Differential Modulation of Cytochrome P450 Activity and the Effect of 1-Aminobenzotriazole on Hepatic Transport in Sandwich-Cultured Human Hepatocytes

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ABSTRACT:
Sandwich-cultured human hepatocytes (SCHH) have been widely used for in vitro assessments of biliary clearance. However, the modulation of metabolism enzymes has not been fully evaluated in this system. The present study was therefore undertaken to determine the activity of cytochrome P450 (P450) 1A2, 2C8, 2C9, 2C19, 2D6, and 3A and to evaluate the impact of 1-aminobenzotriazole (ABT) on hepatic uptake and biliary excretion in SCHH. The SCHH maintained integrity and viability as determined by lactate dehydrogenase release and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assays conducted over the culture period. Although all assessed P450 activity decreased in day 2 SCHH, the extent of the decrease and the subsequent rebound in activity varied across the different isoforms. Day 5 CYP1A2 activity was approximately 2.5-fold higher than day 1 activity, whereas the CYP3A and CYP2C9 activities were 90 and 60% of the day 1 levels, respectively. In contrast, the initial CYP2C8, CYP2C19, and CYP2D6 activity losses did not rebound over the 5-day culture period. Furthermore, ABT was not found to have an effect, whether directly or indirectly as a P450 inactivator, with respect to the hepatic transport of rosuvarstatin, atrovastatin, and midazolam in SCHH. Taken together, these results suggest that the SCHH model is a reliable tool to characterize hepatic uptake and biliary excretion. Due to the differential modulation of P450 activity, SCHH may not be considered a suitable tool for metabolic stability assessments with compounds predominantly cleared by certain P450 enzymes.

Introduction
Human hepatocyte in vitro systems are widely accepted as a valuable tool to screen new chemical entities and to investigate metabolic liability, gene induction, and toxicity (Gebhardt et al., 2003). Despite the utility of hepatocytes, the characterization of each in vitro system remains an active area of research with respect to the advancement of hepatic drug disposition and drug-drug interaction predictions. Freshly isolated and cryopreserved hepatocyte suspensions preserve the overall metabolic function of cytochrome P450 (P450) enzymes and therefore can be used to predict drug metabolism in vivo (Lu et al., 2008). However, it is important to note that the hepatocyte polarity that allows for vectorial transport in vivo is rapidly disrupted when cells are isolated from intact liver (Roelofs et al., 1995; Bow et al., 2008). Furthermore, in contrast to hepatocyte suspension assays, hepatocyte culture models exhibit a rapid decline in liver-specific functions, including both P450 enzyme activity and gene expression patterns (Richert et al., 2006). These issues represent confounding factors with respect to the use of hepatocytes to predict transporter- and P450-mediated drug clearance (Balls, 2002; Boess et al., 2003). Hepatocytes cultured in a sandwich configuration result in cellular repolarization and the development of an intact bile canalicular network that imparts the proper three-dimensional orientation and localization of hepatobiliary transporters, which thereby provides a useful model for the prediction of in vivo intrinsic biliary clearance rates for drugs (Liu et al., 1999a,b,c). However, the expression of at least a subset of enzymes and transporters can be altered over time in

ABBREVIATIONS: P450, cytochrome P450; SCH, sandwich-cultured hepatocytes; SCHH, sandwich-cultured human hepatocytes; ABT, 1-aminobenzotriazole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; LDH, lactate dehydrogenase; CV, coefficient of variation; ANOVA, analysis of variance.
sandwich-cultured hepatocytes (SCH) (Kern et al., 1997; Hoffmaster et al., 2004; Li et al., 2009). Thus, a more thorough understanding of the model is needed as these alterations undermine confidence in vivo predictions and confound its application as an in vitro model for the assessment of transporter–enzyme interplay in the liver.

We previously demonstrated the ability to use cryopreserved human hepatocytes for SCH and established that the uptake and efflux transporter activities were maintained at levels comparable to those in fresh hepatocytes under the conditions used (Bi et al., 2006). Recently, we also reported that the integration of a scaling factor, which addressed the hepatobiliary transporter expression differences between in vitro and in vivo, largely improved the prediction of in vivo clearance from the SCH model (Li et al., 2010). In addition to these findings, a better understanding of P450 modulation and associated effects on hepatic elimination profiles in sandwich-cultured human hepatocytes (SCHH) is imperative for the successful use of these in vitro models in in vivo pharmacokinetic predictions. In the present study, time-dependent profiles of six major P450s were measured in SCHH by monitoring the metabolites formed from selected probe substrates. The effect of 1-aminobenzotriazole (ABT), a P450 inactivator, was also assessed with respect to the hepatic uptake and biliary excretion of rosuvastatin, atorvastatin, and midazolam to further evaluate the reliability of this model in hepatic transport characterizations.

