In vitro assessment of drug-drug interaction potential of boceprevir associated with drug metabolizing enzymes and transporters

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Boceprevir, BOC; HCV, hepatitis C virus; NS3, nonstructural protein 3; DDI, drug-drug interactions; ADME, absorption, distribution, metabolism and excretion; CYP, cytochrome P450s; UGT, UDP-glucuronosyltransferases, AKR, aldo-keto reductase; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; NTCP, sodium/taurocholate co-transporting peptide, Pgp, P-glycoprotein; MRP, multidrug resistance protein; BCRP, breast cancer resistance protein; HBSS, Hanks' balanced salt solution; HLM, human liver microsomes; LC-MS/MS, liquid chromatography-tandem mass spectrometry.
Abstract:

In this paper, the inhibitory effect of boceprevir (BOC), an inhibitor of hepatitis C virus (HCV) nonstructural protein 3 (NS3) protease, was evaluated in vitro against a panel of drug metabolizing enzymes and transporters. BOC, a known substrate for cytochrome P450 (CYP) 3A and aldo-ketoreductases, was a reversible time-dependent inhibitor (kinact = 0.12 min⁻¹, Kᵢ = 6.1 µM) of CYP3A4/5, but not an inhibitor of other major CYPs, nor of UDP-glucuronosyltransferases (UGT) 1A1 and 2B7. BOC showed weak to no inhibition of breast cancer resistance protein (BCRP), P-glycoprotein (Pgp), and multidrug resistance protein 2 (MRP2). It was a moderate inhibitor of organic anion transporting polypeptide (OATP) 1B1 and -1B3, with an IC₅₀ of 18 and 4.9 µM, 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, -1B3, -2B1, organic cation transporter (OCT1), or sodium/taurocholate co-transporting peptide. Overall, our data suggest that BOC has 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 CYPs and transporters tested are less likely 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 HCV genotype 1 infections. BOC is specifically designed to inhibit hepatitis C virus (HCV) nonstructural protein 3 (NS3) 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 NS3 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 (Figure 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 CYP3A4/5-mediated oxidation, and ketoreduction by cytosolic aldo-keto reductases AKR1C2 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 altered therapeutic effect of a drug. Pharmacokinetic DDIs attributable to alterations of drug absorption, distribution, metabolism and excretion (ADME) 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 considering concomitant medications commonly prescribed for this patient population with varying stages of disease, including cirrhosis, organ...
transplantation and co-infection with human 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 CYP 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 co-transporting peptide NTCP (SLC10A1), as well as the efflux transporters MDR1 P-glycoprotein (Pgp, ABCB1), multidrug resistance protein MRP2 (ABCC2), and breast cancer resistance protein BCRP (ABCG2) (Griffin et al., 2011).

Recently, it has been increasingly recognized that drug transporters have significant impact on DDIs by modulating the ADME 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 HMG-CoA 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.

In this paper, 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 CYP and UGT enzymes; 2) inhibition of major human drug transporters (OATP1B1, -1B3, -2B1, Pgp, BCRP, and MRP2); and 3) impact on the enzyme-transporter interplay between CYP3A4 or UGTs and OATP1B in human hepatocytes. The effort was also extended to evaluate if BOC is transported by efflux transporters Pgp, BCRP, MRP2, and hepatic uptake transporters OATP1B, -1B3, -2B1, NTCP, and OCT1. Where applicable, the results are compared to clinical DDI observations 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% measured by high-performance liquid chromatography (HPLC)) was synthesized by the Radiochemistry Department, Merck Research Laboratories, Kenilworth, NJ. Unlabeled BOC, SCH-534128, SCH-534129, SCH-629144, and Ko143 were synthesized by the Chemistry Department, Merck Research Laboratories, Kenilworth, NJ. \(^{[3H]}\) Estradiol 17 β-D-Glucuronide (E217βG) and \(^{[3H]}\) cholecystokinin Octapeptide (CCK-8), \(^{[3H]}\) prazosin, \(^{[3H]}\) verapamil were purchased from PerkinElmer Life Sciences, Inc (Boston, MA). \(^{[3H]}\) Pitavastatin, unlabeled pitavastatin, \(^{[3H]}\) taurocholic acid (TCA), and \(^{[14C]}\) tetraethylammonium chloride (TEA) were purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). \(^{[3H]}\) Methotrexate was purchased from Moravek (Brea, CA). \(^{[14C]}\) Ethacrynic acid glutathione conjugate (EA-SG) was synthesized by the Radiochemistry Department, Merck Research Laboratories, Kenilworth, NJ. Prazosin, cyclosporine A (CsA), testosterone, 6β-hydroxytestosterone, cortisol, glucose 6-phosphate, NADP, glucose 6-phosphate dehydrogenase, UDP-glucuronic acid (UDPGA), alamethicin, estradiol, 3’-azido-3’-deoxythimidine, nicardipine, diclofenac, thymidine and labetolol were purchased from Sigma (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 5 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

MDCKII, MDCKII cells stably transfected with human BCRP (MDCKII-hBCRP), CHO-K1, and CHO-K1 cells stably transfected with human OCT1 (CHO-K1-hOCT1) were obtained from Solvo Biotechnology (Budapest, Hungary) under license agreement and evaluation agreement, respectively. MDCKII, LLC-PK1 cells and MDCKII or LLC-PK1 cells expressing cDNAs 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 (Monteagudo et al., 2010).

Inhibition studies with human CYP 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 CYP enzymes was conducted as described previously (Madan et al., 2002; Walsky and Obach, 2004; Parkinson et al., 2011). Briefly, incubations were conducted at 37°C in 0.4 mL incubation mixtures (pH 7.4) containing potassium phosphate buffer (50 mM), MgCl2 (3 mM), EDTA (1 mM), an NADPH-generating system, and 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. Kᵢ value for reversible inhibition of CYP3A4/5 was determined using midazolam as probe substrate (1.5-50 µM) with several BOC concentrations (2.5-100 µM). Reactions were initiated with the addition of an aliquot of an NADPH-generating system and were carried out 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′-hydroxymephenytoin, d3-dextrorphan, d2-6-hydroxychlorzoxazone, d3-6β-hydroxytestosterone and d3-1′-hydroxymidazolam.

