Bosentan Is a Substrate of Human OATP1B1 and OATP1B3: Inhibition of Hepatic Uptake as the Common Mechanism of Its Interactions with Cyclosporin A, Rifampicin, and Sildenafil

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ABSTRACT:

The elimination process of the endothelin receptor antagonist bosentan (Tracleer) in humans is entirely dependent on metabolism mediated by two cytochrome P450 (P450) enzymes, i.e., CYP3A4 and CYP2C9. Most interactions with concomitantly administered drugs can be rationalized in terms of inhibition of these P450 enzymes. The increased bosentan concentrations observed in the presence of cyclosporin A, rifampicin, or sildenafil, however, are incompatible with this paradigm and prompted the search for alternative mechanisms governing these interactions. In the present article, we identify bosentan and its active plasma metabolite, Ro 48-5033 (4-(2-hydroxy-1,1-dimethyl-ethyl)-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-[2,2’]bipyrimidinyl-4-yl]-benzenesulfonamide), as substrates of the human organic anion transporting polypeptides (OATP) OATP1B1 and OATP1B3. Bosentan uptake into Chinese hamster ovary cells expressing these OATP transporters was efficiently inhibited by cyclosporin A and rifampicin with IC_{50} values significantly below their effective plasma concentrations in humans. The phosphodiesterase-5 inhibitor sildenafil was also shown to interfere with OATP-mediated transport, however, at concentrations above those achieved in therapeutic use. Therefore, inhibition of bosentan hepatic uptake may represent an alternative/complementary mechanism to rationalize some of the pharmacokinetic interactions seen in therapeutic use. A similar picture has been drawn for drugs like pitavastatin and fexofenadine, drugs that are mainly excreted in unchanged form. Bosentan elimination, in contrast, is entirely dependent on metabolism. Therefore, the described interactions with rifampicin, cyclosporin A, and, to a lesser extent, sildenafil represent evidence that inhibition of hepatic uptake may become the rate-limiting step in the overall elimination process even for drugs whose elimination is entirely dependent on metabolism.

Bosentan (Tracleer) is a dual endothelin receptor antagonist (Clozel et al., 1994; Neidhart et al., 1996) approved as the first oral treatment for pulmonary arterial hypertension (PAH) (Rubin et al., 2002). Its pharmacokinetic profile in humans is characterized by a low systemic plasma clearance of 17 l/h, a volume of distribution of about 30 l, and an oral bioavailability of about 50% (Dingemanse and van Giersbergen, 2004). At the maintenance dose of 125 mg b.i.d., bosentan trough concentrations decrease during the first days of treatment as a result of autoinduction of metabolizing enzymes, leading to an about 40% lower exposure at steady state. Bosentan is metabolized in the liver (Fig. 1), mediated to a similar extent by CYP2C9 and CYP3A4, followed by subsequent biliary excretion.

Hydroxylation at the r-butyl group by CYP2C9 and CYP3A4 yields metabolite Ro 48-5033, a metabolite that retains pharmacological activity and is present in human plasma at levels of about 10% compared with parent bosentan. Ro 47-8634 is formed by oxidative demethylation of the guaiacol ether, catalyzed by CYP3A4, to the corresponding phenol, whereas metabolite Ro 64-1056 is formed as a minor product from both primary metabolites. Renal clearance of bosentan is negligible (Hopfgartner et al., 1996; Weber et al., 1999b). Based on preclinical data, the first-pass effect of bosentan is small. Bosentan is neither a substrate nor an inhibitor of the intestinal efflux pump MDR1 (P-glycoprotein, ABCB1) (Weber et al., 1999a; Treiber et al., 2004).

Most of the pharmacokinetic drug-drug interactions observed with bosentan can be rationalized by inhibition of its metabolizing enzymes. For example, concomitant administration of ketoconazole as a prototypical potent CYP3A4 inhibitor led to a 2-fold increase in the exposure to bosentan (van Giersbergen et al., 2002), in line with predictions from in vitro data with human liver microsomes (A. Treiber, unpublished results). There is today, however, a number of observations that appear to be incompatible with this paradigm. Concomitant dosing of...

