Evaluation of Hepatic Disposition of Paroxetine Using Sandwich-Cultured Rat and Human Hepatocytes

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ABSTRACT

Paroxetine, a selective serotonin reuptake inhibitor, is metabolized in the liver and excreted into bile and urine as metabolites, but species differences have been observed in hepatic disposition between rats and humans. A major metabolite in rats is M1-glucuronide, whereas M1-glucuronide and M1-sulfate are found in humans. The primary excretion route of paroxetine-derived radioactivity in rats and humans is bile and urine, respectively. The aim of this study was to examine the usefulness of sandwich-cultured hepatocytes (SCH) to evaluate in vivo species differences of the hepatic disposition of paroxetine between rats and humans. The metabolite profile of[^H]paroxetine in SCH was similar to that in hepatocytes in suspension, and the in vitro metabolite profiles were similar to the published in vivo metabolic pathways for both species. Furthermore, the biliary excretion index (BEI) of formed M1-glucuronide in rat SCH (25.8–50.9%) was higher than that in human SCH (15.1–16.7%). The BEI of formed M1-sulfate (16.4–29.1%) was comparable to that of M1-glucuronide in human SCH, whereas the BEIs of paroxetine were negligible in SCH of both species. Moreover, M1-glucuronide was demonstrated to be a multidrug resistance-associated protein 2 substrate in both species, as determined by its uptake into ATP-binding cassette transporter-expressing membrane vesicles. SCH should prove to be useful to evaluate the processes of hepatic uptake and metabolism of parent drugs and the simultaneous examination of the biliary excretion of both parent drug and liver-derived metabolites.

Introduction

Paroxetine is a selective serotonin reuptake inhibitor widely used for the treatment of major depressive disorder, panic disorder, obsessive-compulsive disorder, social anxiety disorder, posttraumatic stress disorder, and generalized anxiety disorder (Marks et al., 2008). Figure 1 shows the metabolic pathway of paroxetine. Paroxetine is initially oxidized by cytochrome P450 (P450) to an unstable catechol intermediate, and the intermediate is promptly methylated by catechol-O-methyltransferase (COMT) to M1 and M2. M1 and M2 are further conjugated by UDP-glucuronosyltransferases (UGT) and sulfotransferases (SULT) to glucuronide and sulfate, respectively, and eliminated from the body (Haddock et al., 1989; Kaye et al., 1989; Jornil et al., 2010). In rats, a major metabolite of paroxetine is M1-glucuronide, as shown by radiolabeled studies, and the radioactivity is mainly excreted into bile, up to approximately 75% of the dose after oral administration (Haddock et al., 1989). The major metabolites in humans are M1-sulfate and M1-glucuronide and the radioactivity after oral administration of radiolabeled paroxetine is mainly excreted into urine, up to approximately 60% of the dose (Haddock et al., 1989; Kaye et al., 1989). Thus, there are species differences in the hepatic disposition of paroxetine between rats and humans.

Liver is a key organ that functions to eliminate endogenous and exogenous compounds, including drugs. Hepatic intrinsic clearance consists of four factors: basolateral uptake, basolateral efflux, intracellular metabolism, and biliary excretion. Metabolism is conventionally studied using liver microsomal fractions. The widespread use of liver microsomal fractions is attributable to their ease of preparation, and thus, they are a powerful tool to predict metabolic intrinsic clearance (Obach, 1999; Naritomi et al., 2001). However, the use of hepatocytes is gaining more attention because of their increased availability and quality as a result of the improved isolation techniques. Furthermore, metabolic stability and pathway studies with hepatocytes have been recognized as useful methods for the estimation of metabolic clearance (Lau et al., 2002, Chiba et al., 2009). In addition, hepatic uptake and biliary excretion studies have generally been conducted with hepatocytes (Hoffmaster et al., 2005; Paine et al., 2008; Li et al., 2010). Therefore, hepatocytes are ideal models for estimating hepatic disposition.

Sandwich-cultured hepatocytes (SCH) that are cultivated between two layers of gelled collagen maintain polarity, morphology, and liver-specific metabolic activities (Kern et al., 1997; Slaus et al., 2001; Tuschl et al., 2009). In addition, SCH develop functional canalicular networks sealed by tight junctions, and hepatic transporter proteins are expressed and localized to the correct membrane domains, allowing for assessment of vectorial transport function (LeCluyse et al., 1994).