The preincubation-dependent inhibition of BOC was also determined for CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5 by comparison of IC₅₀ values with and without 30 minute pre-incubation in the presence of NADPH. The probe substrates and test concentrations were the same as reversible inhibition conducted without pre-incubation. To determine kinetic parameters (kᵢᵣₑᵣᵤₑ and Kᵢ) 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 min. The incubation mixtures were diluted 10-fold with the same buffer containing 250 µM testosterone (CYP3A probe substrate) and an NADPH-generating system. The incubation was continued for an additional 10 min to monitor the extent of testosterone 6β-hydroxylation.

To determine if BOC formed a spectrophotometrically detectable metabolite inhibitory complex (MIC) with cytochrome P450 (i.e., peaks at approximately 452 nm), an individual human liver microsomal sample (H0079, lot # 0010127, XenoTech, LLC) containing high levels of CYP3A4/5 activity (final protein concentration of 1 mg/mL, 1.7 nmol P450/mg protein) was added to the sample and reference cuvettes in a buffer mixture consisting of potassium phosphate (50 mM), and MgCl₂ (3 mM) for a final volume of 980 µL. Baseline scans from 380 to 520 nm were recorded on a Varian Cary 100 BIO UV/Vis dual beam spectrophotometer. BOC was then added to the sample
cuvette in 10 µL of methanol for a final incubation concentration of 3 µM. A corresponding volume of the solvent (10 µL of methanol) was added to the reference cuvette. The reactions were initiated with 10 µL of β-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 β-NADPH. All scans were conducted at approximately 37°C. Troleandomycin, at a final concentration of 25 µM, was used as a positive control using the same procedure, except that the reference cuvette received a 10-µL aliquot of acetonitrile.

The time-dependent inhibition of CYP3A4/5 (as measured by testosterone 6β-hydroxylation and midazolam 1′-hydroxylation) was further evaluated by determining if the increased inhibition observed after 30 minute pre-incubation with NADPH was reversible after dilution. In this evaluation, BOC (final concentration 3 µM) was pre-incubated first with HLM (0.05 mg/mL for midazolam and 0.1 mg/mL for testosterone) for 0, 15 and 30 minutes, in the presence and absence of an NADPH-generating system, without a dilution step. Substrate (100 µM for testosterone and 5 µM for midazolam) was then added and the incubation was carried out for 5 minutes. Second, BOC (0 and 3 µM) were pre-incubated with human liver microsomes (1.25 mg/mL for midazolam and 2.5 mg/mL for testosterone, which is approximately 25 times the typical incubation concentration) in the presence of an NADPH-generating system, for 0, 15 and 30 minutes. The samples were then diluted 25-fold, prior to being incubated with marker substrate (200 µM for testosterone and 50 µM for midazolam). The incubation (at 1/25 the pre-incubation 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’-deoxythimidine (AZT) glucuronidation was evaluated using HLM. Pooled HLM (0.5 mg/mL) were incubated at 37°C for 20 min. 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 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 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 thymidine (UGT1A1) or labetolol (UGT2B7) as the internal standards. The samples were spun in a centrifuge at 14,000 x g for 30 min at 4°C. The supernatants were subjected to LC/MS-MS analysis.

**Covalent binding of BOC in HLM**

Pooled human liver microsomes (1 nmol P450/mL) were incubated with [¹⁴C] BOC (20 μM) for 120 min in 0.5 mL of 100 mM potassium phosphate buffer (pH 7.4) containing 3 mM MgCl₂ and an NADPH-generating system (0.5 mM NADP, 5 mM glucose-6-phosphate and 1.5 units/mL glucose-6-phosphate dehydrogenase). After the pre-incubation at 37°C for 2 min, reactions were initiated by addition of BOC. Following the incubation for 120 min, the reactions were terminated by the addition of 0.5 mL of ice-cold acetonitrile with 1% acetic acid. After vortexing and centrifuging (~10,000g) at 4°C for 10 min, the supernatants were analyzed by HPLC/FSA. Microsomal pellets were washed with 0.5 mL of acetonitrile containing 1% acetic acid for 5 times. After the centrifugation following each washing, the supernatants were combined and counted for radioactivity. The final pellet
was dissolved in 1 ml BTS-450 (tissue solubilizer (Beckman Coulter, Brea CA), neutralized with 6N HCl, and counted for radioactivity.

Uptake and inhibition studies in OATP1B1, -1B3, -2B1, and OCT1 transfected cells

OATP1B1-, OATP1B3- and OATP2B1-mediated uptake was determined in MDCKII cells stably transfected with OATP1B1, OATP1B3 or OATP2B1 cDNAs as described previously (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, St. Louis, MO) for 24 hr to increase OATPs expression prior to the experiment. Cells were dislodged with trypsin EDTA and resuspended in Hank's buffered salt solution (HBSS) plus 10 mM HEPES. Cells were then suspended in 96 deep well plates at a density of 0.6 x 10⁶ cells/well. Uptake was initiated by the addition of [¹⁴C] BOC (1 µM) or the positive control substrates [³H] E₂¹7βG (1µM), [³H] CCK8 (2.5nM), and [³H] estrone-3- sulfate (0.1µM), [¹⁴C] TEA (1 µM) for OATP1B1, OATP1B3, 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 min at 3000 rpm at 4°C (Eppendorf, Model 5180R; Hamburg, Germany), and washing of the cell pellets with PBS. Cell pellets were resuspended in 50% acetonitrile, scintillation fluid (Scintisafe Econo 2; Fisher Chemicals, Pittsburgh, PA) was added, and radioactivity was determined by liquid scintillation counting in a LS6500 Multipurpose Scintillation Counter (Beckman Coulter, Brea, CA). Inhibitory effect of BOC on OATP1B1, OATP1B3, and OATP2B1-mediated uptake was also evaluated in OATP1B1, OATP1B3, and OATP2B1 transfected MDCKII cells. [³H] Pitavastatin (0.1 µM), [³H] bromosulfophthalein (BSP) (0.1 µM), and [³H] estrone-3-sulfate (0.1 µM) were used as probe substrates for
OATP1B1, -1B3, and -2B1, respectively. Uptake of all probe substrates tested in transfected cells was at least 5-fold higher than in control cells (data not shown).

