In Vitro Assessment of Drug-Drug Interaction Potential of Boceprevir Associated with Drug Metabolizing Enzymes and Transporters

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ABSTRACT

The inhibitory effect of boceprevir (BOC), an inhibitor of hepatitis C virus nonstructural protein 3 protease was evaluated in vitro against a panel of drug-metabolizing enzymes and transporters. BOC, a known substrate for cytochrome P450 (P450) CYP3A and aldo-ketoreductases, was a reversible time-dependent inhibitor ($k_{inact} = 0.12$ minute$^{-1}$, $K_{i} = 6.1$ mM) of CYP3A4/5 but not an inhibitor of other major P450s, nor of UDP-glucuronosyltransferases 1A1 and 2B7. BOC showed weak to no inhibition of breast cancer resistance protein (BCRP), P-glycoprotein (Pgp), or multidrug resistance protein 2. It was a moderate inhibitor of organic anion transporting polypeptide (OATP) 1B1 and 1B3, with an $IC_{50}$ of 18 and 4.9 mM, respectively. In human hepatocytes, BOC inhibited CYP3A-mediated metabolism of midazolam, OATP1B1-mediated hepatic uptake of pitavastatin, and both the uptake and metabolism of atorvastatin. The inhibitory potency of BOC was lower than known inhibitors of CYP3A (ketoconazole), OATP1B1 (rifampin), or both (telaprevir). BOC was a substrate for Pgp and BCRP but not for OATPB1, OATPB3, OATPB1, organic cation transporter, or sodium/taurocholate cotransporting peptide. Overall, our data suggest that BOC has the potential to cause pharmacokinetic interactions via inhibition of CYP3A and CYP3A/OATP1B interplay, with the interaction magnitude lower than those observed with known potent inhibitors. Conversely, pharmacokinetic interactions of BOC, either as a perpetrator or victim, via other major P450s and transporters tested are less likely to be of clinical significance. The results from clinical drug-drug interaction studies conducted thus far are generally supportive of these conclusions.

Introduction

Boceprevir (BOC), also known as SCH-503034 (Fig. 1), is an antiviral agent for the treatment of hepatitis C virus (HCV) genotype 1 infections. BOC is specifically designed to inhibit HCV nonstructural protein 3 protease (Bacon et al., 2011; Poordad et al., 2011), a serine protease essential for HCV polyprotein processing and therefore viral replication (Chen and Tan, 2005). BOC binds covalently yet reversibly to the nonstructural protein 3 protease active site through a ketoamide functional group, and elicits potent inhibitory activity in the replicon system alone (Malcolm et al., 2006) or in combination with interferon α-2b and ribavirin (Kwo et al., 2010; Foote et al., 2011; Maddur and Kwo, 2011). BOC is a mixture of two diastereomers, SCH-534128 (active isomer) and SCH-534129 (inactive isomer), that differ in the stereochemical configuration at the third carbon atom (Fig. 1) from the ketoamide end group, and elicits potent inhibitory activity in the replicon system alone (Malcolm et al., 2006) or in combination with interferon α-2b and ribavirin (Kwo et al., 2010; Foote et al., 2011; Maddur and Kwo, 2011). BOC is a mixture of two diastereomers, SCH-534128 (active isomer) and SCH-534129 (inactive isomer), that differ in the stereochemical configuration at the third carbon atom (Fig. 1) from the ketoamide end group, and elicits potent inhibitory activity in the replicon system alone (Malcolm et al., 2006) or in combination with interferon α-2b and ribavirin (Kwo et al., 2010; Foote et al., 2011; Maddur and Kwo, 2011). BOC is a mixture of two diastereomers, SCH-534128 (active isomer) and SCH-534129 (inactive isomer), that differ in the stereochemical configuration at the third carbon atom (Fig. 1) from the ketoamide end group, and elicits potent inhibitory activity in the replicon system alone (Malcolm et al., 2006) or in combination with interferon α-2b and ribavirin (Kwo et al., 2010; Foote et al., 2011; Maddur and Kwo, 2011). BOC is a mixture of two diastereomers, SCH-534128 (active isomer) and SCH-534129 (inactive isomer), that differ in the stereochemical configuration at the third carbon atom (Fig. 1) from the ketoamide end group, and elicits potent inhibitory activity in the replicon system alone (Malcolm et al., 2006) or in combination with interferon α-2b and ribavirin (Kwo et al., 2010; Foote et al., 2011; Maddur and Kwo, 2011).
immunodeficiency virus (HIV) (Wilby et al., 2012). Most immunosuppressants and anti-HIV drugs are known to be substrates and/or potent inhibitors of important drug-metabolizing enzymes (Jimenez-Nacher et al., 2011), such as P450 enzymes (Lin, 2006; Zhou, 2008; Obach, 2009) and UDP-glucuronosyltransferases (UGT) (Kiang et al., 2005; Zhang et al., 2005). Numerous in vitro and in vivo studies have demonstrated that these compounds are also substrates, inhibitors, or inducers of various drug transporters, including the hepatic uptake transporters organic anion-transporting polypeptides (OATP1B1) (SLCO1B1), OATP1B3 (SLCO1B3), and OATP2B1 (SLCO2B1), organic cation transporter (OCT1) (SLC22A1), and the sodium/taurocholate cotransporting peptide (NTCP) (SLC10A1), as well as the efflux transporters MDR1 P-glycoprotein (Pgp, ABCB1), multidrug resistance protein (MRP2) (ABCB2), and breast cancer resistance protein (BCRP) (ABCG2) (Griffin et al., 2011).

It is increasingly recognized that drug transporters have significant impact on DDIs by modulating the absorption, distribution, metabolism, and excretion of drugs alone or in interplay with drug-metabolizing enzymes (Giacomini et al., 2010). Hepatic elimination of drugs often is a result of the interplay between hepatic uptake/efflux transporters and drug-metabolizing enzymes. For instance, hepatic elimination of some 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors such as atorvastatin involves OATP-mediated uptake followed by metabolism by CYP3A4 (Lau et al., 2006; Lau et al., 2007). Furthermore, several efflux transporters such as Pgp, BCRP, and MRP2 are also localized in the apical membrane of enterocytes. These transporters, together with the enzymes in the gut, can also modulate the first-pass effect of orally administered drugs and thus influence the manifestation of DDIs.

We describe the comprehensive in vitro assessment of the potential of BOC to cause pharmacokinetic interactions at the levels of drug-metabolizing enzymes and transporters. The evaluations include 1) inhibition of major human P450 and UGT enzymes; 2) inhibition of major human drug transporters (OATP1B1, OATP1B3, OATP2B1, Pgp, BCRP, and MRP2); and 3) impact on the enzyme-transporter interplay between CYP3A4 or UGTs and OATP1B in human hepatocytes. We also evaluated whether BOC is transported by efflux transporters Pgp, BCRP, and MRP2, and hepatic uptake transporters OATP1B, OATP1B3, OATP2B1, NTCP, and OCT1. Where applicable, the results are compared with clinical DDI observations that have been reported, and the implications for the potential for DDIs with these enzymes and transporters are discussed.

Materials and Methods

Materials

[^14C]BOC (specific activity 56.3 mCi/mmol; purity 98.2% as measured by high-performance liquid chromatography [HPLC]) was synthesized by the Radiochemistry Department of Merck Research Laboratories (Kenilworth, NJ). Unlabeled BOC, SCH-534128, SCH-534129, SCH-629144, and Ko143 (3-(6-isobutyl-9-methoxy-1,4-dioxo-1,2,3,4,6,7,12,12a-octahydropyrazino[1’,2’:1,6] pyrido[3,4-b]indol-3-yl)-propionic acid tert-butyl ester) were synthesized by the Chemistry Department, Merck Research Laboratories. [1H]Estradiol-17β-D-glucuronide (E1,βG) and [1H]cholecytokinin octapeptide (CCK-8), [1H]prazosin, and [1H]verapamil were purchased from PerkinElmer Life Sciences (Boston, MA). [1H]Pitavastatin, unlabeled pitavastatin, [1H]taurocholic acid (TCA), and [14C]TEA were purchased from American Radiolabeled Chemicals (St. Louis, MO). [1H]Methotrexate was purchased from Moravek (Brea, CA). [1C]Ethacrynic acid glutathione conjugate (EA-SG) was synthesized by the Radiochemistry Department of Merck Research Laboratories. Prazosin, cyclosporine A (CsA), testosterone, 6β-hydroxysterosterone, cortisol, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADP, UDP-glucuronic acid (UDP-GA), amelaminic, estradiol, 3’-azido-3’-deoxythymidine, nicardipine, dicrofenac, thymidine, and labetalol were purchased from Sigma-Aldrich (St. Louis, MO). Bromosulfophthalein (BSP) was purchased from MP Biomedicals (SOLON, OH). All other reagents were commercially obtained with the highest analytical purity grade.

