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-keto reductases, was a reversible time-dependent inhibitor ($K_i = 0.12$ minute $^{-1}$, $K_d = 6.1 \mu M$) 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 $\mu M$, respectively. In human hepatocytes, BOC inhibited CYP3A-mediated metabolism of midazolam, OATP1B-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), OATP1B (rifampin), or both (telaprevir). BOC was a substrate for Pgp and BCRP but not for OATP1B1, OATP1B3, OATP2B1, 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/OATP1B1 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 $\alpha$-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 of the molecule. There is a rapid interconversion between the two diastereomers, with about 2-fold higher systemic exposure in favor of the active isomer in humans. Biotransformation is a major elimination pathway for BOC across preclinical species and humans. BOC undergoes extensive metabolism involving both cytochrome P450 (P450) CYP3A4/5-mediated oxidation and ketoreduction by cytosolic aldo-keto reductases (AKR)1C2 and AKR1C3 (Ghosal et al., 2011).

Drug-drug interactions (DDIs) caused by changes in pharmacokinetics and/or pharmacodynamics may lead to drug-induced toxicity or an altered therapeutic effect of a drug. Pharmacokinetic DDIs attributable to alterations of drug absorption, distribution, metabolism, and excretion due to inhibition or induction of drug metabolizing enzymes and/or transporters (Muller and Fromm, 2011) have been commonly reported in patients receiving polypharmacy. Managing DDIs is particularly challenging in the treatment of HCV when considering the concomitant medications commonly prescribed for this patient population who have varying stages of disease, including cirrhosis, organ transplantation, and coinfection with human immunodeficiency virus.
Transporters are discussed. The implications for the potential for DDIs with these enzymes and compared with clinical DDI observations that have been reported, and BCRP, MRP2, and hepatic uptake transporters OATP1B, OATP1B3, between CYP3A4 or UGTs and OATP1B in human hepatocytes. We inhibition of major human P450 and UGT enzymes; 2) inhibition of metabolizing enzymes and transporters. The evaluations include 1) BOC to cause pharmacokinetic interactions at the levels of drug-efflux transporters such as Pgp, BCRP, and MRP2 are also localized in the apical membrane of enterocytes. These transporters, together with 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, 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

[^14]CBOC (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]pyridin(3,4-b)indol-3-yl)-propionic acid tert-butyl ester) were synthesized by the Chemistry Department, Merck Research Laboratories. [^3H]Estradiol-17b-D-glucoronide (E17bG) and [^3H]cholecytokinin octapeptide (CCK-8), [^3H]prazosin, and [^3H]verapamil were purchased from PerkinElmer Life Sciences (Boston, MA). [^3H]Pitavastatin, unlabeled pitavastatin, [^3H]taurocholic acid (TCA), and [^14]C]TEA were purchased from American Radiolabeled Chemicals (St. Louis, MO). [^3H]Methotrexate was purchased from Moravek (Brea, CA). [^14]C]Ethacrynic acid glutathione conjugate (EA-SG) was synthesized by the Radiochemistry Department of Merck Research Laboratories. Prazosin, cyclosporine A (CsA), testosterone, 6-9hydroxytestosterone, cortisol, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADP, UDP-glucuronic acid (UDPGA), alamethicin, estradiol, 3'-azido-3'-deoxythymidine, nicardipine, dicyclofemacin, thymidine, and labetalol were purchased from Sigma-Aldrich (St. Louis, MO). Bromosulphthalein (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 (ABCB2) 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 MRD1 Pgp (MDCKII-MRD1 or LLC-MRD1) 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 (Madan et al., 2002; Walsky and Obach, 2004; Parkinson et al., 2011). Briefly, incubations were conducted at 37°C in 0.4 ml of
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 \(K_m\) values, as indicated in Table 1. Both midazolam and testosterone were used as probe substrates for CYP3A4/5. The \(K_i\) value for reversible inhibition of CYP3A4/5 was determined using midazolam as the probe substrate (1.5–50.0 \(\mu\)M) with several BOC concentrations (2.5–100.0 \(\mu\)M). 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-hydroxybupropion, d₅-N-desethylamodiaquine, d₄-4'-hydroxydiclofenac, d₃-4'-hydroxyxymefentanyl, 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 NADPH. The probe substrates and test concentrations were the same as reversible inhibition conducted without preincubation. To determine kinetic parameters (\(K_m\) and \(V_{max}\)) 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 NADPH-generating system for 5 to 30 minutes. The incubation mixtures were diluted 10-fold with the same buffer containing 250 \(\mu\)M testosterone (CYP3A probe substrate) and an NADPH-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 37°C). Troleandomycin, at a final concentration of 25 \(\mu\)M, was added to the sample and reference cuvette. The reactions were initiated with 10 \(\mu\)l of methanol) was added to the reference cuvette. The reactions were initiated with 10 \(\mu\)l of BOC-NADPH added to both cuvettes to give a final volume of 1 ml. Continuous scans were conducted every minute for 15 minutes after the addition of BOC-NADPH. All scans were recorded on a Varian Cary 100 BIO UV/Vis dual beam spectrophotometer (Varian Analytical Instruments, Palo Alto, CA). BOC was then added to the sample cuvette in 10 \(\mu\)l of methanol for a final incubation concentration of 3 \(\mu\)M. A corresponding volume of the solvent (10 \(\mu\)l of methanol) was added to the reference cuvette. The reactions were initiated with 10 \(\mu\)l of BOC-NADPH added to both cuvettes to give a final volume of 1 ml. Continuous scans were conducted every minute for 15 minutes after the addition of BOC-NADPH. All scans were recorded on a Varian Cary 100 BIO UV/Vis dual beam spectrophotometer (Varian Analytical Instruments, Palo Alto, CA).