ABBREVIATIONS: Ro 48-5033, 4-(2-hydroxy-1,1-dimethyl-ethyl)-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-[2,2’]bipyrimidinyl-4-yl]-benzenesulfonamide; Ro 47-8634, 4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-[2,2’]bipyrimidinyl-4-yl]-benzenesulfonamide; Ro 64-1056, 4-(2-hydroxy-1,1-dimethyl-ethyl)-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-[2,2’]bipyrimidinyl-4-yl]-benzenesulfonamide; PAH, pulmonary arterial hypertension; OATP, organic anion transporting polypeptide(s); CHO, Chinese hamster ovary; E3S, estrone-3-sulfate; DHEAS, dehydroepiandrosterone sulfate; E17βG, estradiol-17β-glucuronide; P450, cytochrome P450.
bosentan and rifampicin (600 mg/day) led to the expected reduction in bosentan concentrations by about 40% at steady state. Surprisingly, however, mean exposure of bosentan on the first day of concomitant dosing was 6.5 times higher compared with volunteers receiving bosentan alone. Plasma exposure to the active metabolite Ro 48-5033 was determined in this study and was also increased by 6.6-fold (P.L.M. van Giersbergen, submitted for publication). In a combination trial of Tracleer with sildenafil at a dose of 80 mg t.i.d., i.e., 4 times the recommended dose of the phosphodiesterase-5 inhibitor approved for symptom relief in PAH patients, bosentan concentrations were 50% higher in patients treated with both drugs (Witke et al., 2005). Finally, bosentan at a dose of 500 mg b.i.d. was given together with the immnosuppressant cyclosporin A (300 mg b.i.d.) in a safety and tolerability trial in healthy volunteers early in clinical development. A 30-fold increase in bosentan trough concentrations was observed on day 1 when compared with volunteers receiving bosentan alone. The interaction was initially assigned to the inhibition of CYP3A4-mediated clearance in the liver because cyclosporin A is a potent inhibitor of this enzyme (Wacher et al., 1998), retrospectively a questionable interpretation in light of the only 2-fold increase observed with the much more potent CYP3A4 inhibitor ketoconazole, and pointing again to the existence of additional factors governing these pharmacokinetic interactions.

The early onset of the increase in bosentan levels after coadministration with cyclosporin A or rifampicin is suggestive of an inhibition of hepatic uptake of bosentan. Along these lines, we have shown that bosentan is a substrate of all three organic anion transporting polypeptide (OATP) isoforms expressed in the rat, i.e., Oatp1a1 (formerly Oatp1), Oatp1a4 (Oatp2), and Oatp1b2 (Oatp4). Concomitant dosing of cyclosporin A led to a 90% decrease in bosentan clearance, whereas it had no effect on bosentan metabolism, indicating that the vast majority of drug clearance is dependent on active bosentan uptake into the liver (Treiber et al., 2004). Rifampicin has been shown to inhibit rat Oatp1a4 and is a more potent inhibitor of human OATP1B1 (OATP-C) and OATP1B3 (OATP-B) than OATP2B1 (OATP-B) (Fattinger et al., 2000; Vavricka et al., 2002). Indeed, bosentan and its metabolite Ro 48-5033 are amphipathic monoanions under physiological conditions, with molecular masses of 550 to 570 Da, and are highly bound to human plasma albumin, thus fulfilling all the molecular features currently considered as prerequisites for efficient OATP substrate recognition (Hagenbuch and Meier, 2004).

In the present article, we describe bosentan and Ro 48-5033 as substrates of OATP1B1 and OATP1B3 heterologously expressed in Chinese hamster ovary (CHO) cells. Transport by OATP was efficiently inhibited by rifampicin, cyclosporin A, and sildenafil. In contrast, the effect of bosentan on OATP model substrates was weak, indicating that bosentan is unlikely to act as an OATP inhibitor in clinical use.

**Materials and Methods**

**Materials.** $^{14}$C-Radiolabeled bosentan (specific activity, 22.6 mCi/mmol; lot 12352B65) and its metabolite Ro 48-5033 (specific activity, 52.2 mCi/mmol; lot 16555B58) were from Actelion internal sources. Tritiated estrone-3-sulfate (E3S) (specific activity, 50 Ci/mmol; lot 060111) was purchased from Anawa (Zurich, Switzerland), and $^{3}$H-dehydroepiandrosterone sulfate (DHEAS) (specific activity, 60 Ci/mmol; lot 060111) was purchased from Sigma Aldrich (Buchs, Switzerland). 14C-Labeled warfarin (lot CFA449 batch 68) with a specific activity of 56.0 mCi/mmol and a radiochemical purity of 99.8% was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Liquid scintillation mixture IRGA-Safe-Plus was from Packard (Zurich, Switzerland). All the solvents used for experimental and analytical purposes were of the highest commercially available quality. The cell lines expressing the human transporters OATP2B1 (OATP-B), OATP1B1 (OATP-C), and...
OATP1B3 (OATP-8) were licensed from the University of Zurich together with the respective wild-type cell lines lacking the transport proteins.