Pooled human liver microsomes (HLM) (26 male and 21 female; Lot 37181) were purchased from BD Biosciences Discovery Labware (Woburn, MA). Cryopreserved human hepatocytes (Lot DAC; pooled from five donors) were purchased from Celsis IVT (Chicago, IL). Membrane vesicles isolated from baculovirus infected Spodoptera frugiperda (Sf9) cells containing human BCRP (ABCG2) or MRP2 (ABCC2) were purchased from Invitrogen by Life Technologies (Carlsbad, CA).

Cells

Madin-Darby canine kidney type II (MDCKII) cells, MDCKII cells stably transfected with human BCRP (MDCKII-hBCRP), Chinese hamster ovary K1 (CHO-K1) cells, and CHO-K1 cells stably transfected with human OCT1 (CHO-K1-hOCT1) were obtained from Solvo Biotechnology (Budapest, Hungary) under a license agreement and evaluation agreement, respectively. MDCKII, pig kidney epithelial cells (LLC-PK1), and MDCKII or LLC-PK1 cells expressing complementary DNA (cDNA) encoding human MDR1 Pgp (MDCKII-MDR1 or LLC-MDR1) were obtained from the Netherlands Cancer Institute (Amsterdam, The Netherlands) under a license agreement.

OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), and OATP2B1 (SLCO2B1) stably transfected MDCKII cells (MDCKII-OATP1B1, MDCKII-OATP1B3, and MDCKII-OATP2B1 cells) were generated as described previously elsewhere (Monteagudo et al., 2010).

Inhibition Studies with Human P450 and UGT Enzymes in HLM

Evaluation of BOC as a reversible and time-dependent inhibitor of major P450 (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5) and a reversible inhibitor of UGT (UGT1A1 and UGT2B7) enzymes was conducted in HLM.

Reversible inhibition to the human P450 enzymes was conducted as described elsewhere (Madden et al., 2002; Walsky and Obach, 2004; Parkinson et al., 2011). Briefly, incubations were conducted at 37°C in 0.4 ml of
Covalent Binding of BOC in HLM

Pooled HLM (1 nmol P450/ml) were incubated with [14C]BOC (20 μM) for 120 minutes in 0.5 ml of 100 mM potassium phosphate buffer (pH 7.4) to determine the covalent binding of BOC to HLM. The incubation mixtures (pH 7.4) containing potassium phosphate buffer (50 mM), MgCl2 (3 mM), EDTA (1 mM), an NADPH-generating system, and a probe substrate at concentrations approximately equal to their apparent Km values, as indicated in Table 1. Both midazolam and testosterone were used as probe substrates for CYP3A4/5. The Km value for reversible inhibition of CYP3A4/5 was determined using midazolam as the probe substrate (1.5–50.0 μM) with several BOC concentrations (2.5–100.0 μM). Reactions were initiated with the addition of an aliquot of an NADPH-generating system and were performed in duplicate. Reactions were terminated at 5 minutes by the addition of acetonitrile containing the appropriate internal standard. The internal standards were deuterated metabolites of the probe substrates: d4-acetaminophen, d5-7-hydroxycoumarin, d6-bupropion, d5-N-desethylamodiaquine, d4-4′-hydroxydiclofenac, d3-4′-hydroxyxenethonium, d3-dextrophan, d2-6-hydroxychloroxazone, d3-6β-hydroxytestosterone, and d3-1′-hydroxymidazolam.

The preincubation-dependent inhibition of BOC was also determined for CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP3A4/5 by comparison of IC50 values with and without 30 minutes of preincubation in the presence of NAPDH. The probe substrates and test concentrations were the same as reversible inhibition conducted without preincubation. To determine kinetic parameters (kmax and Km) for time-dependent inhibition by CYP3A, pooled HLM (1 mg/ml) were preincubated (in duplicate) at 37°C with various concentrations of BOC in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 6 mM MgCl2, and an NAPDH-generating system for 5 to 30 minutes. The incubation mixtures were diluted 10-fold with the same buffer containing 250 μM testosterone (CYP3A4 probe substrate) and an NAPDH-generating system. The incubation was continued for an additional 10 minutes to monitor the extent of testosterone 6β-hydroxylation.

To determine whether BOC formed a spectrophotometrically detectable metabolite inhibitory complex with cytochrome P450 (i.e., peaks at approximately 452 nm), an individual HLM sample (H0079, Lot 0010127, XenoTech, LLC) containing high levels of CYP3A4/5 activity (final protein concentration of approximately 452 nm), an individual HLM sample (H0079, Lot 0010127, XenoTech, LLC) containing high levels of CYP3A4/5 activity (final protein concentration of approximately 25 times the typical incubation concentration) in the presence of an NAPDH-generating system, for 0, 15, and 30 minutes in the presence and absence of an NAPDH-generating system, without a dilution step. Substrate (100 μM for testosterone and 5 μM for midazolam) was then added, and the incubation was performed for 5 minutes. Second, BOC (0 and 3 μM) were preincubated with HLM (1.25 mg/ml for midazolam and 2.5 mg/ml for testosterone, which is approximately 2 times the typical incubation concentration) in the presence of an NAPDH-generating system, for 0, 15, and 30 minutes. The samples were then diluted 25-fold before being incubated with marker substrate (200 μM for testosterone and 50 μM for midazolam). The incubation (at 1/25 the preincubation concentration of BOC and microsomal protein) was then continued for 5 minutes (to allow formation of any metabolites of the marker substrate) and stopped by the addition of the internal standard and acetonitrile. The residual CYP3A4/5 activity was determined. In this study, troleandomycin was used as positive control.

The inhibitory effect of BOC on human UGT1A1-mediated estradiol 3-glucuronidation and UGT2B7-mediated 3′-azido-3′-deoxythymidine (AZT) glucuronidation was evaluated using HLM. Pooled HLM (0.5 mg/ml) were incubated at 37°C for 20 minutes. For the UGT1A1 assays, the reaction mixtures contained 20 μM estradiol and 0.78 to 100 μM of BOC in 81 mM HEPES buffer (pH 7.0) with 9 mM MgCl2, 5 mM UDPGA, and 25 μg/ml of alamethicin. Nicardipine was included as positive control inhibitor of UGT1A1 (0.78 to 100 μM). For the UGT2B7 assays, the reaction mixtures contained 750 μM AZT and 0.78 to 100 μM of BOC in 50 mM potassium phosphate buffer (pH 7.4) with 8 mM MgCl2, 5 mM UDPGA, and 25 μg/ml of alamethicin. Diclofenac was included as positive control inhibitor (0.78 to 100 μM). The UGT reactions were terminated by adding 0.2 ml of ice-cold organic solvent (either methanol or acetonitrile containing 0.3% formic acid for UGT1A1 and UGT2B7 assays, respectively) and the supernatants were subjected to liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

Table 1 In vitro evaluation of boceprevir (BOC) as an inhibitor of major cytochrome P450 (P450) and UGT enzymes in human liver microsomes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>CYP/UGT Reaction</th>
<th>Substrate Concentration</th>
<th>Reversible Inhibition, No Preincubation</th>
<th>Time-Dependent Inhibition, 30-Minute Preincubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μM</td>
<td>IC50 (Km) μM</td>
<td>IC50 Inhibition (%) at 100 μM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Phenacetin O-deethylation</td>
<td>60</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>CYP2A6</td>
<td>Coumarin 7-hydroxylation</td>
<td>0.75</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion hydroxylation</td>
<td>50</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Amodiaquine N-dealkylation</td>
<td>2.0</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac 4′-hydroxylation</td>
<td>7.5</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-Mephenytoin 4′-hydroxylation</td>
<td>40</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan O-demethylation</td>
<td>7.5</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chloroxazone 6-hydroxylation</td>
<td>30</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>CYP3A4/5</td>
<td>Testosterone 6β-hydroxylation</td>
<td>100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Midazolam 1′-hydroxylation</td>
<td>5.0</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>Estradiol 3-glucuronidation</td>
<td>20</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>3′-azido-3′-deoxythymidine (AZT) glucuronidation</td>
<td>750</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

NA, not applicable (no value was obtained as the rates at the highest concentration of BOC evaluated [100 μM] were higher than the control rates); ND, not determined.