ABSTRACT:

Paroxetine, a selective serotonin reuptake inhibitor, is metabolized in the liver and excreted into bile and urine as metabolites, but species differences have been observed in hepatic disposition between rats and humans. A major metabolite in rats is M1-glucuronide, whereas M1-glucuronide and M1-sulfate are found in humans. The primary excretion route of paroxetine-derived radioactivity in rats and humans is bile and urine, respectively. The aim of this study was to examine the usefulness of sandwich-cultured hepatocytes (SCH) to evaluate in vivo species differences of the hepatic disposition of paroxetine between rats and humans. The metabolite profile of[^H]paroxetine in SCH was similar to that in hepatocytes in suspension, and the in vitro metabolite profiles were similar to the published in vivo metabolic pathways for both species. Furthermore, the biliary excretion index (BEI) of formed M1-glucuronide in rat SCH (25.8–50.9%) was higher than that in human SCH (15.1–16.7%). The BEI of formed M1-sulfate (16.4–29.1%) was comparable to that of M1-glucuronide in human SCH, whereas the BEIs of paroxetine were negligible in SCH of both species. Moreover, M1-glucuronide was demonstrated to be a multidrug resistance-associated protein 2 substrate in both species, as determined by its uptake into ATP-binding cassette transporter-expressing membrane vesicles. SCH should prove to be useful to evaluate the processes of hepatic uptake and metabolism of parent drugs and the simultaneous examination of the biliary excretion of both parent drug and liver-derived metabolites.

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Sandwich-cultured hepatocytes (SCH) that are cultivated between two layers of gelled collagen maintain polarity, morphology, and liver-specific metabolic activities (Kern et al., 1997; Slaus et al., 2001; Tuschl et al., 2009). In addition, SCH develop functional canalicular networks sealed by tight junctions, and hepatic transporter proteins are expressed and localized to the correct membrane domains, allowing for assessment of vectorial transport function (LeCluyse et al., 1994).
These unique features make SCH useful for the prediction of biliary intrinsic clearance, drug-drug interactions, and drug-induced hepatotoxicity (Liu et al., 1999a; Marion et al., 2007; Fukuda et al., 2008; Li et al., 2010; Nakanishi et al., 2011). The use of cultured cells that only express a single ATP-binding cassette (ABC) transporter in a transcellular transport study may lead to results that are not representative of in vivo conditions. Because hydrophilic metabolites often have less membrane permeability than their lipophilic parent compound (Pang et al., 1984; de Lannoy and Pang, 1986), the use of polarized cultured cells, such as Madin-Darby canine kidney cells, that coexpress uptake and efflux transporters has increased in studies to evaluate hepatic disposition (Ishiguro et al., 2008). However, the hepatobiliary disposition of a metabolite should be ideally assessed on the basis of the metabolite formed in cells after adding parent compound, rather than from the results of the administered preformed metabolite. For this reason, SCH are superior to double-transfected cells, because they maintain intracellular metabolic activity. Indeed, some researchers have previously conducted metabolic/hepatobiliary transport studies that have proven to be useful to reveal the hepatic disposition of various compounds, such as bilirubin, terfenadine, troglitazone, pafuramidine, and 2,5-bis[5-(N-methoxyamidino)-2-pyridyl] furan (CPD-8068) (Lengyel et al., 2005; Turncliff et al., 2006; Lee et al., 2010; Yan et al., 2011). Moreover, the Food and Drug Administration guidance for the safety testing of drug metabolites and the ICH-M3 (R2) have had a significant impact on drug discovery and development, and toxicology studies have been conducted metabolic/hepatobiliary transport studies using rat liver S9 and human liver cytosolic fractions. The purities of [3H]M1-glucuronide and [3H]M1-sulfate were enzymatically synthesized using rat liver S9 and human liver cytosolic fractions. The purities of [3H]M1-glucuronide and [3H]M1-sulfate were greater than 95%. Paroxetine maleate, S-(S′-adenosyl)-L-methionine (SAM); β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), uridine 5′-diphosphoglucuronic acid, and adenosine 3′-phosphate 5′-phosphosulfate were purchased from Sigma-Aldrich (St. Louis, MO). Cryopreserved hepatocyte recovery medium, cryopreserved hepatocyte plating medium, hepatocyte maintenance supplement pack, and Geltrex were purchased from CellzDirect (Durham, NC), and Williams’ media E (WME) without phenol red and Hanks’ balanced salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). Sprague-Dawley rat liver S9 (pool of 400 animals, male) and human liver cytosolic fractions (pool of 50 donors, mixed sex) were purchased from XENOTECH LLC (Lenexa, KS). MRP2/Mrp2 (multidrug resistance-associated protein 2)-, BCRP/Bcrp (breast cancer resistance protein)-, and BSEP/Bsep (bile salt export pump)-expressing Sf9 membrane vesicles were purchased from Genomembrane Inc. (Yokohama, Japan). All other chemicals and reagents were of higher grade, such as special grade or high-performance liquid chromatography (HPLC) grade.