**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). $[^{14}C]$ BOC (1 µM) were prepared in HBSS 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 to that containing the compound. At 3 hr, 50 µL of sample was taken out 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 (Perkin Elmer, Boston, MA). $[^{3}H]$ Verapamil (1 µM) and cyclosporine A (CsA, 10 µM) was used as the positive control substrate and inhibitor for Pgp, respectively. Inhibitory effect of BOC on MDR1 Pgp-mediated $[^{3}H]$ digoxin (0.1µM) transport was evaluated in LLC-PK1 cells stably transfected with a human MDR1 Pgp cDNA as described previously (Reitman et al., 2011).

MDCKII and MDCKII-BCRP cells were cultured in 24-well transwell culture plates (BD Biosciences, San Jose, CA). Twenty four hours prior to the experiment, cells were treated with 10 mM sodium butyrate to increase BCRP expression. $[^{14}C]$ BOC (1 µM) were prepared in HBSS with 10 mM HEPES. Substrate solution (500 µL) was added to either the apical (A) or the basolateral (B) compartment of the culture plate, and buffer (500 µL) was added to the compartment opposite to that containing BOC. Other procedures are the same as Pgp bidirectional transport assay. $[^{3}H]$ 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 [14C] BOC (final concentration 2 µM) or [14C] 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 min at 37°C. Uptake was initiated by the addition of 20 µL ATP regenerating reagent (5 mM ATP, 10 mM creatine phosphate and 100 µg/mL 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 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 pre-wetted 96-well glass fiber type B filter plate (1.0 µm) (Millipore, Billerica, MA). Filters containing the membrane vesicles were washed with 200 µL ice-cold stop buffer, and dried at room temperature followed by the addition of 25 µL scintillation fluid. Radioactivity was determined in a MicroBeta Wallac Trilux Scintillation Counter. Inhibitory effect of BOC on MRP2- or BCRP-mediated uptake was also evaluated using [14C] ethacrynic acid glutathione (EA-SG) (2 µM) or [3H] methotrexate (10 µM) as 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 (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 (KHB) (Sigma-Aldrich, St. Louis, MO) (pH 7.4) in 96-deep well plates (BD Falcon, San Jose, CA) at a density of 0.2 x 10⁶ cells/well. The cells and dosing solution were preincubated at 37°C or 4°C for 5 min,
respectively. Uptake studies were initiated by the addition of 50 μL of [14C] BOC (final concentration 1 μM) or the positive control substrate [3H] E217βG (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 described above. To study sodium-dependent uptake, uptake of [14C] BOC (1 μM) was determined at 37°C and 4°C in KHB and sodium-free KHB at 37°C. [3H] TCA (0.5 μM) was used as a positive control. To measure the kinetic parameters of BOC, initial uptake rate of [14C] BOC at various concentrations was determined at 0.5 and 3 min 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 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 [3H] pitavastatin (1μM) and [3H] atorvastatin (3μM) was studied with human hepatocytes at 37°C after incubation for 1 and 5 min. The substrate concentrations tested in these studies were well below K_m values for pitavastatin (K_m= 5 μM; unpublished data), and atorvastatin (K_m=18.9 μM) (Lau et al., 2007), measured in OATP1B1 transfected cells. Other experimental procedures were as described above for hepatocyte uptake studies.

To assess the effect of BOC on the metabolism of midazolam, pitavastatin, and atorvastatin acid, 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 DMSO 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 below. Human hepatocytes (1.5 or 2 million cells/mL) were suspended in William's E buffer (0.3 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 min at 37 ºC under a 95% air: 5% CO₂ atmosphere, in the absence (solvent only) or presence of an inhibitor. The 20-min reaction time was chosen to ensure that inhibitory effects on both OATP1B and CYP3A-mediated metabolite formation can 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 µM) and telaprevir (0.25-10 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 CYP enzyme activity**

All CYP probe substrates including 1'-OH midazolam were analyzed by LC-MS/MS with deuterated metabolites as internal standard as described previously (Parkinson et al., 2011).

**Quantitation of UGT1A1 activity**

A Sciex API 4000 triple quadrupole mass spectrometer was used to acquire data. Chromatographic separation was achieved using a Symmetry C₁₈ reversed-phase column (4.6 x 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 min at 20% buffer B, 0.5 to 2.0 min to 40% buffer B, 2.0 to 2.5 min to 70% buffer B, 2.5 to 3.5 min at 70% buffer B and 3.5 to 3.8 min return to 20% buffer B and re-equilibrate until 4.3 min with 20% buffer B. The instrument was operated in the negative ionization mode using the Electrospray™ interface and selected reaction monitoring (SRM) was used to determine 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 x 75 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 (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 min at 5% buffer B, 1.5 to 3.0 min at 5 to 60% buffer B, 3.0 to 3.5 min at 60% buffer B, 3.6 to 4.0 min to 95% buffer B and 4.0 to 4.5 min return to 5% buffer B. The instrument was operated in the negative ionization mode using the Electrospray™ interface and selected reaction monitoring (SRM) was used to determine 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 (QTOF) Xevo mass spectrometer. HPLC analyses consisted of a Waters UPLC HSS T3 column (1.8 um, 2.1x50 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 min, 90%A; 7.5 min, 5%A; the column was washed at 5%A (1 min) and equilibrated at 90%A (1 min) before the next injection. The QTOF mass spectrometer was operated under ESI 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 $K_i$ value of BOC for the competitive inhibition of CYP3A4/5 was calculated by equation 1.