Time-dependent inhibition was determined by comparison of IC50 values with and without preincubation.
containing 3 mM MgCl₂ and an NADPH-generating system (0.5 mM NADP, 5 mM glucose-6-phosphate, and 1.5 units/ml of glucose-6-phosphate dehydrogenase). After the preincubation at 37°C for 2 minutes, reactions were initiated by addition of BOC. After incubation for 120 minutes, the reactions were terminated by the addition of 0.5 mL of ice-cold acetonitrile with 1% acetic acid. After vortexing and centrifuging (10,000 rpm) for 4°C for 10 minutes, the supernatants were analyzed by HPLC/flow scintillation analyzer. Microsomal pellets were washed 5 times with 0.5 mL of acetonitrile containing 1% acetic acid. After the centrifugation following each washing, the supernatants were combined and counted for radioactivity. The final pellet was dissolved in 1 mL of BBS-450 (tissue solubilizer; Beckman Coulter, Brea, CA), neutralized with 6N HCl, and counted for radioactivity.

Uptake and Inhibition Studies in OATP1B1, OATP1B3, OATP2B1, and OCT1 Transfected Cells

Uptake mediated by OATP1B1, OATP1B3, and OATP2B1 was determined in MDCKII cells stably transfected with OATP1B1, OATP1B3, or OATP2B1 cDNA, as previously described elsewhere (Monteagudo et al., 2010). OCT1-mediated uptake was measured in CHO-K1 cells stably transfected with OCT1 cDNA. For OATP transfected cells, cells were treated with 10 mM sodium butyrate (Sigma-Aldrich) for 24 hours to increase the OATP expression before the experiment. Cells were dislodged with trypsin EDTA and resuspended in Hank’s balanced salt solution plus 10 mM HEPES. Cells were then suspended in 96-well plates at a density of 0.6 × 10⁶ cells/well. Uptake was initiated by the addition of [¹⁴C]BOC (1 μM) or the positive control substrates [¹¹²H]E17β-G (1 μM), [¹³C]CCK8 (2.5 μM), [¹²⁵I]estrone-3-sulfate (0.1 μM), or [⁴⁺C]TEA (1 μM) for OATP1B1, OATP1B3, and OATP2B1, and OCT1, respectively. Cells were then incubated for the indicated time at 37°C, and uptake was stopped by the addition of ice cold phosphate-buffered saline (PBS), followed by immediate centrifugation for 1 minute at 300g/2°C, and uptake was determined by liquid scintillation counting in a MicroBeta Wallac Trilux Scintillation Counter (PerkinElmer). The inhibitory effect of BOC on MDR2- or MRP2-mediated uptake was also evaluated using [¹¹²H]TEA-SG (2 μM) or [¹⁴H]nethoxetate (10 μM) as the probe substrate in membrane vesicles containing MRP2 or BCRP, respectively (Chu et al., 2004).

Bidirectional Transport and Inhibition Studies

Bidirectional transport of BOC was assessed in MDCKII, MDCKII-MDR1, and MDCKII-BCRP cells. MDCKII and MDCKII-MDR1 cells were cultured in 96-well transwell culture plates (BD Biosciences, San Jose, CA). [¹⁴C]BOC (1 μM) was prepared in Hank’s balanced salt solution with 10 mM HEPES. Substrate solution (150 μL) was added to either the apical (A) or the basolateral (B) compartment of the culture plate, and buffer (150 μL) was added to the compartment opposite that containing the compound. At 3 hours, 50 μL of sample was removed from both sides, and 200 μL of scintillation fluid was added. Radioactivity was determined by liquid scintillation counting in a MicroBeta Wallac Trilux scintillation counter (Beckman Coulter). Inhibitory effect of BOC on OCT1-mediated uptake was also evaluated in CHO-K1 cells stably transfected with OCT1 cDNA. For OCT1-transfected cells, cells were treated with 10 mM sodium butyrate (Sigma-Aldrich) for 24 hours to increase the OCT1 expression before the experiment. Cells were dislodged with trypsin EDTA and resuspended in Hank’s balanced salt solution plus 10 mM HEPES. Cells were then suspended in 96-well plates at a density of 0.6 × 10⁶ cells/well. Uptake was initiated by the addition of [¹⁴C]BOC (1 μM) or the positive control substrates [¹¹²H]E17β-G (1 μM), [¹³C]CCK8 (2.5 μM), [¹²⁵I]estrone-3-sulfate (0.1 μM), [⁴⁺C]TEA (1 μM) for OATP1B1, OATP1B3, and OATP2B1, respectively. Cells were then incubated for the indicated time at 37°C, and uptake was stopped by the addition of ice cold phosphate-buffered saline (PBS), followed by immediate centrifugation for 1 minute at 300g/2°C, and uptake was determined by liquid scintillation counting in a LS6500 Multipurpose Scintillation Counter (Beckman Coulter). The inhibitory effect of BOC on OCT1-mediated uptake was also evaluated in MDCKII-transfected cells with OATP1B1, OCT1B3, and OATP2B1. [¹³¹I]Pitavastatin (0.1 μM), [¹²⁵I]BSF (0.1 μM), and [¹²⁵I]estrone-3-sulfate (0.1 μM) were used as probe substrates for OATP1B1, OATP1B3, and OATP2B1, respectively. Uptake of all probe substrates tested in transfected cells was at least 5-fold higher than in control cells (unpublished data).

Vesicular Uptake and Inhibition Studies

Time- and ATP-dependent uptake of BOC was conducted in human MRP2 and control vesicles. Membrane vesicles (10 μL) were added to 8-stripe tubes (Corning Inc., Corning, NY) at 20 μg/tube. Then 20 μL of [¹⁴C]BOC (final concentration 2 μM) or [¹¹²H]EA-SG (final concentration 2 μM) dissolved in transport buffer (0.25 M sucrose, 10 mM Tris-HCl buffer [pH 7.4], 10 mM MgCl₂) were added into the tubes containing vesicles. The mixtures of vesicle and dosing solution were preincubated for 3 minutes at 37°C. Uptake was initiated by the addition of 20 μL of ATP-regenerating reagent (5 mM ATP, 10 mM creatine phosphate, and 100 μg/mL of creatine phosphokinase in transport buffer) or 20 μL transport buffer, followed by incubation at 37°C for the indicated time. Uptake was stopped by the addition of 200 μL of ice-cold stop buffer (0.25 M sucrose, 0.1 M NaCl, 10 mM Tris-HCl buffer [pH 7.4]) followed by rapid filtration of the reaction mixture onto a prewettted 96-well glass-fiber type B filter plate (1.0 μm) (Millipore, Billerica, MA). Filters containing the membrane vesicles were washed with 200 μL of ice-cold stop buffer and dried at room temperature, followed by the addition of 25 μL of scintillation fluid. Radioactivity was determined in a MicroBeta Wallac Trilux Scintillation Counter (PerkinElmer). The inhibitory effect of BOC on MRP2- or BCRP-mediated uptake was also evaluated using [¹¹²H]EA-SG (2 μM) or [¹⁴H]nethoxetate (10 μM) as the probe substrate in membrane vesicles containing MRP2 or BCRP, respectively (Chu et al., 2004).