**Cell Culture.** OATP1B1, OATP1B3, and OATP2B1-transfected CHO cell lines and the wild-type cells were cultured at passage numbers 8 to 31. Wild-type cells were grown at 37°C in T-flasks of 175-cm² growth area (Nunc Inc., Roskilde, Denmark) in an atmosphere containing 5% CO2, 95% relative humidity and maintained in Dulbecco’s modified Eagle’s medium containing penicillin/streptomycin (100 IU/ml), 10% fetal calf serum, and t-proline (0.05 mg/ml). The culture medium for the transfected cell lines additionally contained geneticin G-418 (500 µg/ml). All the media supplements were purchased from Invitrogen (Basel, Switzerland). For transport experiments, cells were seeded on Petri dishes of 3.5-cm diameter (Vitaris, Baar, Switzerland), and culture medium was replaced every other day. Transport experiments were usually performed on days 3 to 4 after seeding when cells were grown to confluence. One day before starting the transport experiments, cells were additionally treated with 5 µM sodium butyrate (Sigma Aldrich).

**Transport Studies.** Before the transport experiment, cells were rinsed twice with 2 ml of prewarmed (37°C) sodium uptake buffer (Earle’s balanced salt solution) (Rabito and Karish, 1983) containing 5.3 mM KCl, 1 mM NaH2PO4, 2 H2O, 0.8 mM MgSO4, 7 H2O, 5.5 mM d-glucose, 20 mM HEPES, and 116.4 mM NaCl. Uptake was started by adding 2 ml of uptake buffer containing the radiolabeled substrate. Stock solutions of radiolabeled bosentan, Ro 48-5033, and the OATP model substrates were prepared in ethanol and consisted of 1 µM radiolabeled substrate (final concentration) diluted with various amounts of the nonlabeled analog. The concentration of organic solvent was below 1% and constant in all the inhibition experiments. After incubation for 3 to 5 min at 37°C or 4°C, the uptake buffer with the substrate was quickly removed and the cells rapidly rinsed three times with each 2 ml of ice-cold choline buffer containing 5.3 mM KCl, 1 mM NaH2PO4·2 H2O, 0.8 mM MgSO4·7 H2O, 5.5 mM d-glucose, 20 mM HEPES, and 116.4 mM choline hydrochloride. Cells were then lysed by addition of 550 µl of 1% Triton X-100 solution. A 500-µl aliquot of each lysate was transferred into scintillation vials, whereas another 50-µl aliquot was used for protein determination. Scintillation vials were supplemented with scintillation mixture IRGA Safe Plus and well mixed before analysis using a Tri-Carb 2300 TR liquid scintillation analyzer (Packard Bioscience, Zurich, Switzerland).

**Determination of Protein Concentration.** Total protein was determined using the colorimetric BCA assay kit (Pierce Science, Lausanne, Switzerland) with quantification at a wavelength of 590 nm on a SpectroCount spectrophotometer (Packard Bioscience). Raw data were analyzed using the PlateReader software I-Smart (version 3.0 for Windows, Packard Bioscience).

**Determination of Kinetic Parameters.** Kinetic analysis for the uptake of bosentan and Ro 48-5033 was performed in a substrate concentration range of 1 to 300 µM. Before these experiments, the linearity of cellular uptake over time and microsomal protein concentration. Net uptake rates were calculated as the difference in the uptake rate of the transfected and wild-type cells for each individual concentration. Kinetic parameters, Km and Vmax, were calculated using the Michaelis-Menten equation V = Vmax × S/(Km + S) and the Origin software package (version 6.0, Microcal Software Inc., Northampton, MA).