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

(1)

Where $K_i =$ Inhibition constant; $V =$ rate of reaction in presence of inhibitor at a concentration = [I], and substrate at a concentration = [S]; $K_m$ and $V_{max}$ are the Michaelis Menton constants for a given P450 reaction. The data were fitted in GraFit 4.0 software for $K_i$ determination. 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 % remaining activity versus pre-incubation time. The $k_{inact}$ and $K_i$ values were calculated by nonlinear regression analysis of equation 2 using KaleidaGraph Synergy Software (Reading, PA).

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

(2)
Where $K_i$ represents the inhibitor concentration that produce half-maximal rate of inactivation, $k_{\text{inact}}$ 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 equation 3 by nonlinear regression analysis.

\[
\text{Control(\%)} = \frac{100}{1 + I/\text{IC}_{50}}
\]  

(3)

Where control (\%) represents 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 equation 4.

\[
\text{Control(\%)} = \frac{100 - M}{1 + I/\text{IC}_{50}} + M
\]

(4)

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 (equation 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:

\[
R = 1 + \frac{f_u \times I_{\text{in,\text{max}}}}{\text{IC}_{50}}
\]

(5)

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

$$I_{\text{m,\text{max}}} = I_{\text{max}} + \frac{F_a \times \text{Dose} \times k_a}{Q_h}$$ (6)

Where $I_{\text{max}}$ is the maximum plasma concentration of the inhibitor, $f_a$ is the fraction of the dose of the inhibitor that is absorbed, $k_a$ 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 $I_{\text{in},\text{max}}$ value, $F_a$ was set at 1, $k_a$ was set at 0.03 min$^{-1}$, and the blood-to-plasma concentration ratio was assumed to be 1.

\textbf{Kinetic analysis}

Kinetic parameters for uptake of BOC into human hepatocytes were estimated using nonlinear least-squares data fitting from the equation 7

$$v = \frac{V_{\text{max}} \times S}{K_m + S} + P_{\text{dif}} \times S$$ (7)

Where $v$ is the initial uptake velocity of BOC (pmol/min/10$^6$ cells); $S$ is BOC concentration in the reaction mixture (µM), $K_m$ is the Michaelis Menton constant (µM), $V_{\text{max}}$ is the maximum uptake rate (pmol/min/10$^6$ cells), and $P_{\text{dif}}$ is the non-saturable uptake clearance (µl/min/10$^6$ cells)
Results

Evaluation of BOC as an inhibitor of major CYP 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 CYP2C19, whereas it demonstrated 45% inhibition of CYP2D6. However, its inhibitory effect to CYP2D6, if assumed that IC$_{50}$=100 µM as 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 significant inhibition of estradiol 3-glucuronidation by UGT1A1 and 3'-azido-3'-deoxythimidine (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), 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 prior to the addition of CYP3A probe substrates. The IC$_{50}$ 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 CYPs tested. Kinetic analysis showed that BOC caused a time-dependent inhibition (TDI) of CYP3A activity (measured by testosterone 6β-hydroxylation) with $k_{\text{inact}}$ and $K_i$ values of 0.12 min$^{-1}$ 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 (MIC) 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 (Figure 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 following a 25-fold dilution (Figure 2B-2D). Similar results were also observed when testosterone was used as the probe substrate (data not shown).

**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, -1B3, and -2B1 was evaluated in MDCKII-OATP1B1, MDCKII-OATP1B3, and MDCKII-OATP2B1 cells. BOC inhibited uptake for OATP1B1, -1B3, and -2B1 with an estimated IC$_{50}$ 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 IC$_{50}$ of 81 ± 28 µM (Table 2). Likewise, no inhibition
of MRP2-mediated ATP-dependent uptake of [14C] EA-SG (2 µM) was observed (IC_{50} >100 µM; Table 2).

**Evaluation of BOC as a potential perpetrator for OATP1B1-mediated DDIs**

As OATP1B1 is a major contributor to hepatic uptake of several clinically used drugs, such as statins (Shitara 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 with several known OATP inhibitors with various clinical DDI effects as benchmarking compounds. These included cyclosporine A (CsA), rifampin, lopinavir, telaprevir, amprenavir, and ritonavir. R-values of all tested compounds at the clinically relevant dose were estimated and compared to clinical DDI data using atorvastatin, pitavastatin, or rosuvastatin as victim drugs (Table 3). At the clinically relevant dose (800mg, TID), the IC_{50} of BOC for OATP1B1 inhibition (IC_{50} = 18 ± 2.4 µM) was 18- 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 800mg TID) 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 amprenavir (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 (OATP1B and UGTs), midazolam (CYP3A), and atorvastatin (OATP1B and CYP3A). For the uptake experiments, studies were performed under linear conditions (< 5 minutes) where metabolism was minimal. As shown in Figure 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 (Figure 3A, Table 4). Interestingly, estrone sulfate and estropipate, potent prototypical inhibitors for OATP1B1 (Gui 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) (Figure 3A, 3B, 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 employed for the hepatocyte uptake studies (Figures 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 Cmax at the clinically relevant dose, and corrected for the nonspecific binding of inhibitors in hepatocytes due to the relatively high hepatocyte
density (1.5–2 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\textsubscript{50} = 11 ± 1.0 μM), BOC at 3 μM demonstrated minimal inhibition of the metabolism of midazolam (Figure 4A). The results also suggested that the 20-min 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\textsubscript{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\textsubscript{i} = 0.006 μM) (Fahmi et al., 2009), and telaprevir at 3 μM (IC\textsubscript{50} = 3.3 μM) (Garg et al., 2012), showed a relatively greater inhibition of CYP3A-mediated midazolam oxidation (Figure 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\textsubscript{inact} = 0.097 min\textsuperscript{-1}, K\textsubscript{i} = 0.54 μM, respectively; unpublished data).