Uptake Studies in Cryopreserved Human Hepatocytes

Uptake of BOC was evaluated as described previously elsewhere (Monteagudo et al., 2010) in cryopreserved human hepatocyte suspension. Briefly, uptake of [¹⁴C]BOC into human hepatocytes was determined at 37°C or 4°C, respectively. Cells were resuspended in Krebs-Henseleit modified buffer (KBH) (Sigma-Aldrich) (pH 7.4) in 96-well deep wells (BD Falcon, San Jose, CA) at a density of 0.2 × 10⁶ cells/well. The cells and dosing solution were preincubated at 37°C or 4°C for 5 minutes, respectively. Uptake studies were initiated by the addition of 50 μL of [¹⁴C]BOC (final concentration 1 μM) or the positive control substrate [¹¹²H]E17β-G (final concentration 1 μM). The reaction mixtures were incubated at 37°C or 4°C for the time indicated, and uptake was stopped by the addition of ice-cold PBS. Other experimental procedures are the same as the uptake assay in transfected cells as described previously. To study sodium-dependent uptake, the uptake of [¹⁴C]BOC (1 μM) was determined at 37°C and 4°C in KHB and sodium-free KHB at 37°C. [¹¹²H]TCA (0.5 μM) was used as a positive control. To measure the kinetic parameters of BOC, the initial uptake rate of [¹⁴C]BOC at various concentrations was determined at 0.5 and 3.0 minutes at 37°C. Inhibitory effect of several compounds on initial uptake rate of [¹⁴C]BOC (0.5 μM) was measured at 37°C in the presence and absence of various concentrations of the compounds tested.

Inhibition of BOC on Hepatic Uptake and Metabolism in Human Hepatocytes

The inhibitory effect of BOC on the uptake and metabolism of several probe substrates, including pitavastatin (OATP1B and UGT1s), midazolam (CYP3A), and atorvastatin (OATP1B and CYP3A), was evaluated using the same lot of hepatocytes. For the uptake studies, the impact of BOC on the initial uptake rate of [¹³¹I]pitavastatin (1μM) and [¹²⁵I]atorvastatin (3μM) was studied with human hepatocytes at 37°C after incubation for 1 and 5 minutes. The substrate concentrations tested in these studies were well below Km values for pitavastatin (Km = 5 μM; unpublished data) and atorvastatin (Km = 18.9 μM) (Lau et al., 2007) measured in OATP1B1 transfected cells. Other experimental procedures were as described earlier for the hepatocyte uptake studies.

To assess the effect of BOC on the metabolism of midazolam, pitavastatin, and atorvastatin acid, the formation of their major metabolites, 1-OH-midazolam, pitavastatin-glucoronide, p- and o-OH-atorvastatin acid and atorvastatin lactone, was evaluated in the presence of BOC and positive control inhibitors. Stock solutions of the substrates and inhibitors were prepared in dimethylsulfoxide followed by dilution with 50% acetonitrile. A 3-μL aliquot of each diluted stock solution was added to the incubation mixtures to give final substrate and inhibitor concentrations, as indicated later. Human hepatocytes (1.5 or 2 million cells/ml) were suspended in William’s E buffer (0.5 mL) containing L-glutamine. Incubations (n = 3 for each inhibitor-substrate pair) were
conducted with midazolam (1 μM), pitavastatin (1 μM), or atorvastatin acid (3 μM) for 20 minutes at 37°C under a 95% air/5% CO₂ atmosphere in the absence (solvent only) or presence of an inhibitor. The 20-minute reaction time was chosen to ensure that the inhibitory effects on both OATP1B and CYP3A-mediated metabolite formation could be measured. Ketoconazole (2 μM), rifampin (20 μM), and telaprevir (3 μM) were used as positive control inhibitors for CYP3A, OATP1B, and both, respectively. In addition, a range of BOC (0.5–10.0 μM) and telaprevir (0.25–10.0 μM) concentrations was tested to assess the concentration-dependent inhibition on the formation of metabolites of atorvastatin. The metabolites formed in the solvent control incubations were expressed as 100%. Reactions were terminated by the addition of 0.5 volumes of ice-cold acetonitrile containing the internal standard labetalol (0.2 or 0.5 μM), followed by vortex-mixing and centrifugation. The resultant supernatant was analyzed by HPLC-MS.

LC-MS/MS Analysis

Quantification of P450 Enzyme Activity. All P450 probe substrates including 1′-OH midazolam were analyzed by LC-MS/MS with deuterated metabolites as internal standard as described previously elsewhere (Parkinson et al., 2011).

Quantitation of UGT1A1 Activity. A Sciex API 4000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA) was used to acquire data. Chromatographic separation was achieved using a Symmetry C18 reversed-phase column (4.6 × 100 mm, 3.5 μm; Waters Corp., Milford, MA) and eluted at a flow rate of 1 ml/min with a mobile phase consisting of A (10% methanol in deionized water containing 0.05% formic acid) and B (10% deionized water in acetonitrile containing 0.05% formic acid). The gradient conditions were as follows: 0 to 0.5 minutes at 20% buffer B, 0.5 to 2.0 minutes to 40% buffer B, 2.0 to 2.5 minutes to 70% buffer B, 2.5 to 3.5 minutes at 70% buffer B, and 3.5 to 3.8 minutes return to 20% buffer B and re-equilibrate until 4.3 minutes with 20% buffer B. The instrument was operated in the negative ionization mode using the electrospray interface, and selected reaction monitoring was used to determine the specific precursor-ion to product-ion transitions for the glucuronide (447.1/113.0).

Quantitation of UGT2B7 Activity. A Sciex API 4000 triple quadrupole mass spectrometer was used to acquire data. Chromatographic separation was achieved using a Zorbax SB reversed-phase column (4.6 × 75 mm, 3.5 μm; Waters Corp.) and eluted at a flow rate of 1 ml/min with a mobile phase consisting of A (deionized water containing 0.01% formic acid) and B (acetonitrile containing 0.01% formic acid). The gradient conditions were as follows: 0 to 1.5 minutes at 5% buffer B, 1.5 to 3.0 minutes at 5 to 60% buffer B, 3.0 to 3.5 minutes at 60% buffer B, 3.6 to 4.0 minutes to 95% buffer B, and 4.0 to 4.5 minutes return to 5% buffer B. The instrument was operated in the negative ionization mode using the electrospray interface, and selected reaction monitoring was used to determine the specific precursor-ion to product-ion transitions for the glucuronide (442.0/125.0).

Quantitation of Metabolites for Midazolam, Pitavastatin, and Atorvastatin. LC-MS/MS analysis was performed using a Waters AcquITY Ultra Performance LC system coupled to a Waters quadrupole time-of-flight Xevo mass spectrometer. HPLC analyses consisted of a Waters Ultra-Performance Liquid Chromatography High-Strength Silica T3 column (1.8 μm, 2.1 × 50 mm) and a mobile phase with (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile (constant flow rate of 0.15 ml/min). Hepatocyte incubates were analyzed using the following gradient elution: 0–0.5 minutes, 90% A; 7.5 minutes, 5% A; the column was washed at 5% A (1 minute) and equilibrated at 90% A (1 minute) before the next injection. The quadrupole time-of-flight mass spectrometer was operated under electrospray ionization positive-ion mode. The source temperature was set at 100°C while the desolvation temperature was 600°C. The resolution of the time-of-flight detection was approximately 8000. Formation of major metabolites of midazolam, pitavastatin, and atorvastatin were monitored to assess the effect of inhibitors.