**Inhibition of Bosentan and Ro 48-5033 Uptake.** Inhibition experiments on the cellular uptake of 14C-labeled bosentan and Ro 48-5033 were performed at a single substrate concentration of 10 µM, prepared as a 1 + 9 mixture of labeled and nonlabeled compound. Inhibitor solutions were prepared in ethanol in a final concentration range from 0.1 to 100 µM. The concentration of organic solvent was below 1% and constant in all inhibition experiments. Inhibition experiments were started by addition of 2 ml of prewarmed uptake buffer containing the labeled substrate and the inhibitor, and uptake of radioactivity was measured after 3 to 5 min of incubation time. IC50 values were determined by plotting the log inhibitor concentration against the net uptake rate and nonlinear regression of the dataset using the equation:

\[ y = \frac{a}{1 + \left(\frac{IC50}{IC50} \right)^b} \]

in which y is the net uptake rate (pmol/min · µg protein), I is the inhibitor concentration (µM), s is the slope at the point of inversion, and a and b are the maximum and minimum values for cellular uptake. Net uptake was calculated for each inhibitor concentration as the difference in the uptake rates of the transporter-expressing and wild-type cell lines.

**Effect of Bosentan on OATP Model Substrates.** The potential inhibitory effect of bosentan on OATP transport was investigated using known substrates for the different transport proteins, i.e., E3S for OATP2B1, E179G for OATP1B1, and DHEAS for OATP1B3. Bosentan was used in a concentration range from 0.1 to 100 µM, whereas the OATP model substrates were used at a concentration of 10 µM, i.e., around their respective Km values. Experiments were performed in triplicate and evaluated as described above.

**Inhibition of CYP3A4 and CYP2C9 Activity by Sildenafil.** To rule out a role of metabolism in the interaction between bosentan and sildenafil, the effect of sildenafil on the activities of CYP2C9 and CYP3A4 was investigated by using human liver microsomes and cytochrome P450 (P450)-specific marker reactions, i.e., midazolam 1′-hydroxylation for CYP3A4 (Walsky and Obach, 2004) and diclofenac 4′-hydroxylation for CYP2C9 (Leemann et al., 1993). Experiments were performed under initial rate conditions at substrate concentrations around the respective Km value, i.e., 5 µM for both substrates, and sildenafil as inhibitor at concentrations up to 50 µM. Metabolite formation was quantified by triple stage mass spectrometry coupled to liquid chromatography with an electrospray interface operating in selected reaction monitoring mode. Metabolic rates were determined by normalization for incubation time and microsomal protein concentration.

**Results.** A number of different in vitro experiments have been performed to identify which OATP isoforms are involved in the hepatic uptake of bosentan and its active metabolite Ro 48-5033 and to estimate their individual contribution to the overall OATP transport. In addition, we have investigated the effect of three drugs, i.e., cyclosporin A, rifampicin, and sildenafil, on OATP-mediated transport and, vice versa, the effect of bosentan on the transport of OATP model substrates.

As exemplified for OATP1B3 in Fig. 2, initial experiments indicated a marked uptake of bosentan at 37°C not only into OATP-expressing but also wild-type CHO cells. At a clinically relevant concentration of 1 µM, uptake into transfected cells was enhanced by less than a factor of 2 over uptake into wild-type cells. The difference in uptake rates was absent when experiments were performed at 4°C, pointing to the existence of endogenous bosentan transport activity in the wild-type CHO cell line. Similar observations have recently been published with another endothelin receptor antagonist, atrasentan. Using OATP transporters expressed in HeLa cells, uptake experiments were also compromised by the presence of an endogenous transport activity in the wild-type cell line (Katz et al., 2006). In contrast, control experiments, including
known substrates for the different OATP transporters and warfarin as a negative control, yielded outcomes consistent with published literature (Table 1) (Kullak-Ublick et al., 2001).

As a consequence, the uptake experiments with bosentan and Ro 48-5033 required individual optimization for each OATP-expressing cell line. The effects of cell number, incubation volume, substrate concentration, and incubation time were studied systematically before each uptake experiment with the aim to maximize the OATP-mediated uptake over the uptake mediated by the presumed endogenous transporter. As a compromise between maximizing uptake rates and working at clinically relevant conditions, a concentration of 10 μM was selected for the inhibition experiments with bosentan and Ro 48-5033. All the reported uptake rates are corrected for the corresponding values in the wild-type cells determined separately under exactly the same experimental conditions.