**Materials and Methods**

**Chemicals.** [3H]Paroxetine (22.9 Ci/mmol; >97%) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). [3H]M1-glucuronide and [3H]M1-sulfate were enzymatically synthesized using rat liver S9 and human liver cytosolic fractions. The purities of [3H]M1-glucuronide and [3H]M1-sulfate were greater than 95%. Paroxetine maleate, S-(S′-adenosyl)-L-methionine (SAM); β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), uridine 5′-diphosphoglucuronic acid, and adenosine 3′-phosphate 5′-phosphosulfate were purchased from Sigma-Aldrich (St. Louis, MO). Cryopreserved hepatocyte recovery medium, cryopreserved hepatocyte plating medium, hepatocyte maintenance supplement pack, and Geltrex were purchased from CellzDirect (Durham, NC), and Williams’ media E (WME) without phenol red and Hanks’ balanced salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). Sprague-Dawley rat liver S9 (pool of 400 animals, male) and human liver cytosolic fractions (pool of 50 donors, mixed sex) were purchased from XENOTECH LLC (Lenexa, KS). MRP2/Mrp2 (multidrug resistance-associated protein 2)-, BCRP/Bcrp (breast cancer resistance protein)-, and BSEP/Bsep (bile salt export pump)-expressing Sf9 membrane vesicles were purchased from Genomembrane Inc. (Yokohama, Japan). All other chemicals and reagents were of higher grade, such as special grade or high-performance liquid chromatography (HPLC) grade.

**Hepatocytes.** Cryopreserved Sprague-Dawley rat and human hepatocytes were purchased from CellzDirect and Celsis In Vitro Technologies (Baltimore, MD), respectively. Characteristics of the hepatocyte lots are shown in Table 1. **Hepatocytes Sandwich Culture.** Cryopreserved rat or human hepatocytes were thawed in accordance with CellzDirect’s standard methods: hepatocytes were thawed at 37°C and decanted into cryopreserved hepatocyte recovery medium. The hepatocytes were then centrifuged at 60g for 10 minutes, and the supernatant was discarded. After the hepatocytes were resuspended to 4 × 10^5 viable cells/ml (rat) or 7 × 10^5 viable cells/ml (human) in cryopreserved hepatocyte plating medium, the hepatocytes suspension was added to collagen I-coated BioCoat 24-well plates (BD Biosciences, Bedford, MA) in a volume of 0.5 ml/well. After 4 hours of static incubation under an atmosphere of 5% carbon dioxide in air at 37°C, the medium was replaced with Geltrex (350 μg/ml) containing WME with hepatocyte maintenance supplement. Cultures were maintained in Geltrex-free WME with hepatocyte maintenance supplement, which was replaced every 24 hours.

**Metabolite Profile of Paroxetine.** For incubation with hepatocytes in suspension, cryopreserved rat or human hepatocytes were thawed and resuspended to 6 or 10 × 10^5 viable cells/ml in WME, respectively. Two hundred fifty microliters of the hepatocyte suspension was added in advance and incubated statically at 37°C in a humidified chamber in a 95%/5% air/carbon dioxide atmosphere for 0, 0.25, 0.5, 1, 2, and 3 hours. For incubation with SCH, day 4 hepatocytes were incubated statically with 0.5 ml of 1 μM [3H]paroxetine in WME in the incubator (37°C, 5% carbon dioxide) for 0, 0.25, 0.5, 1, 3, 6, and 24 hours. Each incubation was terminated by adding 0.5 ml of

**Table 1. Characteristics of cryopreserved hepatocytes**

<table>
<thead>
<tr>
<th>Species</th>
<th>Lot number</th>
<th>Sex</th>
<th>Age</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD Rat</td>
<td>R587</td>
<td>M</td>
<td>7 weeks</td>
<td>Pool of 4 animals</td>
</tr>
<tr>
<td></td>
<td>R609</td>
<td>M</td>
<td>7 weeks</td>
<td>Pool of 4 animals</td>
</tr>
<tr>
<td></td>
<td>R616</td>
<td>M</td>
<td>7 weeks</td>
<td>Pool of 4 animals</td>
</tr>
<tr>
<td>Human</td>
<td>KOG</td>
<td>F</td>
<td>38 years old</td>
<td>Single donor, white</td>
</tr>
<tr>
<td></td>
<td>GHA</td>
<td>F</td>
<td>1 year old</td>
<td>Single donor, white</td>
</tr>
<tr>
<td></td>
<td>RTM</td>
<td>F</td>
<td>61 years old</td>
<td>Single donor, white</td>
</tr>
</tbody>
</table>

SD, Sprague-Dawley.
pretreatment solvent (acetonitrile/ethanol/methanol mixture, 1:1:1, v/v/v), and the mixture was collected in a glass tube. Each well was washed with the same pretreatment solvent twice, and the wash was combined with the mixture. The resultant solution was evaporated to dryness under reduced pressure, and the residue was dissolved in a 10 mM ammonium acetate (pH 4.0) and acetonitrile mixture (9:1, v/v) and injected into a radio-HPLC.