Data Analysis

The Kᵢ value of BOC for the competitive inhibition of CYP3A4/5 was calculated by eq. 1:

\[
\frac{1}{V} = \frac{K_m}{V_{max}} \left(1 + \frac{[I]}{K_i}\right) \left(\frac{1}{[S]} + \frac{1}{V_{max}}\right)
\]

where Kᵢ is the inhibition constant; V is the rate of reaction in presence of inhibitor at a concentration [I] and substrate at a concentration [S]; and K_m and V_max are the Michaelis-Menten constants for a given P450 reaction. The data were fitted in GraFit 4.0 software for Ki determination (Erithacus Software, Horley, United Kingdom). Visual inspection was performed using Eadie-Hofstee plots to confirm the nature of the inhibition.

The first-order rate constants (k_{obs}) of BOC for inactivation of CYP3A4/5 at various concentrations were estimated from the initial slopes of a natural logarithm plot of the percentage of remaining activity versus the preincubation time. The k_{max} and K_i values were calculated by nonlinear regression analysis of eq. 2 using KaleidaGraph Synergy Software (Reading, PA):

\[
k_{obs} = \frac{k_{max}}{K_i + [I]}
\]
Results

Evaluation of BOC as an Inhibitor of Major P450 and UGT Enzymes in HLM. Evaluation of BOC reversible inhibition of major human CYPs (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5) was conducted in HLM with selective probe substrates. We also assessed the reversible inhibition of BOC to UGT1A1 and UGT2B7, two major UGT isoforms responsible for glucuronidation of most clinically used drugs (Williams et al., 2004).

As shown in Table 1, BOC showed minimal inhibition of CYP2B6, CYP2C9, or CYP2E1. At the highest concentration tested (100 μM), BOC showed only 20–25% inhibition of CYP1A2, CYP2A6, CYP2C8, and CYP2C9, whereas it demonstrated 45% inhibition of CYP2D6. However, its inhibitory effect on CYP2D6, if we assume that IC50 = 100 μM is the worst-case scenario, is not likely to be clinically significant, as the IC50 value was ~33-fold higher than the total plasma Cmax (~3 μM) of BOC at the clinically relevant dose. There was also no inhibition of estradiol 3-glucuronidation by UGT1A1 or AZT glucuronidation by UGT2B7 in HLM (IC50 > 100 μM).

However, BOC exerted an appreciable inhibition of CYP3A4/5 (measured by midazolam 1’-hydroxylation) with an IC50 of 11 ± 1.0 μM. The inhibition of midazolam hydroxylation was further confirmed to be competitive with a Ki value of 7.7 ± 0.8 μM. Interestingly, BOC did not inhibit testosterone 6β-hydroxylation, another functional marker activity for CYP3A4/5 (IC50 > 100 μM). Such substrate-dependent inhibition with CYP3A4/5 has been reported previously for atorvastatin, erythromycin, and quimidine (Obach et al., 2006), and is attributable to the binding of multiple substrates within the active site of the enzyme (Kenworthy et al., 1999).

The CYP3A4/5 inhibition by BOC was also time dependent, as indicated by the increased inhibitory potency upon 30-minute preincubation with NADPH before the addition of CYP3A probe substrates. The IC50 values were 2.3 ± 0.2 and 1.0 ± 0.1 μM for the inhibition of testosterone 6β-hydroxylation and midazolam 1’-hydroxylation, respectively (Table 1). Time dependency was not observed for any of the other P450s tested.

Kinetic analysis showed that BOC caused a time-dependent inhibition (TDI) of CYP3A activity (measured by testosterone 6β-hydroxylation) with kmax and Ks values of 0.12 minute⁻¹ and 6.1 μM, respectively. Several experiments were conducted to investigate possible mechanisms for this TDI. BOC did not form a spectrophotometrically detectable metabolite inhibitory complex with CYP3A4/5, as increases in absorbance between 380 and 520 nm were not observed in spectral readings of the interactions between BOC and an HLM sample from an individual with high levels of CYP3A4/5 activity (Fig. 2A). When HLM were incubated with [14C]BOC (20 μM), no radioactivity was detected in the microsomal pellets after multiple washings, suggesting that BOC did not show covalent binding to HLM. Further studies showed that the observed TDI measured by midazolam 1’-hydroxylation was NADPH-dependent and reversible after a 25-fold dilution (Fig. 2, B-D). Similar results were also observed when testosterone was used as the probe substrate (unpublished data).

Evaluation of BOC as an Inhibitor for Several Uptake and Efflux Transporters in Transfected Cell Lines or Membrane Vesicles. The inhibitory effect of BOC on uptake by human OATP1B1, OATP1B3, and OATP2B1 was evaluated in MDCKII-OATP1B1, MDCKII-OATP1B3, and MDCKII-OATP2B1 cells. BOC inhibited uptake for OATP1B1, OATP1B3, and OATP2B1 with an estimated IC50 of 18 ± 2.4 μM, 4.9 ± 1.3 μM, and >50 μM, respectively (Table 2). The inhibitory effects of BOC on several efflux transporters were also evaluated, including MDR1 Pgp, BCRP, and MRP2. BOC showed no inhibition (<10% decrease in net transport) of MDR1 Pgp-mediated [3H]digoxin (0.1 μM) transport over the concentration range tested (0.3–300 μM). In membrane vesicles containing human BCRP, BOC inhibited ATP-dependent uptake of [3H]methotrexate (10 μM) with an IC50 of 81 ± 28 μM (Table 2). Likewise, no inhibition of MRP2-mediated ATP-dependent uptake of [14C]EIA-SG (2 μM) was observed (IC50 > 100 μM; Table 2).

Evaluation of BOC as a Potential Perpetrator for OATP1B1-Mediated DDIs. As OATP1B1 is a major contributor to the hepatic uptake of several clinically used drugs such as statins (Shirata and Sugiyama, 2006; Muller and Fromm, 2011; Niemi et al., 2011), the potential for BOC as a perpetrator of inhibition of OATP1B1-mediated hepatic uptake was estimated using R-value analysis (Hirano et al., 2006; Giacomini et al., 2010). To validate this approach, we assessed OATP1B1 in vitro inhibition by several known OATP inhibitors with various clinical DDI effects as benchmarking compounds. These included cyclosporine A (CsA), rifampin, lorivapin, telaprevir, ampravir, and ritonavir. R values of all tested compounds at the clinically relevant dose were estimated and compared with clinical DDI data using atorvastatin, pitavastatin, or rosuvastatin as victim drugs (Table 3). At the clinically relevant dose (800 mg, three times daily), the IC50 of BOC for OATP1B1 inhibition (IC50 = 18 ± 2.4 μM) was 18-fold and 2-fold higher than its unbound maximum plasma concentration and unbound maximum concentration at the inlet to the liver, respectively (Table 3). The R value of BOC (R = 1.5 at 800 mg, three times daily) was lower than for CsA, rifampin, lopinavir, and telaprevir, which all have been reported to cause clinically significant DDIs with statins (Table 3). On the other hand, the R value of BOC was higher than for ampravir (600 mg) and ritonavir (100 mg), both of which have shown not to cause clinically significant DDIs with rosuvastatin (Table 3).

