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**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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### **Running Title:**

Bosentan is a human OATP substrate

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Number of text pages: 31

Number of tables: 3

Number of figures: 5

Number of references: 31

Number of words in Abstract: 242

Number of words in Introduction: 776

Number of words in Discussion: 1465

### **ABBREVIATIONS:**

IC<sub>50</sub>, half-inhibition constant; K<sub>i</sub>, inhibition constant; LC-MS/MS, triple stage mass spectrometry coupled to liquid chromatography; OATP, organic anion transporting polypeptide; DHEAS, dehydroepiandrosterone sulfate; E3S, estrone-3-sulfate; E17βG, estradiol-17β-glucuronide; b.i.d., bis in diem (twice daily); CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; K<sub>m</sub>, Michaelis constant; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PAH, pulmonary arterial hypertension; q.d., quaque die (daily); t.i.d., tris in diem (three times a day).

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### Abstract

The elimination process of the endothelin receptor antagonist bosentan (Tracleer<sup>TM</sup>) in man 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 paper, we identify bosentan and its active plasma metabolite, Ro 48-5033, as substrates of the human organic anion transporting polypeptides OATP1B1 and OATP1B3. Bosentan uptake into CHO cells expressing these OATP transporters was efficiently inhibited by cyclosporin A and rifampicin with IC<sub>50</sub> values significantly below their effective plasma concentrations in man. The PDE-5 inhibitor sildenafil was also shown to interfere with OATP-mediated transport, however, at concentrations above those achieved in therapeutic use. Inhibition of bosentan hepatic uptake might therefore 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. The described interactions with rifampicin, cyclosporin A and, to a minor extent sildenafil, therefore represent evidence that inhibition of hepatic uptake might become the rate-limiting step in the overall elimination process even for drugs whose elimination is entirely dependent on metabolism.

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### INTRODUCTION

Bosentan (Tracleer<sup>TM</sup>) 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 man 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 due to auto-induction of metabolizing enzymes, leading to an about 40% lower AUC at steady state. Bosentan is metabolized in the liver (Figure 1), mediated to a similar extent by CYP2C9 and CYP3A4, followed by subsequent biliary excretion.

[Insert Figure 1]

Hydroxylation at the *t*-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 to parent bosentan. Ro 47-8634 is formed by oxidative demethylation of the guajacol ether, catalyzed by CYP3A4, to the corresponding phenol, while metabolite Ro 64-1056 is formed as a minor product from both primary metabolites. Renal clearance of bosentan is negligible (Weber et al., 1999a; Hopfgartner et al., 1996). 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-gP, ABCB1; Weber et al., 1999b; Treiber et al., 2004).

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Most of the pharmacokinetic drug-drug interactions observed with bosentan can be rationalized by inhibition of its metabolizing enzymes. For example, co-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 (unpublished results). There is today, however, a number of observations that appear to be incompatible with this paradigm. Concomitant dosing of bosentan and rifampicin (600 mg q.d.) led to the expected reduction in bosentan concentrations by about 40% at steady state. Surprisingly however, mean AUC of bosentan on the first day of concomitant dosing was 6.5 times higher compared to 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 (van Giersbergen, submitted). 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 (PDE-5) inhibitor approved for the symptom relief in PAH patients, bosentan concentrations were 50% higher in patients treated with both drugs (Wittke et al., 2005). Finally, bosentan at a dose of 500 mg b.i.d. was given together with the immunosuppressant 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 to volunteers receiving bosentan alone. The interaction was initially assigned to the inhibition of CYP3A4-mediated clearance in the liver since 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

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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 co-administration 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 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 while 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, 2004). Rifampicin has been shown to inhibit rat Oatp1a4 and is a more potent inhibitor of human OATP1B1 (OATP-C) and OATP1B3 (OATP-8) than OATP2B1 (OATP-B) (Vavricka et al., 2002; Fattinger et al. 2000). Indeed, bosentan and its metabolite Ro 48-5033 are amphipathic mono-anions under physiological conditions, with molecular weights of 550-570 Daltons, and are highly bound to human plasma albumin, thus fulfilling all molecular features currently considered as prerequisites for efficient OATP substrate recognition (Hagenbuch and Meyer, 2004).

In the present paper, we describe bosentan and Ro 48-5033 as substrates of OATP1B1 and OATP1B3 heterologously expressed in CHO cells. Transport by OATPs 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.