Materials and Methods

Chemicals. Probe substrates for P450 activity assessments and internal standards were selected as described previously (Walsky and Obach, 2004; Linder et al., 2009). All chemicals and solvents were of the highest grade commercially available.

SCHH. Cryopreserved human hepatocyte lot 109 was purchased from BD Biosciences (Woburn, MA). Lots Hu4163 and Hu4168 were purchased from CellzDirect (Pittsboro, NC), and lot RCP was purchased from Celsis In Vitro Technologies, Inc. (Baltimore, MD). In VitroGro-HT (thawing), In VitroGro-CP (plating), and In VitroGro-HI (incubation) media were supplemented with Torpedo Antibiotic Mix (Celsis In Vitro Technologies, Inc.), according to the manufacturer’s instructions. Cryopreserved hepatocytes were thawed and plated as described previously (Bi et al., 2006). The hepatocyte plate-seeding density was 0.75 × 10^6 cells/ml for lots 109 and RCP and 0.85 × 10^6 cells/ml for lots Hu4163 and Hu4168. Day 1 measurements represent 4 h posthepatocyte seeding, and day 2 measurements represent 6 h post-Matrigel overlay.

Assessment of P450 Activity in SCHH on Day 5. P450 activity was assessed by a primary incubation of SCHH with ABT (1 mM). After preincubation with ABT for a designated period (0–60 min), the probe substrate mixtures were added to SCHH and incubated for an additional 30 min.

Lactate Dehydrogenase and MTT Assays. Hepatocyte viability was assessed by lactate dehydrogenase (LDH) release using the LDH Cytotoxicity Assay Kit (Cayman Chemical Company, Ann Arbor, MI). Hepatocyte viability was determined using the MTT Cell Viability Assay Kit (Biotium, Inc., Hayward, CA) according to the manufacturer’s protocol. For LDH release measurements, hepatocytes were washed with incubation media that was subsequently replaced with fresh incubation media with or without 1% Triton X-100. For the MTT assay, hepatocytes were washed in incubation media that was subsequently replaced with fresh incubation media containing the MTT solution.

Initial Hepatic Uptake and Biliary Excretion in SCHH on Day 5. To assess hepatic uptake and biliary excretion in SCHH, rosuvastatin, atorvastatin, and midazolam—which are cleared via active hepatic uptake and biliary excretion—were incubated in the presence of 10 mM CaCl_2 and MgCl_2. The initial uptake rate was calculated from the slope between 0.5 and 5 min, whereas biliary clearance was estimated as previously reported (Liu et al., 1999b). Metabolites of rosuvastatin and atorvastatin were not evaluated.

Time-Dependent P450 Modulation in SCHH. P450 activity in SCHH was evaluated in probe substrate incubations (500 μM/well). The reactions were terminated at designed time points (5, 10, 15, 30, 45, and 60 min) by the addition of 500 μl of acetonitrile. After shaking, solutions were transferred to 96-well plates and dried down. The internal standards were added either with the acetonitrile or with the sample reconstitution mixture. The sample preparation and analysis were conducted as described previously (Walsky and Obach, 2004; Bi et al., 2006; Zientek et al., 2008).

Data Analysis. Metabolite formation rates in SCHH were obtained from the slope of the linear regression. Data are representative of a minimum of two in vitro experiments performed on different days. Two data points were collected in each experiment with coefficients of variation (CV%) less than 15%. The enzyme activity in the various lots of hepatocytes are reported as mean ± S.D., and the data were analyzed using a repeated measures analysis of variance (ANOVA) followed by a post hoc analysis using GraphPad Prism version 5.01 for Windows (GraphPad Software Inc., San Diego, CA).

Results and Discussion

The hepatic elimination of drugs consists of hepatic uptake, metabolic transformation, and/or biliary excretion. Because considerable overlap in the substrate specificity of metabolism enzymes and transporters has been reported in the literature, the P450 enzyme-transporter interplay has been hypothesized to play a pivotal role in the determination of drug disposition (Wacher et al., 1995; Zhang and Benet, 2001).