Formation of pitavastatin glucuronides (the major metabolites of pitavastatin formed by UGT1A3 and -2B7) was inhibited moderately by both BOC and telaprevir (Figure 4B). Neither BOC nor telaprevir are potent inhibitors of UGT1A3, and -2B7 (Table 1 and data not shown). Rifampin, a known inhibitor for OATP1B, but not UGT1A3 and -2B7, displayed the most potent inhibition (>80%), while ketoconazole did not show significant inhibition (Figure 4B) of pitavastatin glucuronidation. This is consistent with ketoconazole not being a potent inhibitor of OATP1B, UGT1A3 and -2B7 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 (Figure 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 towards the atorvastatin oxidative metabolism by CYP3A, as compared to 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 (Figure 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 to 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 to telaprevir (Figure 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 ($P_{\text{app}} = 4.5 \times 10^{-6}$ cm/s). In both MDCKII-MDR1 (Figure 5A) and MDCKII-BCRP (Figure 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 to 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 (Figures 5D and 5E). 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 (data not shown).

Similar to control vesicles, uptake of $[^{14}\text{C}]$ BOC (2 µM) into MRP2 containing vesicles was not time- and ATP-dependent (Figure 5C), suggesting that BOC was not a substrate of MRP2. $[^{14}\text{C}]$ 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 (Figure 5F), indicating the functional activity of MRP2 in these vesicles. SCH-534128 and SCH-534129 were also not substrates for MRP2 (data not shown).

**Hepatic uptake of BOC by human hepatocytes and uptake transporters NTCP, OATP1B1, -1B3, and -2B1, and OCT1**

To assess if BOC was a substrate for hepatic uptake transporters, uptake of BOC was determined in cryopreserved human hepatocytes. Uptake of $[^3\text{H}]$ E$_2$17βG (1 µM), $[^3\text{H}]$ CCK-8 (10 nM), $[^3\text{H}]$ estrone sulfate (ES) (100 nM), $[^3\text{H}]$ TCA (1 µM), and $[^{14}\text{C}]$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 (data not shown).
This indicated that the human hepatocytes used in this study had retained the functional activities of these transporters. As shown in Figure 6A, the uptake of \( ^{[14]C} \) BOC (1 \( \mu M \)) into human hepatocytes was time- and temperature-dependent. Initial uptake of BOC at \( 37^\circ C \) was saturable (\( K_m = 12.4 \pm 7.4 \mu M \), \( V_{max} = 343 \pm 150 \text{ pmol/min/10}^6 \text{ cells} \), and \( P_{dif} = 7.6 \pm 1.5 \mu l/min/10^6 \text{ cells} \); Figure 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-, temperature-dependent, and saturable uptake (data not shown). To assess if hepatic uptake of BOC was mediated by NTCP, uptake of 1 \( \mu M \) \( ^{[14]C} \) BOC into human hepatocytes was also conducted at \( 37^\circ C \) in sodium-free buffer. As shown in Figure 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 \( ^{[14]C} \) BOC (1\( \mu M \)) into none of the transfected cell lines was significantly greater than in control MDCKII or CHO-K1 cells, respectively (Figures 7A, 7B, 7C, and 7D), suggesting that BOC was not a substrate of these transporters. Compared to control cells, uptake of positive control substrates \( [^3H] \) E\(_217\) \( \beta \)G (1 \( \mu M \)), \( [^3H] \) CCK8 (2.5 nM), \( [^3H] \) ES (0.1 \( \mu M \)), and \( [^{14}C] \) TEA (1 \( \mu M \)) was significantly greater in OATP1B1, -1B3, -2B1, or OCT1 transfected cells, indicating the presence of functional transporters in these cell lines (Figures 7E, 7F, 7G, 7H, respectively).

Consistent with above findings, uptake of \( ^{[14]C} \) BOC (0.5 \( \mu M \)) 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 (Figure 8) (Hirano et al., 2006). These findings confirm that hepatic OATPs do not contribute to uptake of BOC. Quinidine (1-100 \( \mu M \)), 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 (Figure 8).
Discussion

Our studies indicate that except for CYP3A4/5, BOC did not significantly inhibit any of the CYP 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, since very low levels of these oxidative metabolites were observed in human plasma following a therapeutic dose of BOC (data on file). 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 6β-hydroxylation, respectively; supplementary Table 1) to BOC, and exhibited a time-dependent inhibition to CYP3A4/5 with Kᵢ/kᵣᵢᵦ ratio ~4-fold higher than that for BOC (supplementary Figure 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 (800mg TID) increased plasma AUC and Cₘₐₓ of orally administered midazolam (4mg) 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/kᵣᵢᵦ parameters and the clinically observed concentrations of BOC, without taking into consideration for potential inhibitory effect of SCH-629144 (Prueksaritanont et al.; submitted). Admittedly, the fact that the model provided reasonable prediction does not eliminate the possibility for the involvement of 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 cyclosporine (2.7-fold) and tacrolimus (17-fold) in humans when coadministered with BOC, since both are substrates of CYP3A (Hulskotte et al., 2012b).