**Accumulation of Paroxetine and Metabolites in SCH.** Modulation of Ca\(^{2+}\) in the incubation buffer was used to manipulate the tight junctions sealing the canalicular networks between cells. In Ca\(^{2+}\)-containing standard buffer (HBSS), tight junction integrity is preserved and substrates are excreted into the bile canaliculi, whereas incubation of cells in Ca\(^{2+}\)-free buffer (HBSS modified) disrupts the tight junctions and opens the canalicular spaces, and canalicular contents are released into the incubation medium (Lee et al., 2010).

Metabolic/hepatobiliary transport studies with SCH were conducted according to the method described previously with minor modifications (Lee et al., 2010). In brief, rat or human SCH were incubated statically under an atmosphere of 5% carbon dioxide in air at 37°C for 0.25, 0.5, 1, 4, and 24 hours with \[^{3}H\]paroxetine at the initial concentration of 1 \(\mu\)M in WME. At each time point, the medium was removed, and cells were rinsed twice and incubated statically for 5 minutes in the incubator (37°C, 5% carbon dioxide), with Ca\(^{2+}\)-containing buffer (HBSS[Ca/Mg\(^{2+}\)] containing 20 mM HEPES) or Ca\(^{2+}\)-free buffer (HBSS[Ca/Mg\(^{2+}\)]- containing 20 mM HEPES and 1 mM EGTA). The buffer was removed, and cells were washed three times with the same ice-cooled buffer. Cells were lysed with 0.5 ml of pretreatment solvent, and the lysate was collected in a glass tube. Each well was washed with the same pretreatment solvent twice, and the wash was combined with the lysate. The resultant solution was evaporated to dryness under reduced pressure, and the residue was dissolved in a 10 mM ammonium acetate (pH 4.0) and acetonitrile mixture (9:1, v/v) and injected into a radio-HPLC.

**Uptake by Membrane Vesicles.** \[^{3}H\]M1-glucuronide and \[^{3}H\]M1-sulfate were enzymatically formed from \[^{3}H\]paroxetine. \[^{3}H\]M1-glucuronide was obtained from the reaction mixture consisting of 10 \(\mu\)M \[^{3}H\]paroxetine, 2 mg/ml rat liver S9, cofactors (1 mM NADPH, 1 mM SAM, and 5 mM uridine 5'-diphosphoglucuronic acid), 25 \(\mu\)g/ml alamethicin, 5 mM MgCl\(_2\), and 100 mM potassium phosphate buffer at pH of 7.4. In the case of \[^{3}H\]M1-sulfate, \[^{3}H\]M1 was generated from the reaction mixture consisting of 10 \(\mu\)M \[^{3}H\]paroxetine, 2 mg/ml rat liver S9, cofactors (1 mM NADPH and 1 mM SAM), 5 mM MgCl\(_2\), and 100 mM potassium phosphate buffer at pH of 7.4. Then, 10 \(\mu\)M \[^{3}H\]M1 was incubated with 1 mg/ml human liver cytosol, 0.6 mM adenosine 3'-phosphate 5'-phosphosulfate, 5 mM MgCl\(_2\), and 100 mM potassium phosphate.

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**Fig. 2.** Product ion spectra of M1-glucuronide (A) and M1-sulfate (B). M1-glucuronide was obtained from the incubation of paroxetine with NADPH-, SAM-, and UDPGA (uridine 5'-diphosphoglucuronic acid)-supplemented rat liver S9 fractions. M1-sulfate was obtained from the incubation of M1, which was generated from the reaction mixture containing paroxetine and NADPH- and SAM-supplemented rat liver S9 fractions, with PAPS (adenosine 3'-phosphate 5'-phosphosulfate)-supplemented human liver cytosolic fractions. The origins of the characteristic ions of M1-glucuronide and M1-sulfate are also shown. MH+, protonated molecule; MS, mass spectrometry.
buffer at pH of 7.4, and [3H]M1-sulfate was obtained. All incubations were done at 37°C over night. [3H]M1-glucuronide, [3H]M1, and [3H]M1-sulfate were separated by HPLC, and the eluates were collected at their corresponding retention times (M1-glucuronide, 15.7 minutes; M1, 23.2 minutes; M1-sulfate, 21.7 minutes). Each compound was further purified using solid-phase extraction with OASIS HLB (Waters, Milford, MA; 6 cc, 500 mg).