More than 10 P450 isoforms have been detected in human liver (Venkataraman et al., 2001). Of these, six major P450 isoforms (CYP3A4, -2D6, -2C9, -2C19, -2C8, and -1A2) were reported to be the key enzymes that are responsible for drug metabolism and drug-drug interactions (Nebert and Russell, 2002). To assess the overall P450 activity in SCHH, we adopted an incubation procedure that has been described previously and measured the residual enzyme activity after preincubation with 1 mM ABT, which is known to be a nonselective P450 enzyme inactivator (Linder et al., 2009). Hydroxy-midazolam formation, used as a marker of CYP3A activity, was essentially abolished (≈3% of control) by preincubation with ABT. The residual activity of CYP2C9, -2D6, and -1A2 remained between 35 and 35% with ABT preincubations up to 60 min (Fig. 1), which is consistent with a previous report (Linder et al., 2009). These results reconfirmed the existence and activity of P450 enzymes in SCHH.

The formation of acetaminophen, which is generated by the CYP1A2-catalyzed O-deethylation of phenacetin, was linear up to 60 min in day 1 SCHH incubations. Assessment of CYP1A2 indicated the activity decreased immediately postseeding and then increased after overlay with Matrigel. The activity of CYP1A2 reached the highest level observed on day 5 with approximately a 2.5-fold increase over the activity of day 1 measurements (Fig. 2). To assess whether the up-regulation of CYP1A2 was related to stress generated
under the current culture conditions, LDH release and MTT assays were conducted. As shown in Fig. 3, the LDH release at day 5 was significantly lower than day 1 and day 2. Furthermore, there was not a significant change in formazan formation between day 1 and day 5 SCHH. In total, these results are consistent with those noted in a recent review, which indicates the hepatocyte culture model maintains cell integrity and viability (Swift et al., 2010) and suggests the increase in CYP1A2 activity is unlikely to be due to cellular stress generated during long culture periods. Although the mechanism underlying the increase in CYP1A2 activity was not defined in the present study, the findings conflict with a recent study that reports a substantial decrease in all P450 enzyme activity in 5-day SCHH (Liao et al., 2010). Because CYP1A2 is a highly inducible protein, we speculate that this discrepancy can potentially be attributed to the culture conditions, and further investigation is needed to elucidate the mechanism of CYP1A2 modulation in SCHH.

The CYP3A enzymes, which represent the most abundantly expressed P450 enzymes in the liver and intestine, are responsible for the metabolism of over 50% of all clinically used drugs including antidepressants, benzodiazepines, immunosuppressive agents, imidazole antimycotics, and macrolide antibiotics (Thummel et al., 1994; Li et al., 1995). The CYP3A activity measured by 1’-hydroxymidazolam formation in SCHH was decreased on day 2 and increased after day 3. On day 5, CYP3A activity reached approximately 90% of the day 1 value (Fig. 2).

CYP2C9 is the major CYP2C isoform found in human liver and exclusively converts diclofenac to 4-hydroxydiclofenac (Krausz et al., 2001). The CYP2C9 activity measured by 4’-hydroxydiclofenac formation in SCHH was decreased on day 2, whereas approximately 60% of the activity was detected in SCHH on day 5 (Fig. 2). The retention of CYP3A and CYP2C9 activity in SCHH could be beneficial in the characterization of the overall hepatic disposition of compounds that have a dual involvement of transporters and CYP3A and/or CYP2C9 enzymes.

Paclitaxel is a prototypical CYP2C8 substrate with a low $K_m$ and relatively high turnover due to CYP2C8-catalyzed 6a-hydroxylation (Totah and Rettie, 2005), whereas (S)-mephenytoin is metabolized by CYP2C19-catalyzed 4-hydroxylation and dextromethorphan is metabolized by CYP2D6-catalyzed O-demethylation to form dextrorphan (Funck-Brentano et al., 2005). As such, the formation of 6a-hydroxy placlitaxel, 4-hydroxymephenytoin, and dextromethorphan was determined to represent the CYP2C8, CYP2C19, and CYP2D6 activity in SCHH, respectively. CYP2C8 activity decreased to negligible levels by day 3; however, it recovered to approximately 30% of the day 1 value on day 5 (Fig. 2). Both CYP2C19 and CYP2D6 activity decreased over time in SCHH, with less than 20% of CYP2C19 and CYP2D6 activity remaining on day 5 (Fig. 2).

