroids expressed several hepatocyte-specific proteins including albumin, CYP2E1, CYP3A4, and MRP-2. The expression of albumin appeared higher on day 21 versus day 10 corresponding to the high-albumin production rate on day 21. The CYP2E1 protein was expressed in the 3D HepaRG cultures. Moreover, the organotypic cultures maintained CYP2E1 enzyme activity throughout the 3 weeks of cultivation period, and the activity was consistently higher (approximately 11 fold) in the 3D versus the 2D cultures. The activity of CYP2E1 decreased slightly from days 10 to 21 but remained at least 8-fold higher than in the 2D cultures. The CYP2E1 enzyme metabolizes numerous xenobiotics of toxicological interest, including ethanol, carbon tetrachloride, and acetaminophen (Knockaert et al., 2011). A recent study with 3D HepaRG aggregates demonstrated higher phase I and II enzyme activities (Leite et al., 2012) compared with 2D cultures for longer periods of time.

The 3D organotypic HepaRG cultures show both high expression and functional activity of the phase III efflux transporter MRP-2. The transporter which is located in the apical membrane of polarized cells including hepatocytes plays an imperative role in the detoxification of xenobiotics (Jedlitschky et al., 2006) and therefore is crucial in any in vitro hepatic model used to study drug-induced toxicity. The result of the functional activity assay indicates a liver-like cellular polarization of the hepatocytes within the spheroids.
The toxicity of acetaminophen, troglitazone, and rosiglitazone was assessed in the early and late phases of cultivation. At both time points, the 3D organotypic cultures were significantly more sensitive ($p < 0.001$) to acetaminophen-induced toxicity than the 2D cultures. In the liver, acetaminophen is metabolized to the corresponding glucuronide and sulfate conjugates via phase II enzymes and to N-acetyl-p-benzoquinone imine (NAPQI) mainly by CYP2E1 (McGill et al., 2011; Mitchell et al., 1973). Our results illustrate that the 3D organotypic cultures have higher CYP2E1 functional activities. Enhanced production of NAPQI via CYP2E1 will likely result in the depletion of intracellular glutathione and increased cytotoxicity. On day 5, the EC$_{50}$ value of acetaminophen in the 3D organotypic cultures was 2.7mM, whereas it was 10.1 mM on day 22. This is in good
FIG. 6. MRP-2 protein expression in 3D HepaRG cultures and MRP-2 transporter activity in 2D and 3D cultures. MRP-2 protein expression is shown for 3D HepaRG cultures on cultivation day 10 (a) and 21 (b). Scale bars represent 100 µm. MRP-2 activity, assessed by CMFDA assay, is shown for 2D (c) and 3D (d) HepaRG cultures on cultivation day 10.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>EC_{50} Values and Corresponding Ranges for Acetaminophen, Troglitazone, and Rosiglitazone Assessed on 2D and 3D HepaRG Cultures Upon 24 h Exposure on Day 5</th>
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<td>2D</td>
<td>3D</td>
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<td>Troglitazone</td>
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p-values indicate significant statistical differences between EC_{50} values in 2D and 3D cultures.

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<tr>
<th>TABLE 2</th>
<th>EC_{50} Values and Corresponding Ranges for Acetaminophen, Troglitazone, and Rosiglitazone Assessed on 2D and 3D HepaRG Cultures Upon 24 h of Exposure on Day 22</th>
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p-Values indicate significant statistical differences between EC_{50} values in 2D and 3D cultures.
aUndeterminable.
agreement with the CYP2E1 activity which was highest during the early cultivation period. The 2D HepaRG cultures showed much higher EC$_{50}$ values in the range of 25–34µM which is in agreement with a previous report (McGill et al., 2011). We show that 3D organotypic spheroid cultures reflect the in vivo EC$_{50}$ values that could be directly co-related to the CYP2E1 activity.