### TABLE 1

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

| Enzyme                  | CYP/UGT Reaction          | Substrate Concentration | Reversible Inhibition, No Preincubation | Time-Dependent Inhibition, 30-Minute Preincubation
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<tr>
<td></td>
<td></td>
<td>(\mu)M</td>
<td>(\mu)M IC₅₀ ((K_i)) inhibition (%) at 100 (\mu)M</td>
<td>(\mu)M IC₅₀ IC₅₀ ((K_i)) inhibition (%) at 100 (\mu)M</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Phenacetin O-deethylation</td>
<td>60</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<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>
</tr>
<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>11 ± 1 (7.7 ± 0.8)</td>
<td>91</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)</td>
<td>750</td>
<td>&gt;100</td>
<td>&gt;100</td>
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NA, not applicable (no value was obtained as the rates at the highest concentration of BOC evaluated [100 \(\mu\)M] 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 g) at 4°C for 10 minutes, the supernatants were analyzed by HPLC/flow scintillation analysis. 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 pellets was dissolved in 1 ml of BTS-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 [¹⁷βH]E₂₁₇G (1 μM), [¹⁴C]CCSK-8 (2.5 mM), [¹⁷βH]estrone-3-sulfate (0.1 μM), or [¹⁷βH]TEA (1 μM) for OATP1B1, OATP1B3, 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 3000g at 4°C (Model 5180R; Eppendorf, Hamburg, Germany), and washing of the cell pellets with PBS. Cell pellets were resuspended in 50% acetonitrile, scintillation fluid (Scintisafe Econo 2; Fisher Scientific, Pittsburgh, PA) was added, and radioactivity was determined by liquid scintillation counting in a LS6500 Multifunctional Scintillation Counter (Beckman Coulter). Inhibitory effect of BOC on OCT1, OATP1B3, and OATP2B1-mediated uptake was also evaluated in MDCKII cells transfected with OATP1B1, OATP1B3, and OATP2B1. [¹⁴C]Pitavastatin (0.1 μM), [¹⁷βH]BSP (0.1 μM), and [¹⁷βH]estrone-3-sulfate (0.1 μM) were used as probe substrates for OATP1B1, OATP1B3, and OCT1, respectively. Uptake of all probe substrates tested in transfected cells was at least 5-fold higher than in control cells (unpublished data).

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 culture plates (BD Biosciences). Twenty-four hours before the experiment, the MDR1 Pgp cDNA, as described previously elsewhere (Reitman et al., 2011). compartment of the culture plate, and buffer (150 μl) was added to the compartment opposite that containing BOC. The other procedures were the same as in the Pgp bidirectional transport assay. [¹⁷βH]Prazosin (5 μM) and Ko143 (1 μM) were used as the positive control substrate and inhibitor, respectively.