Kinetic parameters for bosentan and Ro 48-5033 uptake were determined with all three OATP-transporters (Fig. 3) in a concentration range of 1 to 300 μM. Initial uptake rates of bosentan and Ro 48-5033 into cells expressing OATP1B1, OATP1B3, and OATP2B1 were saturable with increasing substrate concentrations as shown in Fig. 3. Michaelis-Menten parameters for both substrates are summarized in Table 2.

The effect of rifampicin, cyclosporin A, and sildenafil on the cellular uptake of bosentan and Ro 48-5033 was investigated with OATP1B1. All three potential inhibitors were used in a concentration range from 0.1 to 100 μM (Fig. 4). Whereas bosentan and Ro 48-5033 were used at a single concentration of 10 μM. All three substances significantly inhibited bosentan uptake, whereas the effect on Ro 48-5033 uptake was much less obvious. Therefore, IC50 values were only calculated for bosentan and are summarized in Table 3. They show the following order of potency for OATP1B1 inhibition: cyclosporin A > sildenafil > rifampicin.

The results on the inhibition of OATP1B3-mediated bosentan and Ro 48-5033 transport are given in Fig. 5. As for OATP1B1, all the tested drugs inhibited bosentan transport markedly. Importantly, and in contrast to OATP1B1, transport of Ro 48-5033 by OATP1B3 was also subject to inhibition by all the drugs tested. The calculated IC50 values are given in Table 3. The rank order of inhibition for OATP1B3-mediated bosentan transport was cyclosporin A > sildenafil > rifampicin and for Ro 48-5033 transport, cyclosporin A > rifampicin > sildenafil.

To investigate the possible effect of bosentan on endogenous and exogenous OATP substrates, inhibition experiments were performed with model substrates of these three transport proteins, i.e., E17βG for OATP1B1, DHEAS for OATP1B3, and E3S for OATP2B1. At a concentration of 100 μM, bosentan inhibited the uptake of E3S, E17βG, and DHEAS to about the same extent, i.e., 49 to 62% compared with controls in the absence of bosentan. In contrast, cyclosporin A and rifampicin as known OATP inhibitors markedly inhibited the uptake of all three OATP (data not shown).

Finally, the effect of sildenafil on the activity of the two P450 enzymes involved in bosentan metabolism, i.e., CYP3A4 and CYP2C9, was investigated using human liver microsomes and P450 isoform-specific marker reactions. Up to the highest inhibitor concentration of 50 μM, sildenafil had no effect on CYP3A4 activity and inhibited CYP2C9 by only 26% compared with the control without inhibitor (data not shown).

**Discussion**

The observed pharmacokinetic interactions between bosentan and rifampicin, cyclosporin A, and sildenafil cannot be explained by inhibition of drug metabolism, although bosentan elimination is entirely dependent on the activity of CYP3A4 and CYP2C9. Important to note, there is no correlation between the inhibitory potency on CYP3A4 and the magnitude of the drug-drug interaction. For example, ketoconazole as the most potent CYP3A4 inhibitor (Ki, 0.015 μM) (Gibbs et al., 1999) led to an only 2-fold increase in bosentan levels (van Giersbergen et al., 2002), whereas rifampicin, the weakest inhibitor in this series (Ki, 18.5 μM) (Kajosaari et al., 2005) with no clinical record of increased drug concentrations caused by CYP3A4 inhibition, showed a mean increase of 6.5-fold. Moreover, none of the three drugs is a known CYP2C9 inhibitor. Bosentan has an oral bioavailability of about 50% in humans (Dingemans and van Giersbergen, 2004) and, based on unpublished preclinical data, has only a small first-pass effect. Therefore, changes in the oral absorption process of bosentan would lead to an increase in plasma levels not exceeding a factor of 2, thus insufficient to explain the much higher increases observed with rifampicin and cyclosporin A. Along these lines, bosentan has been shown to be neither a substrate nor an inhibitor of the intestinal efflux pump MDR1 (Weber et al., 1999a; Treiber et al., 2004). We have recently shown that the interaction between bosentan and cyclosporin A is also present in the rat after p.o. and i.v. dosing (Treiber et al., 2004), and we have identified inhibition of Oatp transport in the liver as a systemic rather than presystemic origin of this effect.