Vesicular transport studies were performed using a rapid filtration technique according to the manufacturer’s protocol. In brief, MRP2/Mrp2-expressing Sf9 membrane vesicles (1 mg/ml) were incubated with 0.5 mM [3H]M1-glucuronide or [3H]M1-sulfate at 37°C in the presence of 2 mM glutathione and cofactor (4 mM ATP or AMP) in transport buffer consisting of 70 mM KCl, 7.5 mM MgCl2, and 50 mM MOPS-Tris (pH 7.0). In the case of BCRP/Bcrp, the composition of reaction mixture was the same as that for MRP2/Mrp2, except for the absence of glutathione. In the case of BSEP/Bsep, 0.5 mM [3H]M1-glucuronide or [3H]M1-sulfate was incubated with membrane vesicles (1 mg/ml) at 37°C in the presence of 4 mM ATP or AMP in transport buffer consisting of 100 mM KNO3, 10 mM Mg(NO3)2, 50 mM sucrose, and 10 mM HEPES-Tris (pH 7.4). An aliquot of the reaction mixture was collected and added into 5-times volume of ice-cold stop buffer consisting of 70 mM KCl and 40 mM MOPS-Tris (pH 7.0) for MRP2/Mrp2 and BCRP/Bcrp or 100 mM KNO3, 50 mM sucrose, and 10 mM HEPES-Tris (pH 7.4) for BSEP/Bsep to terminate the reaction at 10, 30, 60, and 120 seconds. The terminated reaction mixture was passed through a presoaked 0.45-μm pore size membrane filter (Millipore, Bradford, MA) and then washed five times with 0.35 ml of ice-cold stop buffer. The amount of radioactivity retained on the filter was measured using a liquid scintillation counting TRI-CARB 2900TR (PerkinElmer Life and Analytical Sciences).

Sample Analysis. An Agilent 1100 HPLC system equipped with a binary pump, column oven, and auto sampler (Agilent Technologies, La Jolla, CA) was connected to a flow scintillation analyzer 625TR (PerkinElmer Life and Analytical Sciences) and was used for radio-HPLC analysis. A SunFire-C18 5 μm, 4.6 × 250 mm (Waters), kept at 40°C, was used as the analytical column. A gradient with mobile phase A (10 mM ammonium acetate; pH 4.0) and mobile phase B (100% acetonitrile) was used at a flow rate of 1.0 ml/min. The initial composition of mobile phase B was maintained at 5% for 5 minutes and then increased in a linear manner as follows: 20% at 10 minutes, 25% at 20 minutes, 35% at 35 minutes, 50% at 55 minutes, 70% at 65 minutes, and 100% at 65.1 minutes. It was then maintained at 100% for up to 70 minutes. The column was allowed to equilibrate at 5% mobile phase B for 7 minutes before the next injection. Mass spectrometric analyses were performed on a QTRAP ion trap mass spectrometer (AB Sciex, Foster City, CA), which was interfaced to an Agilent HPLC system and equipped with an electrospray ionization source. Eluate from the HPLC column split at a ratio of 1:4 using a splitter was delivered to an MS detector at a flow rate of approximately 0.2 ml/min. The parameters for electrospray ionization were as follows: curtain gas, 40 psi; ionspray voltage, 5.5 kV; temperature, 550°C; ion source gas 1, 50 psi; ion source gas 2, 80 psi; declustering potential, 30 V; entrance potential, 10 V; and collision energy, 30 V. The mass spectrometer was operated in a positive ion mode. For the mass spectrometer (MS)2 scan, products of M1-glucuronide and M1-sulfate were set at mass-to-charge ratio (m/z) 508.2 and m/z 412.2, and the ions were monitored over the full mass ranges of m/z 100–550, and m/z 412–550.

Fig. 3. Typical radio-chromatograms of the incubation mixture of [3H]paroxetine (1 μM) with rat hepatocytes in suspension for 3 hours (A) or SCH for 24 hours (B). For SCH, day 4 hepatocytes were used.