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-strip tubes (Corning Inc., Corning, NY) at 20 μg/tube. Then 20 μl of [¹³C]BOC (final concentration 2 μM) or [¹³C]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 μM/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, 10 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 [¹³C]EA-SG (2 μM) or [¹⁷H]mexothretate (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 [¹³C]BOC into human hepatocytes was determined at 37°C or 4°C, respectively. Cells were resuspended in Krebs-Henseleit modified buffer (KHB) (Sigma-Aldrich) (pH 7.4) in 96-well deep well plates (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 [¹³C]BOC (final concentration 1 μM) or the positive control substrate [¹⁷βH]E₂₁₇G (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 [¹³C]BOC (1 μM) was determined at 37°C and 4°C in KHB and sodium-free KHB at 37°C. [¹⁷H]TCA (0.5 μM) was used as a positive control. To measure the kinetic parameters of BOC, the initial uptake rate of [¹³C]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 [¹³C]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 UGTs), 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 [¹³C]pitavastatin (1μM) and [¹⁷βH]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-glucuronide, 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 mi) containing 1-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 OATP1B1 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, OATP1B1, 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).

**Quantification 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 at 40% buffer B, 2.0 to 2.5 minutes at 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).

**Quantification 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 at 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).

**Quantification 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 inhibition constant ($K_i$) for the competitive inhibition of CYP3A4/5 was calculated by eq. 1:

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

where $K_i$ 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 $K_a$ and $V_{max}$ are the Michaelis-Menten constants for a given P450 reaction. The data were fitted in GraFit 4.0 software for $K_i$ 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):

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

where $K_i$ represents the inhibitor concentration that produces a half-maximal rate of inactivation, $k_{max}$ represents the maximum inactivation rate constant, and $[I]$ is the inhibitor concentration.

The $IC_{50}$ values for inhibition of metabolism or transporter-mediated uptake/efflux in microsomes and transporter transfected cells were obtained by fitting the data to eq. 3 by nonlinear regression analysis:

$$
Control(\%) = \frac{100}{1 + I/IC_{50}}
$$

where control (%) represents the metabolism or transporter-mediated uptake/efflux measured in the presence of various concentrations of inhibitor to that in the absence of inhibitor.

$IC_{50}$ values for inhibition of uptake in human hepatocytes were obtained by fitting the data to eq. 4:

$$
Control(\%) = \frac{100 - M}{1 + I/IC_{50}} + M
$$

where $M$ represents residual control (%) not affected by the inhibitors tested.

**Prediction of Potential for OATP1B1-Mediated DDIs.** The degree of inhibition of OATP1B1 in humans was estimated by calculating the $R$ value (eq. 5) (Hirano et al., 2006; Giacomini et al., 2010), which represents the ratio of the uptake clearance in the absence of inhibitor to that in its presence:

$$
R = 1 + \frac{f_u \times I_{max}}{K_{i50}}
$$

where $f_u$ represents the blood unbound fraction of the inhibitor, $I_{max}$ represents the estimated maximum inhibitor concentration at the inlet to the liver, and $IC_{50}$ was obtained from the in vitro OATP1B1 inhibition study in transfected cell lines. $I_{max}$ was calculated based on eq. 6 (Hirano et al., 2006; Giacomini et al., 2010):

$$
I_{max} = I_{max} + \frac{f_s \times \text{Dose} \times k_u}{Q_h}
$$

where $I_{max}$ is the maximum plasma concentration of the inhibitor, $f_s$ is the fraction of the dose of the inhibitor that is absorbed, $k_u$ is the absorption rate constant of the inhibitor, and $Q_h$ is the hepatic blood flow rate in humans (1500 ml/min). To estimate the $I_{max}$ value, $F_s$ was set at 1, $k_u$ was set at 0.03 minute⁻¹, and the blood-to-plasma concentration ratio was assumed to be 1.

**Kinetic Analysis.** Kinetic parameters for uptake of BOC into human hepatocytes were estimated using nonlinear least-squares data fitting from eq. 7:

$$
v = \frac{V_{max} \times S}{K_m + S} + P_{diff} \times S
$$

where $v$ is the initial uptake velocity of BOC (nmol/min/10⁶ cells), $S$ is BOC concentration in the reaction mixture (μM), $K_m$ is the Michaelis-Menten constant (μM), $V_{max}$ is the maximum uptake rate (nmol/min/10⁶ cells), and $P_{diff}$ is the nonsaturable uptake clearance (μl/min/10⁶ cells).
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, CYP3A4, 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 IC_{50} = 100 μM is the worst-case scenario, is not likely to be clinically significant, as the IC_{50} value was ~33-fold higher than the total plasma C_{max} (~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 (IC_{50} > 100 μM).