To investigate the relevance of these rat findings and to identify the human OATP isoforms responsible for the hepatic uptake of bosentan, we used cell lines stably expressing these OATP. As shown in Fig. 3 and Table 2, bosentan exhibited saturable uptake kinetics with OATP1B1, OATP1B3, and OATP2B1. The highest affinity was observed to OATP1B1 with a Ki of 44 μM, whereas the affinities to OATP1B3 and OATP2B1 were lower. Intrinsic transport capacities, expressed as Vmax/Km were similar for OATP1B1 and OATP1B3, whereas the OATP2B1 contribution was lower. For an estimation of the individual contributions of the three OATP isoforms to the overall bosentan uptake, information on the relative hepatic expression levels is needed. Published expression levels of OATP transport proteins in human liver show conflicting data. Whereas analysis of mRNA levels seemed to indicate higher expression of OATP1B1 over OATP1B3 in two publications (Keitel et al., 2005; Briz et al., 2006), another article...
reported the opposite rank order (Ho et al., 2006). On a protein level, these differences seem to be less pronounced (Keitel et al., 2005), which may be because of the interindividual variability in the OATP levels. In contrast, there seems to be consensus that the relative expression of OATP2B1 is significantly lower (Hirano et al., 2006). Bosentan peak plasma concentrations are about 2 μM and thus well below the K_m values of any of the three transporters. Because the intrinsic transport capacities of OATP1B1 and OATP1B3 are comparable, both OATP transporters are likely to contribute to about the same extent to the overall uptake of bosentan into human liver. In contrast, the role of OATP2B1 in the hepatic uptake of bosentan appears to be limited. Similar properties were observed for metabolite Ro 48-5033 with the exception that the metabolite is not a substrate for OATP2B1. Available literature suggests that hepatic transport by OATP proteins is a common feature of endothelin receptor antagonists. The cyclic pentapeptide BQ-123 has been reported to be a selective substrate of OATP-8/OATP1B3 (Kullak-Ublick et al., 2001), whereas the nonpeptidic
endothelin receptor antagonist atrasentan is a substrate of OATP1B1 (Katz et al., 2006).

Our data (Table 2) show that the most likely explanation for the increased bosentan levels on concomitant administration of rifampicin, cyclosporin A, and sildenafil is reduced hepatic uptake, caused by the inhibitory action of these drugs on OATP1B1- and/or OATP1B3-mediated transport. Rifampicin inhibited bosentan uptake mediated by OATP1B1 and OATP1B3 with IC50 values of 3.2 and 1.6 μM, respectively, and had a similarly strong effect on the OATP1B3-mediated uptake of the metabolite Ro 48-5033 (IC50 0.8 μM). Plasma concentrations of rifampicin at the therapeutic dose of 600 mg/day are in the range of 8 to 15 μM (Acocella, 1978; Loos et al., 1985), thus sufficiently above the determined IC50 values to inhibit the hepatic uptake of bosentan and its metabolite into the liver. In fact, rifampicin has been described as a potent inhibitor of OATP transport, affecting the hepatic uptake of drugs like pitavastatin (Ki on OATP1B1, 0.5 μM) (Hirano et al., 2006) and model compounds such as bromosulphthalein (Ki values on OATP1B1 and OATP1B3, 17 and 5 μM, respectively) (Vavricka et al., 2002). Plasma concentrations of metabolite Ro 48-5033 were elevated to a similar extent as parent bosentan on concomitant dosing of rifampicin. Ro 48-5033 is produced in the liver by CYP2C9 and CYP3A4. Although no information is available on how Ro 48-5033 crosses from liver into sinusoidal blood, its hepatic uptake is, based on these in vitro data, equally vulnerable to OATP inhibition, which may explain the parallel increase in plasma levels.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>IC50 OATP1B1 μM</th>
<th>IC50 OATP1B3 μM</th>
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<tbody>
<tr>
<td>Bosentan</td>
<td>Rifampicin</td>
<td>3.2 ± 1.6</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Cyclosporin A</td>
<td>0.3 ± 0.1</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Sildenafil</td>
<td>1.5 ± 0.5</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Ro 48-5033</td>
<td>Rifampicin</td>
<td>~50</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Cyclosporin A</td>
<td>&gt;100</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Sildenafil</td>
<td>&gt;100</td>
<td>4.0 ± 1.3</td>
</tr>
</tbody>
</table>
Bosentan trough concentrations were 30-fold elevated on concomitant dosing of cyclosporin A, and the combination of both drugs is contraindicated in PAH therapy. As shown in Table 2, cyclosporin A was a very potent inhibitor of OATP transport activity. IC$_{50}$ values for OATP1B1- and OATP1B3-mediated bosentan uptake were 0.3 and 0.8 µM, respectively, and 0.5 µM for the OATP1B3-mediated uptake of Ro 48-5033. This strong inhibitory effect is consistent with literature data of cyclosporin A on cerivastatin uptake into OATP2/OATP1B1-expressing cells (K$_i$ = 0.2 µM) (Shitara et al., 2003). Similar to rifampicin, cyclosporin A had no effect on the uptake of Ro 48-5033 into OATP1B1-expressing cells. Peak plasma concentrations of cyclosporin A determined in the same study were 1.1 to 1.3 µM, in agreement with published data (Min et al., 2000) and above the IC$_{50}$ values determined for cyclosporin A in vitro. Unlike rifampicin, cyclosporin A is also a potent inhibitor of CYP3A4, with a K$_i$ value in the range of 0.7 to 2 µM (Paine et al., 2000; Racha et al., 2003). Therefore, the significantly increased bosentan concentrations in the presence of cyclosporin A may result from a combined effect on hepatic uptake through inhibition of OATP1B1 and OATP1B3 transport together with inhibition of CYP3A4-mediated metabolism.