The hepatic clearance process consists of three steps: 1) passive or active basolateral transport into hepatocytes, which may be accompanied by a return to the blood via passive permeation or active trans-
port; 2) phase I and/or phase II drug metabolism; and 3) biliary excretion exclusively governed by hepatobiliary transporters, which is also known as phase III metabolism. In an in vitro setting, the activation of other compensatory pathways may occur when one pathway is modified. As a result, it is possible that the decrease of P450 enzyme activity could potentially alter the overall elimination profile of certain compounds through compensatory effects on uptake/biliary excretion in SCHH, which could ultimately affect the reliability of the SCHH model. To investigate this issue, the active uptake and biliary excretion of rosuvastatin, atorvastatin, and midazolam in day 5 SCHH was examined in the presence or absence of ABT. Rosuvastatin undergoes active uptake into the liver where it is then mainly eliminated by biliary excretion in vivo. Atorvastatin is transported into hepatocytes by active uptake, metabolized by CYP3A4, and eliminated by biliary excretion, whereas midazolam is metabolized by CYP3A enzymes after its passive diffusion into hepatocytes. As shown in Table 1, the initial uptake clearance and biliary intrinsic clearance of rosuvastatin, atorvastatin, and midazolam were not significantly affected by the presence or absence of ABT in SCHH. Because hepatic transport of rosuvastatin and atorvastatin are mediated by organic anion transporting polypeptides, multidrug resistance protein 2, breast cancer resistance protein, and P-glycoprotein, the results reveal that ABT did not have an inhibitory effect on transporter functions in SCHH. Furthermore, these data suggest that the modulation of P450 activity did not contribute to changes in the elimination profile in the SCHH model. The 1-hydroxy metabolite of midazolam was simultaneously monitored to ensure metabolite formation occurred during the relevant time frame (data not shown). Because midazolam metabolism (and inhibition of metabolism) was detected within 5-min incubations, the inactivation of P450 enzymes in SCHH, at least CYP3A, does not affect the active hepatic uptake assessed herein.

In summary, the culture of human hepatocytes in a sandwich configuration had a significant impact on the functional expression of P450 enzymes with differential effects observed with respect to the modulation of specific P450 isoform activity. Preincubation with ABT, and the inactivation of P450 enzymes by ABT, did not affect the hepatic uptake or hepatobiliary transport of rosuvastatin, atorvastatin, and midazolam. These findings further suggest that the SCHH model is a reliable tool to characterize hepatic disposition. However, due to the loss of several key P450 enzymes, SCHH in culture for over 24 h would not be suitable for metabolic liability assessments with compounds that are cleared primarily by P450 enzymes, particularly CYP2C8, CYP2D6, and CYP2C19.

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Authorship Contributions
Participated in research design: Kimoto, Yang, Linder, Fenner, and Lai.
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Wrote or contributed to the writing of the manuscript: Kimoto, Iseki, Fenner, El-Kattan, and Lai.

References

FIG. 3. LDH release and formazan formation in SCHH. LDH release and formazan formation were determined in hepatocytes (lot Hu4163). Data represent the mean with S.D. from a single study (n = 4–7). **, P < 0.01 by ANOVA with comparison to the day 1 value.

TABLE 1
Initial uptake and biliary clearance of test compounds in day 5 SCHH

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibitor</th>
<th>Uptake CL</th>
<th>Biliary CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosuvastatin (0.2 μM)</td>
<td>Control</td>
<td>6.7</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>+ABT</td>
<td>6.6</td>
<td>2.2</td>
</tr>
<tr>
<td>Atorvastatin (0.2 μM)</td>
<td>Control</td>
<td>12</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>+ABT</td>
<td>10</td>
<td>1.3</td>
</tr>
<tr>
<td>Midazolam (1 μM)</td>
<td>Control</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+ABT</td>
<td>14</td>
<td>0</td>
</tr>
</tbody>
</table>

CL, clearance.

Compounds that are cleared primarily by P450 enzymes, particularly CYP2D6 and CYP3A4, would not be suitable for metabolic liability assessments with compound...


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