However, BOC exerted an appreciable inhibition of CYP3A4/5 (measured by midazolam 1'-hydroxylation) with an IC_{50} value of 11 ± 1.0 μM. The inhibition of midazolam hydroxylation was further confirmed to be competitive with a K_{i} value of 7.7 ± 0.8 μM.

Interestingly, BOC did not inhibit testosterone 6β-hydroxylation, another functional marker activity for CYP3A4/5 (IC_{50} > 100 μM). Such substrate-dependent inhibition with CYP3A4/5 has been reported previously for atorvastatin, erythromycin, and quinidine (Obach 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, lopinavir, telaprevir, ampranavir, 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 IC_{50} of BOC for OATP1B1 inhibition (IC_{50} = 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 ampranavir (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 OATP1B1 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 CYP3A). 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 IC_{50} 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 IC_{50} of 16.1 ± 1.1 μM. In contrast, rifampin, ritonavir, lopinavir, and telaprevir showed more potent inhibition of uptake of pitavastatin with IC_{50} 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 IC_{50} of 7.2 ± 1.4 μM, while ketoconazole, rifampin, ritonavir, lopinavir, and telaprevir inhibited uptake of atorvastatin with IC_{50} 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 estriopregnate, potent prototypical inhibitors for OATP1B1 (Gui et al., 2010), inhibited uptake of pitavastatin (IC_{50} 0.6 ± 0.1 μM, 0.8 ± 0.4 μM), but not atorvastatin (IC_{50} > 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 \( C_{\text{max}} \) 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\(_{50} = 1.1\) 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 (\( K_i = 18.5 \) μM) (Maeda et al., 2011), also showed minimal inhibition of midazolam metabolism in human hepatocytes. As expected, ketoconazole at 2 μM (\( K_i = 0.006 \) μM) (Fahmi et al., 2009) and telaprevir at 3 μM (IC\(_{50} = 3.3 \) μM) (Garg et al., 2012) showed a relatively greater inhibition of CYP3A-mediated midazolam oxidation (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 (\( K_i = 18.5 \) μM) (Maeda et al., 2011), also showed minimal inhibition of midazolam metabolism in human hepatocytes. As expected, ketoconazole at 2 μM (\( K_i = 0.006 \) μM) (Fahmi et al., 2009) and telaprevir at 3 μM (IC\(_{50} = 3.3 \) 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 (\( k_{\text{proct}} = 0.097 \text{ minute}^{-1}, K_i = 0.54 \) 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;
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 potent 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.

### Table 3

<table>
<thead>
<tr>
<th>Perpetrator (dose)</th>
<th>IC₅₀ (μM)</th>
<th>Cmax, u</th>
<th>tₐₘᵢₜ, u</th>
<th>R value</th>
<th>Victim (Clinical DDIs)</th>
<th>Clinical DDIs (Fold Increase of AUC)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOC (800 mg, three times daily)</td>
<td>18 ± 2.4</td>
<td>0.79</td>
<td>8.92</td>
<td>1.5</td>
<td>Atorvastatin</td>
<td>2.3</td>
<td>Hulskotte EG et al., 2011</td>
</tr>
<tr>
<td>CsA (100 mg)</td>
<td>0.3 ± 0.1</td>
<td>0.06</td>
<td>0.23</td>
<td>1.8</td>
<td>Pitavastatin</td>
<td>4.5</td>
<td>Shiina and Sugiyama, 2006</td>
</tr>
<tr>
<td>Rifampin (600 mg)</td>
<td>1.4 ± 0.2</td>
<td>0.87</td>
<td>2.47</td>
<td>2.8</td>
<td>Atorvastatin</td>
<td>8</td>
<td>He et al., 2009</td>
</tr>
<tr>
<td>Lopinavir (400 mg)</td>
<td>0.4 ± 0.1</td>
<td>0.3</td>
<td>0.49</td>
<td>2.4</td>
<td>Rosuvastatin</td>
<td>2</td>
<td>Kiser et al., 2008</td>
</tr>
<tr>
<td>Telaprevir (750 mg)</td>
<td>3.4 ± 0.1</td>
<td>1.01</td>
<td>9.84</td>
<td>3.9</td>
<td>Atorvastatin</td>
<td>7.9</td>
<td>Lee et al., 2011</td>
</tr>
<tr>
<td>Amprenavir (600 mg)</td>
<td>10.0 ± 1.6</td>
<td>1.2</td>
<td>3.57</td>
<td>1.4</td>
<td>Rosuvastatin</td>
<td>1.1</td>
<td>Karlgren et al., 2012</td>
</tr>
<tr>
<td>Ritonavir (100 mg, twice daily)</td>
<td>0.8 ± 0.2</td>
<td>0.03</td>
<td>0.09</td>
<td>1.1</td>
<td>Rosuvastatin</td>
<td>No</td>
<td>Busti et al., 2008</td>
</tr>
</tbody>
</table>

AUC, area under the curve; CsA, cyclosporine A; DDIs, drug-drug interactions; OATP, organic anion-transporting polypeptide.