With respect to drug transporters, BOC was not an inhibitor of P-gp (IC$_{50}>$300 µM) in LLC-MDR1 cells. In contrast, studies in Caco-2 cells indicated that BOC was an inhibitor of digoxin (IC$_{50} = 25$ µM; data not shown). The reason for the discrepancy between these two assay systems is unclear. Since Caco-2 cells express multiple transporters (Xia et al., 2007), 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 inhibition data obtained in LLC-MDR1 cells should more accurately predict the effect of BOC on P-gp-mediated efflux. As such, BOC is unlikely to have a notable inhibitory effect on the P-gp transport at the systemic level ($C_{\text{max}} \sim 3$ µM, 800 mg TID) (Foote et al., 2011). However, local concentrations of BOC in the gut could potentially reach a level of 1 mM following the recommended therapeutic dose, a concentration not assessed in vitro. In a clinical DDI study, co-administration of BOC (800mg TID) with digoxin (0.25mg, SD) increased digoxin exposure (AUC and $C_{\text{max}}$) slightly (~20%), but not half-life, presumably due to inhibition of intestinal, not systemic P-gp (Jumes et al., 2012). Likewise, BOC was also a weak or non-inhibitor of BCRP (IC$_{50} = 81$ µM) and MRP2 (IC$_{50} >$100 µM), respectively, and therefore significant interactions mediated via these transporters are not likely.

Based on the static R-value model, and by comparing to known inhibitors of OATP1B1 causing clinically significant DDIs with statins (Table 3), BOC may be classified as a moderate inhibitor of OATP1B1, and may have 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 hepatic uptake of statins based on data from *in vitro* relative expression/activity factors (Hirano et al., 2006), and 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) demonstrated that genetic polymorphisms of UGT1A3, but not OATP1B3 (*SLCO1B3*), impacted the pharmacokinetics of telmisartan, a selective substrate of OATP1B (Ishiguro et al., 2006).

In the case of drugs which are dual substrates of both CYP 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 demonstrated 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 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 to rifampin, a clinically known OATP1B inhibitor. This finding suggests that BOC should have lesser impact (*vs.* 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<sub>max</sub> 2.3- and 2.7-fold, respectively (Hulskotte et al., 2011). It is noteworthy that atorvastatin has low intestinal availability (Fa*fg=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<sub>max</sub> of pravastatin (40mg) 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 towards both OATP1B and CYP3A, as demonstrated in the present study, also agrees with clinical DDI results that telaprevir (750 mg TID) increased plasma AUC and C<sub>max</sub> 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 <i>in vivo</i> 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 (100mg), 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 aldoketoreductases (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 $V_{\text{max}}/K_m$ accounted for ~79% of the total uptake, suggesting that transporter(s) is/are involved in the hepatic uptake of BOC. Interestingly, BOC was not a substrate for the major hepatic uptake transporters tested, and the (novel) transporter(s) contributing to hepatic uptake of BOC remain(s) to be identified. Although possible interactions from the unidentified transporter(s) can not be ruled out, BOC is likely to have low potential as a victim for drug interactions with major hepatic uptake transporters tested. This speculation is supported by the finding that BOC exposure is unaffected in healthy volunteers when co-dosed with cyclosporine or tacrolimus (Hulskotte et al., 2012b), both broad spectrum inhibitors of drug transporters, including OATPs (Oswald et al., 2011).

Recently, drug interactions between BOC and several ritonavir-boosted HIV protease inhibitors (atazanavir, lopinavir, darunavir) have been reported (Hulskotte et al., 2012a). Unexpectedly, the exposure of all agents (BOC, HIV agents and ritonavir) was decreased. These results cannot be explained simply by inhibition of drug metabolizing enzymes or transporters. While ritonavir, atazanavir, lopinavir, and darunavir are inhibitor and/or inducers of multiple enzymes and transporters (Griffin et al., 2011; Jimenez-Nacher et al., 2011), BOC is not a potent inducer of CYP3A, 1A2 and 2B6 (data not shown), and therefore is not expected to be an inducer of Pgp. The underlying mechanism for this complex DDI remains to be investigated, but may involve several interplays at the level of metabolic enzymes and transporters, including involvement of extrahepatic, minor or yet to be identified pathways.

In summary, our in vitro studies together with clinical DDI observations suggest that BOC is a relatively potent reversible time-dependent inhibitor of CYP3A 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 CYPs and transporters, or to be a victim of inhibitors of these known transporters.
Acknowledgement

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Authorship Contributions

Participated in research design and result interpretations: Chu, Cui, Tang, Ghosal, Palamanda, Evers, Prueksaritanont

Conducted experiments: Cai, Chan, Green, Kuo, Liang, Maciolek

Contributed new reagents or analytic tools: NA

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protease inhibitors and the relationship of this property to in vivo bilirubin glucuronidation. *Drug Metab Dispos* **33**:1729-1739.

Figure Legends

Fig. 1. Chemical structure of Boceprevir (BOC), SCH-534128 and SCH-534129

Chemical structures of boceprevir (BOC) (A) and and its diastereomers SCH-534128 (active) (B), and SCH-534129 (inactive) (C)

Fig. 2. Mechanistic evaluation of BOC as a reversible time-dependent inhibitor to CYP3A4/5

A. Spectral determination of the potential for BOC to form a MIC in human liver microsomes.

Spectrophenotmetrical scan (380-520nm) was conducted every minute for 15 minutes to determine the potential for BOC (3 µM) to form a MIC with cytochrome P450 (i.e., peaks at approximately 452 nm) using human liver microsomes (H0079, lot # 0010127). The experimental details are described in Materials and Methods.

B-D Inhibition of CYP3A4/5 (midazolam 1'-hydroxylation) by BOC: Determination of NADPH-dependence and effects of dilution

The time-dependent inhibition of BOC (3 µM) to CYP3A4/5 was measured by midazolam 1'-hydroxylation in HLM following preincubation at 0 (black bar), 15 (white bar), and 30 min. (lined bar) under the following incubation conditions: in the presence of an NADPH-generating system, without a dilution step (B), in the absence of an NADPH-generating system, without a dilution step (C), and in the presence of an NADPH-generating system, with 25-fold dilution of incubation mixture (D). The experimental details are described in Materials and Methods. The studies were conducted in duplicate measurements.