The toxicity assessment of glitazones (or thiazolidinediones) on the 3D HepaRG organotypic cultures also gave very interesting results. Troglitazone is toxic in most in vitro tests, and various cellular mechanisms have been proposed. These include reactive metabolite formation by CYP3A4-mediated formation of quinone and o-quinone methide (Dixit and Bharatam, 2011; He et al., 2004; Kassahun et al., 2001; Tettey et al., 2001; Yamamoto et al., 2002); inhibition of the hepatic drug transporter, bile salt export pump, or organic anion–transporting polypeptide by troglitazone and troglitazone sulfate (Funk et al., 2001; Nozawa et al., 2004); mitochondrial dysfunction caused by the parent compound by respiratory chain inhibition and reactive oxygen species (ROS) generation (Kim et al., 2012; Lim et al., 2008; Masubuchi et al., 2006); troglitazone-mediated apoptosis (Bae et al., 2003; Shiau et al., 2005); and inflammatory responses involving Kupffer cells (Edling et al., 2009; Sigrist et al., 2000). However, most of these studies were carried out at concentrations well above the pharmacological concentrations (1–2 magnitude) and therefore these in vitro studies could not provide a directly relevant mechanism for the idiosyncratic nature of troglitazone toxicity (Chojkier, 2005; Isley, 2003). Troglitazone toxicity could not be predicted in any preclinical study (Watanabe et al., 1999). Moreover, after the withdrawal of troglitazone from the market, several animal studies were conducted to reproduce troglitazone toxicity, but almost all of these studies were unsuccessful (Bedoucha et al., 2001; Jia et al., 2000; Watanabe et al., 2000). Our 3D HepaRG system was not sensitive to troglitazone toxicity on both days 5 and 22. To the best of our knowledge, this is the first report on an in vitro system that could not show troglitazone toxicity. Although we tend to explain this by detoxification of troglitazone via extensive CYP3A4 metabolism (our results show good expression of CYP3A4 throughout the cultivation period) and phase II metabolism as shown in a previous study (Leite et al., 2012), the poor penetration of troglitazone into the spheroids cannot be completely ruled out. However, this may seem unlikely as rosiglitazone (a structural analog of troglitazone)-induced toxicity in our 3D HepaRG cultures. In addition, we cannot exclude phenotype differences translating into abolishment of troglitazone toxicity, which may be dependent on other mechanisms. Our result supports the idiosyncratic toxicity of troglitazone (Jaeschke, 2007) where genetic and environmental factors are contributing factors. Nevertheless, further studies on troglitazone and its metabolites in the culture medium are needed. Furthermore, we are also convinced that extension of the 3D spheroid system such as using cocultures or bile acids as was reported recently (Ogihara et al., 2011) would give interesting insights into the relevant mechanism(s) involved in troglitazone toxicity.

Rosiglitazone is not considered hepatotoxic in vivo. Until now, there has not been any direct report of clinical toxicity of rosiglitazone (Isley, 2003). However, using in vitro methods, the mean EC$_{50}$ value of rosiglitazone in PHHs from 35 donors (EC$_{50}$ value with PHH from 2 donors was > 500µM) is 140µM (Lloyd et al., 2002). Rosiglitazone is considered to be cytotoxic by forming GSH adducts, ROS production, and oxidative stress (Perez-Oritz et al., 2007; Saha et al., 2012). Our data do support previous literature reports on the cytotoxicity of rosiglitazone by mechanisms which are probably not shared by troglitazone in causing toxicity in vivo.

In summary, our results suggest that the 3D organotypic HepaRG cultures permitted more accurate assessment of acute toxicity of tested compounds, which is similar to in vivo hepatotoxicity. These 3D systems, in addition, will be of great value in assessing chronic toxicity because they are functional for long periods. Future studies for the improvement of the model could include cocultivation of the HepaRG cells with nonparenchymal cells (e.g., Kupffer cells) that might greatly enhance the utility of the model and would allow the evaluation of immunemediated idiosyncratic drug-induced hepatotoxic effects.

**Supplementary Data**

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

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FIG. 7. Acute toxicity in 2D and 3D HepaRG cultures. The 2D and 3D HepaRG cultures were exposed to acetaminophen (a and b), troglitazone (c and d), and rosiglitazone (e and f) for 24 h on culture days 5 and 22, respectively. Cell viability was assessed by measuring the intracellular ATP content. The results are presented as the mean of triplicates (± SD).
3D HEPARG CULTURES IN IN VITRO TOXICOLOGY