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### METHODS

#### Materials

<sup>14</sup>C-Radiolabeled bosentan (specific activity: 22.6 mCi/mmol, lot no. 12352B65) and its metabolite Ro 48-5033 (specific activity: 52.2 mCi/mmol, lot no. 16555B58) were from Actelion-internal sources. Tritiated estrone-3-sulfate (E3S, specific activity: 50 Ci/mmol, lot no. 060111) was purchased from Anawa (Zürich, Switzerland), while <sup>3</sup>H-dehydroepiandrosterone sulfate (DHEAS, specific activity: 60 Ci/mmol) and <sup>3</sup>H-estradiol-17 $\beta$ -glucuronide (E17 $\beta$ G, specific activity: 45 Ci/mmol) were from Perkin-Elmer (Schwerzenbach, Switzerland). Cyclosporin A, rifampicin, and the non-labeled OATP substrates E3S, DHEAS, and E17 $\beta$ G were purchased from Sigma Aldrich (Buchs, Switzerland). <sup>14</sup>C-labelled 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 cocktail IRGA-Safe-Plus was from Packard (Zürich, Switzerland). All 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 Zürich together with the respective wild-type cell lines lacking the transport proteins.

#### Cell culture

OATP1B1, OATP1B3, and OATP2B1-transfected CHO cell lines as well as the wild-type cells were cultured at passage numbers 8–31. Wild-type cells were grown at 37 °C in T-flasks of 175 cm<sup>2</sup> growth area (Nunc Inc., Roskilde, Denmark) in an atmosphere containing 5% CO<sub>2</sub> and 95% relative humidity and maintained in DMEM containing

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penicillin/streptomycin (100 IU/ml), 10% FCS, and L-proline (0.05 mg/ml). The culture medium for the transfected cell-lines additionally contained geneticin G-418 (500 µg/ml). All 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 day 3–4 after seeding when cells were grown to confluence. One day prior to starting the transport experiments, cells were additionally treated with 5 µM sodium butyrate (Sigma Aldrich, Buchs, Switzerland).

### Transport studies

Prior to the transport experiment, cells were rinsed twice with 2 ml of pre-warmed (37 °C) sodium uptake buffer (Earle's balanced salt solution; Rabito and Karish, 1983) containing KCl (5.3 mM), NaH<sub>2</sub>PO<sub>4</sub> \* 2 H<sub>2</sub>O (1 mM), MgSO<sub>4</sub> \* 7 H<sub>2</sub>O (0.8 mM), D-glucose (5.5 mM), HEPES (20 mM) and NaCl (116.4 mM). 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 non-labeled analogue. The concentration of organic solvent was below 1 % and constant in all transport experiments. After incubation for 3–5 minutes 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 KCl (5.3 mM), NaH<sub>2</sub>PO<sub>4</sub> \* 2 H<sub>2</sub>O (1 mM), MgSO<sub>4</sub> \* 7 H<sub>2</sub>O (0.8 mM), D-glucose (5.5 mM), HEPES (20 mM), and choline hydrochloride (116.4 mM). Cells were then lysed by addition of 550 µl of a 1% Triton X-100 solution. A 500 µl-aliquot of each lysate was

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transferred into scintillation vials, while another 50 µl-aliquot was used for protein determination. Scintillation vials were supplemented with scintillation cocktail IRGA Safe Plus and well mixed prior to analysis using a Tri-Carb 2300 TR liquid scintillation analyzer (Packard Bioscience, Zürich, 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 SpectraCount spectrophotometer (Packard Bioscience, Zürich, Switzerland). Raw data were analyzed using the PlateReader software I-Smart (version 3.0 for Windows, Packard Bioscience, Zürich, Switzerland).

### Determination of kinetic parameters

Kinetic analysis for the uptake of bosentan and Ro-48-5033 was performed in a substrate concentration range of 1–300 µM. Prior to these experiments, the linearity of cellular uptake over time was individually determined for each cell line (data not shown). Cellular uptake rates were determined by normalization for incubation time and total protein content. 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,  $K_m$  and  $v_{max}$ , were calculated using the Michaelis-Menten equation  $v = \frac{v_{max} \times S}{(K_m + S)}$  and the

Origin software package (version 6.0, Microcal Software Inc., Northampton, MA, USA).