Fig. 3. Effect of several compounds on 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 were 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 (closed circles), telaprevir (open circles), BOC (closed squares), ketoconazole (open squares), ritonavir (closed triangle), lopinavir (open triangle), estrone sulfate (closed diamond), and estropipate (open diamond) are summarized in Table 5. Values shown are mean ± SE for experiments performed in triplicate.

**Fig. 4. Inhibition of the metabolism of midazolam, pitavastatin, and atorvastatin in human hepatocytes**

Effects of BOC (3 µM), ketoconazole (2 µM), rifampin (20 µM) and telaprevir (3 µM) on metabolism of midazolam (1 µM) (A), pitavastatin (1µM) (B) and atorvastatin (3 µM) (C) was evaluated in cryopreserved human hepatocytes. The data was expressed as % control of the formation of metabolites. In panel C, % control of the formation of both oxidative (para- and ortho- hydroxylations) (white bars) and total metabolites (para- and ortho- hydroxylations and lactone formation) (black bars) of atorvastatin were measured. In panel D, concentration-dependent inhibition of BOC (squares) and telaprevir (circles) on both oxidative (closed squares or circles) and total (open squares or circles) metabolism of atorvastatin was measured. Values shown are mean ± SE for experiments performed in triplicate.

**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 $[^{14}\text{C}]$ BOC (1 µM) was evaluated in MDCKII-MDR1 (A) and MDCKII-BCRP monolayers (B). $P_{\text{app}}$ B-A/A-B ratio in MDR1 or BCRP transfected cells (closed squares) and control MDCKII cells (open squares) in the absence and presence of cyclosporine A (CsA) (10 µM) or Ko143 (5 µM) was measured at 3 hrs. Panels D and E showed transcellular transport of the prototypical substrates $[^{3}\text{H}]$ verapamil (VER) (1 µM) for MDR1 Pgp and $[^{3}\text{H}]$ prazosin (PRA) (5 µM) for BCRP measured in transfected cells (closed squares) and control MDCKII cells (open squares) in the absence and presence of CsA (10 µM) or Ko143 (1 µM), respectively. Time-dependent uptake of $[^{14}\text{C}]$ BOC (2 µM) was evaluated in MRP2 (squares) and control (circles) membrane vesicles (C) in the presence (closed squares or circles) and absence (open squares or circles) of 5mM ATP and an ATP-regenerating system. Panel F showed uptake rate of $[^{14}\text{C}]$ EA-SG (2 µM), prototypical substrate of MRP2, measured in MRP2 containing vesicles in the presence (closed squares) and absence (open squares) of 5mM ATP and ATP-regenerating system at 5 min. Values shown are means ± SE of experiments performed in triplicate.

**Fig. 6. Uptake of BOC in human hepatocytes**

(A). Time- and temperature-dependent uptake of $[^{14}\text{C}]$ BOC (1 µM) into human hepatocytes at 37°C (closed squares) and 4°C (open squares), respectively. (B). Kinetic analysis for initial uptake rate of BOC was conducted in human hepatocytes at 37°C with obtained $K_m=12.4 \pm 7.4 \mu M$, $V_{\text{max}}=343 \pm 150 \text{ pmol/min/10}^6 \text{ cells}$, and $P_{\text{diff}} = 7.6 \pm 1.5 \mu l/min/10^6 \text{ cells}$. (C). Sodium-dependent uptake of $[^{14}\text{C}]$ BOC (1 µM) into human hepatocytes. Time-dependent uptake of $[^{14}\text{C}]$ BOC (1 µM) was conducted at 37°C in sodium-free (closed triangles) and sodium-containing uptake buffer (closed squares), and at 4°C (open squares) in sodium-containing buffer. Values shown are means ± SE of experiments performed in triplicate.
Fig. 7. Uptake of BOC into human OATP1B1, OATP1B3, OATP2B1, and OCT1 transfected MDCKII cells

Time-dependent uptake of $[^{14}\text{C}]$ BOC (1 µM) was evaluated in MDCKII-OATP1B1 (A), MDCKII-OATP1B3 (B), MDCKII-OATP2B1 (C), and CHO-K1-OCT1 cells (D). Panels E, F and G, and H showed uptake rate of the prototypical substrates $[^{3}\text{H}]$ E$_{2}$17βG (1 µM), $[^{3}\text{H}]$ CCK-8 (2.5 nM), $[^{3}\text{H}]$ estrone-3-sulfate (0.1 µM), $[^{14}\text{C}]$ TEA (1 µM) for OATP1B1, OATP1B3, OATP2B1, and OCT1 measured at 5 min, respectively. Closed squares represent the uptake in transfected cells, and open squares for uptake by control MDCKII or CHO-K1 cells. Values shown are means ± SE of experiments performed in triplicate.