Fig. 4. Disappearance of paroxetine and appearance of M1-glucuronide and M1-sulfate over time from an incubation mixture of [3H]paroxetine with rat (A and C) or human hepatocytes (B and D). For SCH, day 4 hepatocytes were used. Metabolite profiles of radioactivity were analyzed by radio-HPLC after the incubation of [3H]paroxetine (1 μM) with rat or human hepatocytes for 0.25, 0.5, 1, 2, and 3 hours in suspension (A or B, respectively) or 0.25, 0.5, 1, 3, 6, and 24 hours in sandwich culture (C or D, respectively). Data are expressed as the mean ± S.D. of three lots of rat or human hepatocytes. □ percentage remaining paroxetine; ◦ percentage produced M1-glucuronide; ○ percentage produced M1-sulfate.
Accumulation of M1-sulfate reached a peak at 15 and 30 minutes incubation. The BEI of M1-glucuronide and M1-sulfate produced after 3 hours incubation in suspension mixture (Fig. 4, A and C). The percentages of M1-glucuronide and M1-sulfate produced after 3 hours incubation in suspension were 50.7 and 1.0%, and those after 24 hours incubation in SCH were 34.5 and 0.8%, respectively. The percentages of M1-sulfate and other metabolites formed were significantly lower than that of M1-glucuronide in rat hepatocytes and were essentially negligible. The total recovered amounts of paroxetine, M1-glucuronide, and M1-sulfate were more than 81.4 and 71.9% of the dose in the incubation mixture of rat suspended hepatocytes and SCH, respectively.

When 1 µM [3H]paroxetine was incubated with human hepatocytes in suspension or SCH, M1-glucuronide and M1-sulfate were produced to a similar extent and they were the main metabolites in the incubation mixture (Fig. 4, B and D; Fig. 5). The percentages of paroxetine remaining and M1-glucuronide and M1-sulfate produced after 3 hours incubation used in suspension were 71.4, 8.1, and 7.9%, respectively, and those after 24 hours incubation in SCH were 52.8, 12.5, and 13.1%, respectively. The total recovered amounts of paroxetine, M1-glucuronide, and M1-sulfate were more than 87.4 and 78.4% of the dose in the incubation mixture of human suspended hepatocytes and SCH, respectively.

Hepatobiliary Disposition of Paroxetine and its Metabolites in SCH. When 1 µM [3H]paroxetine was incubated with rat SCH, accumulation of M1-glucuronide in cells (+ bile) decreased after peaking at 30 minutes incubation (Fig. 6C). The BEI of M1-glucuronide at 15 and 30 minutes incubation was 50.9 and 25.8%, respectively, and it also decreased after peaking at around 30 minutes incubation. The accumulation of M1-glucuronide in cells + bile (treated with Ca2+-containing buffer) at 15 minutes incubation was not significantly higher than that in cells (treated with Ca2+-free buffer), whereas P = 0.107 was near statistical significance. Because the amount of M1-sulfate formed in rat hepatocytes was quite small, the accumulation and BEI of M1-sulfate could not be calculated. On the other hand, accumulation of paroxetine, which was assumed not to be excreted into bile, reached the highest value at 1–4 hours of incubation. Although the accumulation of paroxetine in cells + bile was significantly higher than that in cells at 30 minutes incubation, there were no significant differences of the accumulations between cells + bile and cells at the other time point (Fig. 6A). The BEI of paroxetine in rat SCH was negligible.

When 1 µM [3H]paroxetine was incubated with human SCH, accumulation of M1-glucuronide reached a peak at 30 minutes incubation and then decreased (Fig. 6D). The BEI of M1-glucuronide at 15 and 30 minutes incubation was 50.9 and 25.8%, respectively, and also decreased after peaking at around 30 minutes incubation. The accumulations of M1-glucuronide in cells + bile were significantly higher than those in cells at 30 minutes and 1 hour incubation. Accumulation of M1-sulfate reached a peak at 15–60 minutes incubation (Fig. 6E), and the BEI of M1-sulfate at 15, 30, and 60 minutes incubation was 19.0, 16.4, and 29.1%, respectively. The BEI of M1-glucuronide was comparable to that of M1-sulfate in human SCH. The accumulation of M1-sulfate in cells + bile was significantly higher than that in cells at 30 minutes incubation. Accumulation of paroxetine in cells + bile was similar to that in cells (Fig. 6B), and the accumulation of paroxetine in cells + bile was significantly higher than that in cells at 30 minutes incubation. The BEI of paroxetine in human SCH was negligible. The BEIs of paroxetine, M1-glucuronide, and M1-sulfate at designated time points in both species are summarized in Table 2.
Uptake into ABC Transporter-Expressing Vesicles. Because the results of hepatobiliary dispositions indicated that M1-glucuronide and/or M1-sulfate are likely to be substrates for canalicular efflux transporters, vesicular transport studies were performed to evaluate the involvement of ABC transporters. M1-sulfate was not detectable, and thus, its BEI was unknown in rat SCH; however, the transport of M1-sulfate was also evaluated using rat ABC transporter-expressing membrane vesicles. A significant ATP-dependent uptake of M1-glucuronide into both rat Mrp2 and human MRP2 membrane vesicles was observed (Fig. 7, A and B). At 1 minute incubation, a significant uptake of M1-glucuronide in both rat Bcrp and human BCRP was found in the presence of ATP, whereas it was not time dependent (Fig. 7, C and D). Moreover, no differences were observed in the uptake of M1-glucuronide into BSEP/Bsep-expressing vesicles regardless of the presence or absence of ATP (Fig. 7, E and F). For M1-sulfate, an obvious involvement of the transporters was not observed in any of MRP2/Mrp2, BCRP/Bcrp, and BSEP/Bsep-expressing membrane vesicles (Fig. 8).