Effects of BOC on Uptake and Metabolism of CYP3A and OATP1B Substrates in Human Hepatocytes. To further understand the impact of BOC on hepatic uptake, metabolism, and their potential interplay, uptake and metabolism studies were conducted in human hepatocytes with functional activity of both hepatic uptake transporters and drug-metabolizing enzymes. The probe substrates used in these studies included pitavastatin (OATP1B1 and UGTs), midazolam (CYP3A), and atorvastatin (OATP1B1 and CYP3A4). For the uptake experiments, studies were performed under linear conditions (<5 minutes) where metabolism was minimal. As shown in Fig. 3B and Table 4, BOC inhibited hepatic uptake of [3H]pitavastatin (1 μM) with an IC50 of 6.3 ± 2.1 μM. In comparison, ketoconazole, not reported to be an OATP inhibitor at clinically relevant concentrations, inhibited pitavastatin uptake with an IC50 of 16.1 ± 1.1 μM. In contrast, rifampin, ritonavir, lopinavir, and telaprevir showed more potent inhibition of uptake of pitavastatin with IC50 values of 1.5 ± 0.3 μM, 1.4 ± 0.2 μM, 1.1 ± 0.1 μM, and 3.4 ± 0.9 μM, respectively (Table 4). Similarly, BOC inhibited uptake of [3H]atorvastatin (3 μM) in human hepatocytes with an IC50 of 7.2 ± 1.4 μM, while ketoconazole, rifampin, ritonavir, lopinavir, and telaprevir inhibited uptake of atorvastatin with IC50 values of 11.9 ± 3.1 μM, 3.0 ± 0.5 μM, 1.0 ± 0.1 μM, 1.8 ± 0.2 μM, and 4.1 ± 0.9 μM, respectively (Fig. 3A; Table 4). Interestingly, estrone sulfate and estriopropionate, potent prototypical inhibitors for OATP1B1 (Gu et al., 2010), inhibited uptake of pitavastatin (IC50 = 0.6 ± 0.1 μM, 0.8 ± 0.4 μM), but not atorvastatin (IC50 > 50 μM, >10 μM) (Fig. 3, A and B; Table 4). This apparent substrate dependency may be attributable to multiple binding sites in OATPs (Noe et al., 2007) or to the contribution of transporters other than OATPs to the hepatic uptake of these drugs.
Parallel experiments were also conducted to evaluate the inhibitory effect of BOC on the metabolism of midazolam, pitavastatin, and atorvastatin in the same human hepatocytes preparation as was employed for the hepatocyte uptake studies (Fig. 4). In these studies, ketoconazole (2 μM), rifampin (20 μM), and telaprevir (3 μM) were used as selective inhibitors for CYP3A, OATP1B, or both. Inhibitor concentrations used were within the range of unbound C<sub>max</sub> at the clinically relevant dose, and were corrected for the nonspecific binding of inhibitors in hepatocytes due to the relatively high hepatocyte density (1.5–2.0 million cells/ml) used. The unbound fraction of inhibitors in hepatocytes was estimated as described (Kilford et al., 2008). Consistent with BOC being a moderate reversible CYP3A inhibitor using midazolam as the substrate (IC<sub>50</sub> = 1 1.0 μM), BOC at 3 μM demonstrated minimal inhibition of the metabolism of midazolam (Fig. 4A). The results also suggested that the 20-minute incubation used in this study might not be sufficient for BOC to exhibit a TDI effect on CYP3A under these experimental conditions. Similarly, rifampin, a weak inhibitor of CYP3A (K<sub>i</sub> = 18.5 μM) (Maeda et al., 2011), also showed minimal inhibition of midazolam metabolism in human hepatocytes. As expected, ketoconazole at 2 μM (K<sub>i</sub> = 0.006 μM) (Fahmi et al., 2009) and telaprevir at 3 μM (IC<sub>50</sub> = 3.3 μM) (Garg et al., 2012) showed a relatively greater inhibition of CYP3A-mediated midazolam oxidation (Fig. 4A). As a reference, telaprevir was evaluated along with BOC in the same studies and was shown to be a more potent TDI of CYP3A (k<sub>onact</sub> = 0.097 minute<sup>-1</sup>, K<sub>i</sub> = 0.54 μM, respectively; unpublished data).

Formation of pitavastatin glucuronides (the major metabolites of pitavastatin formed by UGT1A3 and UGT2B7) was inhibited moderately by both BOC and telaprevir (Fig. 4B). Neither BOC nor telaprevir are potent inhibitors of UGT1A3 or UGT2B7 (Table 1; TABLE 2 In vitro evaluation of boceprevir (BOC) as an inhibitor of several uptake and efflux transporters

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Probe Substrate</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1B1</td>
<td>Pitavastatin (0.1 μM)</td>
<td>18.0 ± 2.4</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>Bromosulphophthalein (BSP) (0.1 μM)</td>
<td>4.9 ± 1.3</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>Estrone-3-sulfate (0.1 μM)</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Pgp</td>
<td>Digoxin (0.1 μM)</td>
<td>&gt;300</td>
</tr>
<tr>
<td>MRP2</td>
<td>Ethacrynic acid glutathione (EA-SG) (2 μM)</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

BCRP, breast cancer resistance protein; MRP2, multidrug resistance protein 2; OATP, organic anion-transporting polypeptide; Pgp, P-glycoprotein.
unpublished data). Rifampin, a known inhibitor for OATP1B, but not UGT1A3 and UGT2B7, displayed the most potent inhibition (80%), while ketoconazole did not show significant inhibition (Fig. 4B) of pitavastatin glucuronidation. This is consistent with ketoconazole not being a potent inhibitor of OATP1B, UGT1A3, or UGT2B7 at the concentration tested (2 μM) (Takeda et al., 2006). These results suggest that the observed inhibition of pitavastatin glucuronidation by BOC, telaprevir and rifampin (in increasing order) is likely a consequence of their inhibition of the OATP1B-mediated hepatic uptake rather than direct inhibition of UGT-mediated glucuronidation.

For atorvastatin, a dual substrate for both OATP1B and CYP3A, BOC showed moderate inhibition of the formation of both oxidative (para- and ortho-hydroxylations, both mediated by CYP3A) and total metabolites (para- and ortho-hydroxylations and lactone formation partly via glucuronidation and other metabolic pathways (Prueksaritanont et al., 2002) to a similar degree (Fig. 4C). Similarly, rifampin equally decreased the oxidative and total metabolism of atorvastatin, suggesting that under the current experimental conditions both BOC and rifampin inhibited the metabolism of atorvastatin mainly by blocking atorvastatin uptake into the hepatocytes. Being a more potient inhibitor of OATP1B, rifampin inhibited atorvastatin metabolism (both oxidative and total metabolism) to a greater degree than BOC. In contrast, ketoconazole and telaprevir exhibited more potent inhibition toward the atorvastatin oxidative metabolism by CYP3A, as compared with their effect on the total metabolism of atorvastatin. Additionally, the inhibition of the oxidative metabolism of atorvastatin was strongest with ketoconazole followed by telaprevir and then BOC, in agreement with the rank order of their inhibitory potency of CYP3A activity as measured by midazolam hydroxylation (Fig. 4A). Interestingly, rifampin, which showed minimal inhibition of midazolam metabolism by CYP3A, demonstrated a 20–30% more potent inhibition of total metabolism of atorvastatin as compared with ketoconazole and telaprevir. This further suggested that inhibition by rifampin of the metabolism of atorvastatin was primarily driven by its inhibition of OATP1B. Across the concentration range tested, BOC showed less potent and incomplete inhibition of both oxidative and total metabolism of atorvastatin as compared with telaprevir (Fig. 4D), consistent with it being a less potent inhibitor of both CYP3A and OATP1B.

**Transport of BOC by Efflux Transporters Pgp, BCRP, and MRP2 in Transfected Cell Lines or Membrane Vesicles.** In MDCKII control cells, BOC showed low passive permeability (P_{app} =
In both MDCKII-MDR1 (Fig. 5A) and MDCKII-BCRP (Fig. 5B) monolayers, BOC exhibited a greater B-A/A-B ratio (transport from basal to apical [B-A] divided by transport from apical to basal [A-B]), compared with control cells. This transport was strongly inhibited by the Pgp inhibitor CsA (10 μM) and weakly inhibited by the BCRP inhibitor Ko143 (5 μM), respectively.

Verapamil and prazosin, prototypical substrates for Pgp and BCRP, respectively, showed significant higher B-A/A-B ratios in MDCKII-MDR1 and MDCKII-BCRP cells than in control cells, and this transport was inhibited strongly by CsA and Ko143 (Fig. 5, D and E).

Taken together, these data indicated that despite the endogenous transport observed in MDCKII cells, BOC was a substrate of MDR1 Pgp and BCRP. Similarly, SCH-534128 and SCH-534129, the two diastereomers of BOC, were also substrates for MDR1 Pgp and BCRP (unpublished data).