Fig. 8. Effect of various compounds on uptake of BOC into human hepatocytes

Effect of various compounds, including BSP, rifampin, rifamycin SV, ritonavir, lopinavir, cyclosporine A, quinidine, and ketoconazole on initial uptake rate of $[^{14}\text{C}]$ BOC (0.5 µM) into 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. Values shown are means ± SE of experiments performed in triplicate.
Table 1 *In vitro* evaluation of BOC as an inhibitor of major CYP and UGT enzymes in human liver microsomes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>CYP/UGT reaction</th>
<th>Substrate concentration (µM)</th>
<th>Reversible inhibition</th>
<th>Time-dependent inhibition&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No pre-incubation IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td>Inhibition (%) at 100 µM IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Phenacetin O-deethylation</td>
<td>60</td>
<td>&gt;100</td>
<td>22</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin 7-hydroxylation</td>
<td>0.75</td>
<td>&gt;100</td>
<td>20</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion hydroxylation</td>
<td>50</td>
<td>&gt;100</td>
<td>2.3</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Amodiaquine N-dealkylation</td>
<td>2.0</td>
<td>&gt;100</td>
<td>25</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac 4'-hydroxylation</td>
<td>7.5</td>
<td>&gt;100</td>
<td>3.6</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-Mephenytoin 4'-hydroxylation</td>
<td>40</td>
<td>&gt;100</td>
<td>25</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan O-demethylation</td>
<td>7.5</td>
<td>&gt;100</td>
<td>45</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorozoxzone 6-hydroxylation</td>
<td>30</td>
<td>&gt;100</td>
<td>NA</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Testosterone 6β-hydroxylation</td>
<td>100</td>
<td>&gt;100</td>
<td>41</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Midazolam 1'-hydroxylation</td>
<td>5.0</td>
<td>11 ± 1.0 (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>40</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>3'-azido-3'-deoxythimidine (AZT) glucuronidation</td>
<td>750</td>
<td>&gt;100</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Time-dependent inhibition was determined by comparison of IC<sub>50</sub> values with and without pre-incubation.

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.
Table 2. *In vitro* evaluation of 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 (0.1µM)</td>
<td>18.0 ± 2.4</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>Bromosulfophthalein (BSP) (0.1µM)</td>
<td>4.9 ± 1.3</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>Estrone-3-sulfate (0.1µM)</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>Pgp</td>
<td>Digoxin (0.1µM)</td>
<td>&gt;300</td>
</tr>
<tr>
<td>BCRP</td>
<td>Methotrexate (10 µM)</td>
<td>81 ± 28</td>
</tr>
<tr>
<td>MRP2</td>
<td>Ethacrynic acid glutathione (EA-SG) (2µM)</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>
Table 3: *In vitro* evaluation of BOC as a potential perpetrator for OATP1B1-mediated drug-drug interactions (DDIs)

<table>
<thead>
<tr>
<th>Perpetrator (dose)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM) OATP1B1</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;, u (µM)</th>
<th>I&lt;sub&gt;in&lt;/sub&gt;, max, u (µM)</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 (800mg tid)</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 EGJ, 2011)</td>
</tr>
<tr>
<td>CsA (100mg)</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>(Shitara and Sugiyama, 2006)</td>
</tr>
<tr>
<td>Rifampin (600mg)</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 (400mg)</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 (750mg)</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>Amprenavira&lt;sup&gt;a&lt;/sup&gt; (600mg)</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&lt;sup&gt;b&lt;/sup&gt; (100mg bid)</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>

<sup>a</sup>: The prodrug fosamprenavir was used in the study  
<sup>b</sup>: Co-administered with fosamprenavir
Table 4. IC\textsubscript{50} values for inhibitory effects of several compounds on uptake of [\textsuperscript{3}H] atorvastatin acid (3 µM) and [\textsuperscript{3}H]pitavastatin (1µM) in human hepatocytes

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC\textsubscript{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Probe Atorvastatin acid (3µM)</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>11.9 ± 3.1</td>
</tr>
<tr>
<td>BOC</td>
<td>7.2 ± 1.4</td>
</tr>
<tr>
<td>Rifampin</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>Telaprevir</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Lopinavir</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Estrone sulfate</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>Estropipate</td>
<td>&gt; 10</td>
</tr>
</tbody>
</table>
Figure 1

Boceprevir (BOC)

SCH-534128

SCH-534129
Figure 2

A

B

With NADPH, no dilution

C

Without NADPH, no dilution

D

With NADPH, 25x dilution

Midazolam 1'-hydroxylation (pmol/mg protein/min)

1% Methanol control  3 µM BOC

Midazolam 1'-hydroxylation (pmol/mg protein/min)

1% Methanol control  3 µM BOC

Midazolam 1'-hydroxylation (pmol/mg protein/min)

1% Methanol control  3 µM BOC
Figure 3

A

[3H]Atorvastatin acid (3µM) uptake rate (% Control)

Inhibitor concentrations (µM)

0 10 100

0 20 40 60 80 100

B

[3H]Pitavastatin (1µM) uptake rate (% Control)

Inhibitor concentrations (µM)

0 0.01 0.1 1 10 100

Rifampicin
Telaprevir
BOC
Ketoconazole
Ritonavir
Lopinavir
Estrone sulfate
Estropipate
Figure 4

Formation of metabolites of midazolam (% Control)

Ketoconazole 2 µM
Rifampin 20 µM
BOC 3 µM
Telaprevir 3 µM

Formation of metabolites of pitavastatin (% Control)

Ketoconazole 2 µM
Rifampin 20 µM
BOC 3 µM
Telaprevir 3 µM

Formation of metabolites of atorvastatin (% Control)

BOC on oxidative metabolites
Telaprevir on oxidative metabolites
BOC on total metabolites
Telaprevir on total metabolites

Inhibitor concentration (µM)
Figure 5

A Pgp-BOC

B BCRP-BOC

C MRP2-BOC

D Pgp-Verapamil

E BCRP-Prazosin

F MRP2-EA-SG
Figure 6

A

$[^{14}\text{C}]$ BOC (1 µM) uptake (pmole/10⁶ cells)

- $37^\circ\text{C}$
- $4^\circ\text{C}$

Time (min)

B

$V_o$ (µl/min/10⁶ cells)

- $37^\circ\text{C} + \text{Na}$
- $4^\circ\text{C} + \text{Na}$
- $37^\circ\text{C} - \text{Na}$

Vo (pmole/min/10⁶ cells)

C

$[^{14}\text{C}]$ BOC (1 µM) uptake (pmole/10⁶ cells)

- $37^\circ\text{C} + \text{Na}$
- $4^\circ\text{C} + \text{Na}$
- $37^\circ\text{C} - \text{Na}$

Time (min)
Figure 7
Figure 8

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