Discussion

According to the Food and Drug Administration guidance and the ICH-M3 (R2), the disposition of both the metabolite(s) and the parent drug need to be defined, because the systemic exposure of metabolite(s) in nonclinical animal species has to be compared with that in humans when the metabolite(s) is observed at exposures greater than 10% of parent drug (Food and Drug Administration) or total drug-related exposure (ICH) in humans. In the present study, we evaluated the usefulness of SCH to mimic the hepatic disposition of drugs in animals and humans, especially in terms of predicting the fate of metabolites formed in hepatocytes.

Paroxetine is initially oxidized by P450, promptly methylated by COMT, and further conjugated by UGT and SULT, indicating the involvement of multiple enzymes in its metabolism. Because P450s and UGTs are localized in the endoplasmic reticulum but COMTs and SULTs are intracellular enzymes mainly located in cytosolic fractions (Jancova et al., 2010), hepatocytes are likely to be ideal enzyme sources for the evaluation of the metabolism of paroxetine. Indeed, regardless of the incubation methods, suspension or SCH, M1-glucuronide was the main metabolite from the incubation with rat hepatocytes (Fig. 3) and M1-glucuronide and M1-sulfate were those with human (Fig. 5). In addition, the BEI of M1-glucuronide in rat SCH was higher than that in human SCH (Table 2). These results corresponded with the published in vivo disposition (Haddock et al., 1989; Kaye et al., 1989). Further studies on evaluation of down-regulation rates between phase I and phase II enzymes are required to evaluate the usefulness of SCH for quantitative assessment of the in vivo metabolite pathway.

This is the first report to compare multiple-enzyme metabolism in SCH with that in suspension and the in vivo metabolic pathway. Other researchers have compared individual metabolic enzyme activity between the two methods independently (Kern et al., 1997; Slaus et al., 2001; Lau et al., 2002; Treijtel et al., 2004). SCH is beneficial for the determination of the metabolic intrinsic clearance of slowly metabolizing compounds, because it allows long-term incubation (Treijtel et al., 2004; Treijtel et al., 2005). The incubation period was up to 24 hours in the present study, whereas Treijtel et al. conducted...
metabolic stability studies over 48 hours with SCH for tolbutamide and warfarin and concluded that successful prediction of metabolic intrinsic clearance of these slowly metabolizing drugs was possible. Moreover, they identified minor metabolites of tolbutamide by long-term incubation with SCH (Treijtel et al., 2004). However, liver-specific functions, such as metabolic activity, are dependent on incubation conditions, and some metabolic enzyme activities in cultured hepatocytes were diminished during incubation (Kern et al., 1997; Slaus et al., 2001; Kienhuis et al., 2007). According to an estimate of the percentage of paroxetine remaining at the maximum incubation period corrected by the number of initial viable cells, the metabolic activity of paroxetine in suspension was approximately 7–12-fold higher than that in SCH. Similarly, the metabolic activity of paroxetine was approximately 8–12-fold higher when estimated from the percentage of M1-glucuronide or M1-sulfate produced (Fig. 4). These values were within the reported range, and the difference can be estimated to be smaller, because the number and viability of plated cells in sandwich culture decrease slightly as the incubation time increases (Treijtel et al., 2004). The decrease of metabolic activity could not be fully prevented under the current culture conditions; however, no significant difference was observed in the comprehensive metabolite profiles between the two methods, which indicated that the metabolic activities of UGTs and SULTs decreased at a similar rate.

Because the hepatocytes in suspension can be used for only several hours because of their rapidly diminishing cell viability (Berthiaume et al., 1996; Berry et al., 1997), the SCH system can be useful for providing a sufficient amount of metabolite to determine its chemical structure even if the test article is slowly metabolized.

Our findings also provide information on the possibility of performing P450 induction studies with human SCH. By administering a preformed metabolite to cultured human hepatocytes, an adequate evaluation of its inductive potential may not be possible, because a hydrophilic metabolite often has lower membrane permeability than its lipophilic parent drug (Pang et al., 1984; de Lannoy and Pang, 1987). Moreover, synthesis of a sufficient amount of metabolite(s) and verification of its purity and structure are not easy, particularly in the case of minor or unstable metabolite(s).
findings that metabolites generated in SCH corresponded to in vivo profiles suggest that comprehensive in vitro P450 inductive potential of test article, including the effect of metabolite(s) that are exposed systemically or intrahepatically, can be assessed by the treatment of parent compound with SCH.