Finally, sildenafil at 80 mg t.i.d., i.e., 4 times the recommended dose for the treatment of PAH, increased bosentan plasma concentrations by about 50% (Wittke et al., 2005). Sildenafil did not inhibit either CYP3A4 or CYP2C9 activity. However, sildenafil inhibited the uptake of bosentan into OATP1B1- and OATP1B3-expressing cells with IC$_{50}$ values of 1.5 and 0.8 µM, respectively. The effect on the uptake of Ro 48-5033 into OATP1B3 cells was slightly weaker with an IC$_{50}$ of 4.0 µM. Sildenafil peak plasma concentration at a dose of 80 mg t.i.d. was 1.2 µM and thus in the range of the IC$_{50}$ values determined in vitro. Therefore, inhibition of bosentan hepatic uptake by sildenafil may also explain the pharmacokinetic interaction between these drugs. At the recommended dose of 20 mg t.i.d., however, sildenafil plasma concentrations are expected to be too low to elicit an effect on the pharmacokinetics of bosentan.

The effect of bosentan on the uptake of endogenous OATP substrates was investigated to estimate the potential for drug-drug interactions with coadministered drugs whose pharmacokinetics and pharmacologic action are dependent on hepatic uptake. The effect of bosentan was weak on all three model substrates of OATP transport, and all IC$_{50}$ values were estimated in the range of 100 µM. Peak plasma concentrations of bosentan at the recommended maintenance dose of 125 mg b.i.d. are about 2 µM, i.e., at least 50-fold lower than required for efficient uptake inhibition. Therefore, it is unlikely that bosentan will change the pharmacokinetics

![Fig. 5. Inhibition of bosentan and Ro 48-5033 uptake into OATP1B3-expressing CHO cells by rifampicin, cyclosporin A, and sildenafil.](image-url)
and/or pharmacodynamics of any concomitant drug dependent on OATP activity.

In conclusion, we have shown that the hepatic uptake of the endothelin receptor antagonist bosentan and its metabolite Ro 48-5033 in humans is mediated by OATP1B1 and OATP1B3, whereas OATP2B1 seems to play only a minor role (if any). Beyond inhibition of metabolizing enzymes, interference with bosentan disposition into the liver by drugs inhibiting OATP uptake provides a complementary mechanistic rationale for its drug-drug interactions. Similar conclusions have been drawn for pitavastatin (Hirano et al., 2006) and fexofenadine (Shimizu et al., 2005). Whereas the latter two drugs are eliminated mostly in unchanged form, bosentan is extensively metabolized before excretion. The described interactions with rifampicin, cyclosporin A, and, to a minor extent, sildenafil clearly show that inhibition of hepatic uptake may become the rate-limiting step in the overall elimination process even for drugs whose elimination is mainly dependent on metabolism.

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