* The prodrug fosamprenavir was used in the study.

* Coadministered with fosamprenavir.

**Fig. 3.** Effect of several compounds on the uptake of atorvastatin and pitavastatin in human hepatocytes. Effects of several compounds on initial uptake rate of [³H]atorvastatin (3 μM) (A) and [³H]pitavastatin (1 μM) (B) was evaluated in cryopreserved human hepatocytes. The data are expressed as a percentage of initial uptake rate measured at 1 and 5 minutes at 37°C in the presence and absence of inhibitors. The IC₅₀ values for inhibition of atorvastatin and pitavastatin uptake by rifampin (●), telaprevir (○), BOC (■), ketoconazole (□), ritonavir (▲), lopinavir (△), estrone sulfate (♦), and estropipate (♦) are summarized in Table 4. Values shown are mean ± S.E. for experiments performed in triplicate.
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).

Similar to control vesicles, uptake of $^{[14]}$CBOC (2 μM) into MRP2 containing vesicles was not time or ATP dependent (Fig. 5C), suggesting that BOC was not a substrate of MRP2. $^{[14]}$CJA-EA-SG (2 μM), a positive control substrate for human MRP2 (Chu et al., 2004), showed a significant ATP-dependent uptake in MRP2 containing vesicles (Fig. 5F), indicating the functional activity of MRP2 in these vesicles. SCH-534128 and SCH-534129 were also not substrates for MRP2 (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 $^{[3]}$H₂₁βG (1 μM), $^{[3]}$HCC₇-K (10 nM), $^{[3]}$Hexone sulfate (100 nM), $^{[3]}$HTCA (1 μM), and $^{[14]}$CJEA (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 $^{[14]}$CBOC (1 μM) into human hepatocytes was time and temperature dependent. Initial uptake of BOC at 37°C was saturable ($K_m = 12.4 \pm 7.4 \mu M$, $V_{max} = 343 \pm 150$ pmol/
min/10^6 cells, and P_{diff} = 7.6 ± 1.5 μl/min/10^6 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 1 M [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 (1 μM) 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. P_{app} 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 ○) and absence (□ or ○) 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 K_m = 12.4 ± 7.4 μM, V_max = 343 ± 150 pmol/min/10^6 cells, and P_{diff} = 7.6 ± 1.5 μl/min/10^6 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 $^{14}$C]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 rifampicin 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 TD1 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 6β-hydroxylation, respectively) (Supplemental Table 1) to BOC, and exhibited a time-dependent inhibition to CYP3A4/5 with Kᵢvalues of ~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 Cₘ₉ max 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 Kᵢ/Kᵢmax parameters and the clinically observed concentrations of BOC, without taking into consideration the potential inhibitory effect of SCH-629144 (Pruksaritanont 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. 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 Cₘ₉ max ~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 Cₘ₉ max ~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. 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. 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. 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.
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 ($IC_{50} = 4.9 \mu M$) 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 OATP1B1, 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 casetate 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 $C_{max}$ 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 $C_{max}$ 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 BOCToward both OATP1B1 and CYP3A3, as demonstrated in the previous study, also agrees with clinical DDI results that telaprevir (750 mg, three times daily) increased plasma AUC and \( C_{\text{max}} \) by 7.88-10.6-fold for atorvastatin (Lee et al., 2011), and by 8.96-2.86-fold for midazolam (Garg et al., 2012), respectively.

To aid in understanding the possible contributions of transporters to the in vivo disposition of BOCT and their implications to DDI, we assessed the susceptibility of BOCT as a substrate of various drug transporters. BOCT as well as its two diastereomers SCH-534128 and SCH-534129 were substrates for MDR1 Pgp, and BCRP, but not for MRPs. However, given the high therapeutic dose of BOCT, Pgp/BCRP activity is likely saturated and therefore will not significantly impact the intestinal absorption of BOCT. 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 BOCT. 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 BOCT. 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