Metabolic/hepatobiliary transport studies that provide the hepatic disposition of various compounds have been conducted (Lengyel et al., 2005; Turncliff et al., 2006; Lee et al., 2010; Yan et al., 2011). In this study, we evaluated the hepatic disposition of paroxetine and revealed that the species difference of hepatobiliary disposition of M1-glucuronide between rats and humans using the SCH system could be determined. The primary excretion route of paroxetine-derived radioactivity in rats and humans is bile and urine, respectively (Haddock et al., 1989), and we assumed that this species difference regarding the fate of excreted metabolites depends on the biliary excretion of its major metabolite, because paroxetine is completely eliminated by hepatic metabolism. Indeed, the BEI of M1-glucuronide, which is a major metabolite in both species, was higher in rat SCH than that in human SCH, whereas the BEIs of paroxetine were negligible in both species. In addition, the BEI of M1-sulfate, which is another major metabolite in humans but not in rats, was comparable to that of M1-glucuronide in human SCH (Table 2). These results support our assumption that the species difference of paroxetine with regard to the excretion fate of metabolites depends on the biliary excretion availability of its major metabolites. Furthermore, M1-glucuronide was suggested to be an MRp2/Mrp2 substrate in both species from the results of the vesicular transport studies (Fig. 7). However, our findings indicate a possible involvement of MRp2/Mrp2 in the biliary excretion of M1-glucuronide; thus, additional experiments are needed to demonstrate the effect of Mrp2 on in vivo M1-glucuronide disposition by comparison of the biliary excretion rate between normal and genetically Mrp2-deficient rats. In humans, Mrp2 is encoded by the ABCC2 gene, in which several genetic variations associated with protein function or expression have been found (Suzuki and Sugiyama, 2002). However, because M1-glucuronide and M1-sulfate have markedly weaker pharmacological activities than the parent drug (Haddock et al., 1989), the alteration of effectiveness would not occur even if paroxetine was administered to patients with ABCC2 genetic variations.

The ability of M1-glucuronide transport by rat Mrp2 was higher than that by human MRp2. It is reported that biliary excretion is highly dependent on molecular mass with a certain threshold depending on the animal species. Compounds with molecular mass higher than 325 ± 50 g/mol and 500 ± 50 g/mol are preferentially excreted into bile in rats and humans, respectively (Hirom et al., 1972; Levine, 1978), and MRp2/Mrp2 and BCRP/Bcrp are partly involved in this molecular weight–dependent biliary excretion process and species difference between rats and humans (Kato et al., 2008; Choi et al., 2009). For M1-glucuronide, its molecular mass is around 500 g/mol (Fig. 2). Therefore, the higher biliary excretion availability of M1-glucuronide could be explained by the selectivity of MRp2/Mrp2 for compounds of a certain molecular mass.

Accumulations of M1-glucuronide and M1-sulfate in cells (+bile) reached the maximum values at 15–60 minutes incubation and then decreased (Fig. 6, C–E). We believe that this is caused by the flux from the bile canaliculari to the outer incubation medium. Lee et al. previously observed such a flux and reported a T_{flux} (the occurring time of flux during incubation) of 16.7 minutes (Lee et al., 2010),
which supports our findings. Moreover, the in vitro disposition of a compound is assumed to achieve equilibrium by continuous incubation of medium, cells, and bile canaliculi. Accordingly, the accumulation in the bile canalicular lumen is apparently diminished because of the in vitro equilibrium, as observed in the present study (Fig. 6). For adequate evaluation, the BEI of the metabolites formed in the cells should be assessed at the earliest time that the metabolite amount can be quantified.

In conclusion, we succeeded in mimicking the in vivo species difference of the hepatic disposition of paroxetine between rats and humans using SCH. SCH was found to be useful for evaluating the processes of hepatic uptake and metabolism of parent drugs and the biliary excretion of both parent drugs and formed metabolites in liver at the same time. Data obtained from metabolic/hepatic biopsy transport studies with SCH using radiolabeled compound should allow the synthesis of metabolic pathways to be prioritized.

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Authorship Contributions

Participated in research design: Matsunaga, Nunoya, Ogawa, Tamai. Conducted experiments: Matsunaga, Okada. Performed data analysis: Matsunaga, Nunoya, Ogawa, Tamai. Wrote or contributed to the writing of the manuscript: Matsunaga, Tamai.

References


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