### Inhibition of bosentan and Ro 48-5033 uptake

Inhibition experiments on the cellular uptake of  $^{14}\text{C}$ -labeled bosentan and Ro 48-5033 were performed at a single substrate concentration of 10 µM, prepared as a 1 + 9 mixture

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of labeled and non-labeled compound. Inhibitor solutions were prepared in ethanol in a final concentration range from 0.1–100  $\mu\text{M}$ . The concentration of organic solvent was below 1% and constant in all inhibition experiments. Inhibition experiments were started by addition of 2 ml of pre-warmed uptake medium containing the labeled substrate and the inhibitor, and uptake of radioactivity was measured after 3–5 minutes of incubation time.  $\text{IC}_{50}$  values were determined by plotting the log inhibitor concentration against the net uptake rate and non-linear regression of the dataset using the equation:

$$y = \frac{a}{1 + \left( \frac{I}{(\text{IC}_{50})^s} + b \right)}$$

in which  $y$  is the net uptake rate ( $\text{pmol}/\text{min} \cdot \mu\text{g}$  protein),  $I$  is the inhibitor concentration ( $\mu\text{M}$ ),  $s$  is the slope at the point of inversion, while  $a$  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, E17 $\beta$ G for OATP1B1, and DHEAS for OATP1B3. Bosentan was used in a concentration range from 0.1–100  $\mu\text{M}$ , while the OATP model substrates were used at a concentration of 10  $\mu\text{M}$ , i.e. around their respective  $K_m$  values. Experiments were performed in triplicate and evaluated as described above.

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### **Inhibition of CYP3A4 and CYP2C9 activity by sildenafil**

In order 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-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  $K_m$  value, i.e. 5  $\mu$ M for both substrates, and sildenafil as inhibitor at concentrations up to 50  $\mu$ M. Metabolite formation was quantified by LC/MS-MS with an electrospray interface operating in selected reaction monitoring mode. Metabolic rates were determined by normalization for incubation time and microsomal protein concentration.

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### RESULTS

A number of different in vitro experiments have been performed in order 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 Figure 2, initial experiments indicated a marked uptake of bosentan at 37 °C not only into OATP-expressing but also wild-type CHO cells.

[Insert Figure 2]

At a clinically relevant concentration of 1  $\mu$ M, 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).

[Insert Table 1]

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As a consequence, the uptake experiments with bosentan and Ro 48-5033 required individual optimization for each OATP-expressing cell line. The effect of cell number, incubation volume, substrate concentration, and incubation time were studied systematically prior to 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  $\mu\text{M}$  was selected for the inhibition experiments with bosentan and Ro 48-5033. All 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 (Figure 3) in a concentration range of 1–300  $\mu\text{M}$ . Initial uptake rates

[Insert Figure 3]

of bosentan and Ro 48-533 into cells expressing OATP1B1, OATP1B3, and OATP2B1 were saturable with increasing substrate concentrations as shown in Figure 3. Michaelis-Menten parameters for both substrates are summarized in Table 2.

[Insert 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  $\mu\text{M}$  (Figure 4) while bosentan and Ro 48-5033

[Insert Figure 4]

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were used at a single concentration of 10  $\mu$ M. All three substances significantly inhibited bosentan uptake, while the effect on Ro 48-5033 uptake was much less obvious. IC<sub>50</sub> values were therefore only calculated for bosentan and are summarized in Table 3. They show the following order of potency for OATP1B1 inhibition: cyclosporin A > sildenafil > rifampicin.

[Insert Table 3]

The results on the inhibition of OATP1B3-mediated bosentan and Ro 48-5033 transport are given in Figure 5. As for OATP1B1, all tested drugs inhibited bosentan transport

[Insert Figure 5]

markedly. Importantly, and in contrast to OATP1B1, transport of Ro-48-5033 by OATP1B3 was also subject to inhibition by all drugs tested. The calculated IC<sub>50</sub> 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. estradiol-17 $\beta$ -glucuronide (E17 $\beta$ G) for OATP1B1, dehydroepiandrosterone sulfate (DHEAS) for OATP1B3, and estrone-3-sulfate (E3S) for OATP2B1. At a concentration of 100  $\mu$ M, bosentan inhibited the uptake of E3S, E17 $\beta$ G and DHEAS to about the same extent, i.e. 49–62% compared to controls in the absence of bosentan. In contrast, cyclosporin A and rifampicin as known OATP inhibitors markedly inhibited the uptake of all three OATPs (data not shown).