Hepatic Uptake of BOC by Human Hepatocytes and Uptake Transporters NTCP, OATP1B1, OATP1B3, and OATP2B1, and OCT1.

To assess if BOC was a substrate for hepatic uptake transporters, uptake of BOC was determined in cryopreserved human hepatocytes. Uptake of [3H]E217β (1 μM), [3H]CCK-8 (10 nM), [3H]estrone sulfate (100 nM), [3H]TCA (1 μM), and [14C]TEA (1 μM), prototypical substrates for human OATP1B1, OATP1B3, OATP2B1, NTCP, and OCT1, respectively, showed significant temperature-dependent uptake. This uptake was inhibited by prototypical inhibitors of these transporters (unpublished data). This indicated that the human hepatocytes used in this study had retained the functional activities of these transporters. As shown in Fig. 6A, the uptake of [14C]BOC (1 μM) into human hepatocytes was time and temperature dependent. Initial uptake of BOC at 37°C was saturable (Km = 12.4 ± 7.4 μM, Vmax = 343 ± 150 pmol/
min/10⁶ cells, and Pdif = 7.6 ± 1.5 ml/min/10⁶ cells) (Fig. 6B), suggesting that uptake of BOC into human hepatocytes was a transporter-mediated process. Uptake of SCH-534128, the active form of BOC, also showed time- and temperature-dependent and saturable uptake (unpublished data). To assess whether hepatic uptake of BOC was mediated by NTCP, uptake of [14C]BOC into human hepatocytes was also conducted at 37°C in sodium-free buffer. As shown in Fig. 6C, uptake of BOC was not sodium dependent, indicating that BOC was not a substrate for NTCP.

To further identify potential transporters involved in the hepatic uptake of BOC, we next examined the uptake of BOC using MDCKII-OATP1B1, MDCKII-OATP1B3, MDCKII-OATP2B1, and CHO-K1-OCT1 cells. Uptake of [14C]BOC into none of the transfected cell lines was significantly greater than in control MDCKII or CHO-K1 cells, respectively (Fig. 7, A–D), suggesting that BOC was not a substrate of these transporters. Compared with control cells, uptake of positive control substrates [3H]E217βG (1 μM), [3H]CCK-8 (2.5 nM), [3H]estrone-3-sulfate (0.1 μM), and [14C]TEA (1 μM) was significantly greater in OATP1B1, OATP1B3, OATP2B1, or OCT1 transfected cells, indicating the presence of functional transporters in these cell lines (Fig. 7, E–H, respectively).

Fig. 5. Transport of BOC by MDR1 Pgp, BCRP, and MRP2 in MDCKII-MDR1 and MDCKII-BCRP monolayers and MRP2 containing membrane vesicles. Transcellular transport of [14C]BOC (1 μM) was evaluated in (A) MDCKII-MDR1 and (B) MDCKII-BCRP monolayers. Papp B/A-A-B ratio in MDR1 or BCRP transfected cells (■) and control MDCKII cells (○) in the absence and presence of cyclosporine A (CsA) (10 μM) or Ko143 (5 μM) was measured at 3 hours. (D–E) Transcellular transport of the prototypical substrates [3H]verapamil (VER) (1 μM) for MDR1 Pgp and [3H]prazosin (PRA) (5 μM) for BCRP measured in transfected cells (■) and control MDCKII cells (○) in the absence and presence of CsA (10 μM) or Ko143 (1 μM), respectively. Time-dependent uptake of [14C]BOC (2 μM) was evaluated in MRP2 (squares) and control (circles) membrane vesicles (C) in the presence (●) or absence (○) of 5 mM ATP and an ATP-regenerating system. (F) Uptake rate of [14C]EA-SG (2 μM), prototypical substrate of MRP2, measured in MRP2 containing vesicles in the presence (■) and absence (○) of 5 mM ATP and ATP-regenerating system at 5 minutes. Values shown are mean ± S.E. of experiments performed in triplicate.

Fig. 6. Uptake of BOC in human hepatocytes. (A) Time- and temperature-dependent uptake of [14C]BOC (1 μM) into human hepatocytes at 37°C (■) and 4°C (○), respectively. (B) Kinetic analysis for initial uptake rate of BOC conducted in human hepatocytes at 37°C with obtained Km = 12.4 ± 7.4 μM, Vmax = 343 ± 150 pmol/min/10⁶ cells, and Pdif = 7.6 ± 1.5 ml/min/10⁶ cells. (C) Sodium-dependent uptake of [14C]BOC (1 μM) into human hepatocytes. Time-dependent uptake of [14C]BOC (1 μM) was conducted at 37°C in sodium-free (▲) and sodium-containing uptake buffer (●), and at 4°C (○) in sodium-containing buffer. Values shown are mean ± S.E. of experiments performed in triplicate.
Consistent with above findings, uptake of [14C]BOC (0.5 μM) was not inhibited by BSP, a prototypical inhibitor for anionic transporters. Uptake of BOC was also not inhibited by rifampin, ritonavir, lopinavir, and CsA, but weakly inhibited by rifamycin SV, all known inhibitors for OATPs (Fig. 8) (Hirano et al., 2006). These findings confirm that hepatic OATPs do not contribute to uptake of BOC. Quinidine (1–100 μM), an inhibitor of organic cation transporters, stimulated rather than inhibited the uptake of BOC. The mechanism for this unexpected observation requires further investigation. As a negative control, ketoconazole did not inhibit uptake of BOC (Fig. 8).

Discussion

Our studies indicate that except for CYP3A4/5, BOC did not significantly inhibit any of the P450 and UGT enzymes tested. Considering the reversibility and NADPH-dependent nature of the inhibition on CYP3A4/5, the TDI observed in HLM could be caused by a more potent inhibitory effect of oxidative metabolite(s) of BOC generated in situ. However, the identity of such inhibitory metabolite (s) is currently unknown because very low levels of these oxidative metabolites were observed in human plasma after a therapeutic dose of BOC (unpublished data). Instead, a reductive metabolite formed by AKR, SCH-629144, is the major circulating metabolite in human plasma. Although it is unlikely that SCH-629144 was responsible for the increased TDI activity observed in vitro, nevertheless, its contribution to overall inhibitory effects on CYP3A in vivo could not be ruled out. In vitro, SCH-629144 showed reversible inhibition to CYP3A4/5 with similar IC50 values (9.8 μM and 54 μM for midazolam 1′-hydroxylation and testosterone 6α-hydroxylation, respectively) (Supplemental Table 1) to BOC, and exhibited a time-dependent inhibition to CYP3A4/5 with K1/k inact ratio ~4-fold higher than that for BOC (Supplemental Fig. 1). The finding that BOC and/or the reductive metabolite was a TDI of CYP3A4/5, which are present in both the liver and gut, has been confirmed in clinical DDI studies demonstrating that BOC (800 mg, three times daily) increased the plasma area under the curve (AUC) and Cmax of orally administered midazolam (4 mg) by 5.3- and 2.8-fold, respectively (Kiser et al., 2012). Interestingly, this magnitude of inhibition matched reasonably well with predicted results (~7-fold increase in AUC) using a dynamic mechanistic model-based approach and based simply on the TDI K1/k inact parameters and the clinically observed concentrations of BOC, without taking into consideration the potential inhibitory effect of SCH-629144 (Prueksaritanont et al., unpublished data). Admittedly, the fact that the model provided reasonable prediction does not eliminate the possibility of the involvement of a metabolite in the observed clinical DDI between midazolam and BOC. Also, this TDI effect of BOC could conceivably be attributable in part to the increased AUC of CsA (2.7-fold) and tacrolimus (17-fold) in humans when coadministered with BOC, as both are substrates of CYP3A (Hulskotte et al., 2012b).

With respect to drug transporters, BOC was not an inhibitor of Pgp (IC50 > 300 μM) in LLC-MDR1 cells. In contrast, studies in Caco-2 cells indicated that BOC was an inhibitor of digoxin (IC50 = 25 μM; unpublished data). The reason for the discrepancy between these two assay systems is unclear. Caco-2 cells express multiple transporters (Xia et al., 2007), so BOC may affect another uptake or efflux transporter involved in the transport of digoxin in Caco-2 cells. Therefore, it is reasonable to consider that the inhibition data obtained with LLC-MDR1 cells should more accurately predict the effect of BOC on Pgp-mediated efflux. As such, BOC is unlikely to have a notable inhibitory effect on the Pgp transport at the systemic level (Cmax ~3 μM, 800 mg, three times daily) (Foote et al., 2011). However, local concentrations of BOC in the gut could potentially reach a level of 1 mM after the recommended therapeutic dose, a concentration not assessed in vitro. In a clinical DDI study, coadministration of BOC (800 mg, three times daily) with digoxin (0.25 mg, single dose) increased digoxin exposure (AUC and Cmax) 20-fold (Jumes et al., 2012). Interestingly, this magnitude of inhibition matched reasonably well with predicted results (~7-fold increase in AUC) using a dynamic mechanistic model-based approach and based simply on the TDI K1/k inact parameters and the clinically observed concentrations of BOC, without taking into consideration the potential inhibitory effect of SCH-629144 (Prueksaritanont et al., unpublished data). Admittedly, the fact that the model provided reasonable prediction does not eliminate the possibility of the involvement of a metabolite in the observed clinical DDI between midazolam and BOC. Also, this TDI effect of BOC could conceivably be attributable in part to the increased AUC of CsA (2.7-fold) and tacrolimus (17-fold) in humans when coadministered with BOC, as both are substrates of CYP3A (Hulskotte et al., 2012b).

Fig. 7. Uptake of BOC into human OATP1B1, OATP1B3, OATP2B1, and OCT1 transfected MDCKII cells. Time-dependent uptake of [14C]BOC (1 μM) was evaluated in (A) MDCKII-OATP1B1, (B) MDCKII-OATP1B3, (C) MDCKII-OATP2B1, and (D) CHO-K1-OCT1 cells. (E-H) Uptake rate of the prototypical substrates [3H]E217βG (1 μM), [3H]CCK-8 (2.5 nM), [3H]estrone-3-sulfate (0.1 μM), [3H]TEA (1 μM), and (I) OCT1 measured at 5 minutes, respectively. (■, uptake in transfected cells; □, uptake by control MDCKII or CHO-K1 cells.) Values shown are mean ± S.E. of experiments performed in triplicate.
Based on the static $R$ value model and by comparing to known inhibitors of OATP1B1 that cause clinically significant DDIs with statins (Table 3), BOC may be classified as a moderate inhibitor of OATP1B1, and may have the potential to cause DDIs with statins via its inhibitory effect on OATP1B1. BOC also inhibited OATP1B3 (IC$_{50}$ = 4.9 μM) with an estimated $R$ value of 2.8, assuming that OATP1B3-mediated hepatic uptake is accounting for 100% of the hepatic uptake of drugs. Although studied for completeness, the clinical relevance of this inhibitory effect of BOC on OATP1B3 is currently unclear because OATP1B3 is not a major contributor to the hepatic uptake of statins based on data from in vitro relative expression/activity factors (Hirano et al., 2006), and from clinical studies with individuals with genetic polymorphisms in OATP1B (Niemi et al., 2011). Furthermore, recent clinical studies (Ieiri et al., 2011; Yamada et al., 2011) have demonstrated that genetic polymorphisms of UGT1A3 but not OATP1B3 ($SLCO1B3$) impact the pharmacokinetics of telmisartan, a selective substrate of OATP1B (Ishiguro et al., 2006).

In the case of drugs that are dual substrates of both P450 enzymes and OATP1B, such as atorvastatin and repaglinide, the fact that BOC is an inhibitor of both OATP1B and CYP3A4/5 could further complicate the scenarios of DDIs. In this study, we used human hepatocytes, which demonstrate the functional activity of both hepatic uptake transporters and enzymes, to help provide insight into the impact of BOC on the hepatic elimination of dual OATP1B/CYP3A substrates. Together with several probe substrates and appropriate benchmarking inhibitors, our results suggest that the reduced metabolism of atorvastatin observed in the presence of rifampin or BOC in hepatocytes was largely a consequence of the inhibition of the hepatic uptake, rather than a direct inhibitory effect on CYP3A. These in vitro studies are consistent with a recent clinical cassette microdose study demonstrating that hepatic uptake by OATPs is the rate-determining step in the overall hepatic elimination of atorvastatin in humans (Maeda et al., 2011). Importantly, we demonstrated that BOC at clinically relevant concentrations was a much weaker inhibitor of atorvastatin metabolism in hepatocytes as compared with rifampin, a clinically known OATP1B inhibitor. This finding suggests that BOC should have lesser impact (versus rifampin) on the hepatic elimination of atorvastatin and other dual substrates of OATP1B/CYP3A where uptake is the rate-determining step. Indeed, a recent clinical DDI study showed that BOC increased plasma atorvastatin AUC and $C_{\text{max}}$ 2.3- and 2.7-fold, respectively (Hulskotte et al., 2011). It is noteworthy that atorvastatin has low intestinal availability [Fa*fg (intestinal availability) = 0.24] (Shitara, 2011) in humans, conceivably due to gut CYP3A4 metabolism and Pgp efflux (Hochman et al., 2004). Therefore, the potential of BOC to inhibit atorvastatin gut metabolism might be an additional contributing factor to the increased systemic exposure of atorvastatin. Also consistent with its moderate inhibitory effect on OATP1B1, BOC has been shown to increase the AUC and $C_{\text{max}}$ of pravastatin (40 mg) 1.6- and 1.5-fold, respectively (Hulskotte et al., 2011). In humans, pravastatin is eliminated via hepatobiliary and renal excretion mediated by hepatic OATP/MRP2 and renal OAT3, respectively, with minimal metabolism (Shitara and Sugiyama, 2006). As a reference, rifampin caused higher increase in exposure of atorvastatin (>8-fold) and pravastatin (~2.5-fold) in humans after a single dose of rifampin (Deng et al., 2009; He et al., 2009).
Furthermore, the greater inhibitory potency of telaprevir relative to BOC toward both OATP1B and CYP3A, as demonstrated in the present study, also agrees with clinical DDI results that telaprevir (750 mg, three times daily) increased plasma AUC and \( C_{\text{max}} \) by 7.88- and 10.6-fold for atorvastatin (Lee et al., 2011), and by 8.96- and 2.86-fold for midazolam (Garg et al., 2012), respectively.

To aid in understanding the possible contributions of transporters to the in vivo disposition of BOC and their implications to DDIs, we assessed the susceptibility of BOC as a substrate of various drug transporters. BOC as well as its two diastereomers SCH-534128 and SCH-534129 were substrates for MDR1 Pgp, and BCRP, but not for MRP2. However, given the high therapeutic dose of BOC, Pgp/BCRP activity is likely saturated and therefore will not significantly impact the intestinal absorption of BOC. This notion is supported by a recent clinical DDI study (Hulskotte et al., 2012b) that CsA (100 mg), a potent inhibitor of Pgp and BCRP, did not have a meaningful effect on the pharmacokinetics of BOC.

As BOC has low passive permeability and is eliminated primarily via hepatic metabolism by CYP3A4/5 and aldo-keto reductases (Ghosal et al., 2011), uptake transporters may play an important role in the hepatic elimination of BOC. Our studies indicated that uptake of BOC in human hepatocytes was saturable. The active uptake estimated by hepatic elimination of BOC. Our studies indicated that uptake of BOC in vivo disposition of BOC and their implications to DDIs, we assessed (Garg et al., 2012), respectively.

In summary, our in vitro studies together with clinical DDI observations suggest that BOC is a relatively potent reversible time-dependent inhibitor of CYP3A4 and a moderate inhibitor of dual substrates of CYP3A and OATP1B for which hepatic uptake is the rate-determining step. BOC has low potential to cause pharmacokinetic interactions by inhibition of other major P450s and transporters, or to be a victim of inhibitors of these known transporters.

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