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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  $\mu$ M, sildenafil had no effect on CYP3A4 activity and inhibited CYP2C9 by only 26% compared to the control without inhibitor (data not shown).

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### DISCUSSION

The observed pharmacokinetic interactions between bosentan with rifampicin, cyclosporin A and sildenafil cannot be explained by inhibition of drug metabolism though 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 ( $K_i$  0.015  $\mu$ M; Gibbs et al., 1999) led to an only 2-fold increase in bosentan levels (van Giersbergen, 2002) while rifampicin, the weakest inhibitor in this series ( $K_i$  18.5  $\mu$ M, Kajosaari et al., 2005) with no clinical record of increased drug concentrations due to 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 man (Dingemanse and van Giersbergen, 2004) and, based on unpublished preclinical data, has only a small first-pass effect. Changes in the oral absorption process of bosentan would therefore 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., 1999b; Treiber et al., 2004). We have recently shown, that the interaction between bosentan and cyclosporin A is also present in the rat after oral as well as after intravenous dosing (Treiber et al., 2004), and have identified inhibition of Oatp transport in the liver as a systemic rather than pre-systemic origin of this effect.

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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 OATPs. As shown in Figure 3 and Table 2, bosentan exhibited saturable uptake kinetics with OATP1B1, OATP1B3, and OATP2B1. The highest affinity was observed to OATP1B1 with a  $K_m$  of 44  $\mu$ M while the affinities to OATP1B3 and OATP2B1 were lower. Intrinsic transport capacities, expressed as  $v_{max}/K_m$ , were similar for OATP1B1 and OATP1B3, while 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 are needed. Published expression levels of OATP transport proteins in human liver show conflicting data. While analysis of mRNA levels seemed to indicate higher expression of OATP1B1 over OATP1B3 in two publications (Briz et al., 2006; Keitel et al., 2005), another paper 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 might be due to the inter-individual 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  $\mu$ M and thus well below the  $K_m$  values of any of the three transporters. Since 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

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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) while the non-peptidic endothelin receptor antagonist atrasentan is a substrate of OATP1B1 (Katz et al., 2006).

Our data (Table 2) demonstrate that the most likely explanation for the increased bosentan levels upon concomitant administration of rifampicin, cyclosporin A and sildenafil is reduced hepatic uptake due to the inhibitory action of these drugs on OATP1B1 and/or OATP1B3-mediated transport. Rifampicin inhibited bosentan uptake mediated by OATP1B1 and OATP1B3 with  $IC_{50}$  values of 3.2 and 1.6  $\mu M$ , respectively, and had a similarly strong effect on the OATP1B3-mediated uptake of the metabolite Ro 48-5033 ( $IC_{50}$  0.8  $\mu M$ ). Plasma concentrations of rifampicin at the therapeutic dose of 600 mg q.d. are in the range of 8–15  $\mu M$  (Acocella, 1978; Loos et al., 1985), thus sufficiently above the above  $IC_{50}$  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 ( $K_i$  on OATP1B1: 0.5  $\mu M$ ; Hirano et al., 2006) and model compounds such as bromosulfophthalein ( $K_i$  values on OATP1B1 and OATP1B3: 17  $\mu M$  and 5  $\mu M$ ; Vavricka et al., 2002). Plasma concentrations of metabolite Ro 48-5033 were elevated to a similar extent as parent bosentan upon 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 is crossing from liver into sinusoidal blood, its hepatic uptake is, based on this in vitro data, equally vulnerable to OATP inhibition, which might explain the parallel increase in plasma levels.

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Bosentan trough concentrations were 30-fold elevated upon concomitant dosing of cyclosporin A, and the combination of both drugs is contra-indicated 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  $\mu$ M and 0.8  $\mu$ M, respectively, and 0.5  $\mu$ M 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  $\mu$ M; 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 1.1–1.3  $\mu$ M, 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 0.7–2  $\mu$ M (Paine et al., 2000; Racha et al., 2003). The significantly increased bosentan concentrations in the presence of cyclosporin A might therefore result from a combined effect on hepatic uptake through inhibition by 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  $\mu$ M and 0.8  $\mu$ M, respectively. The effect on the uptake of Ro 48-5033 into OATP1B3 cells was slightly weaker with an  $IC_{50}$  of 4.0  $\mu$ M. Sildenafil peak plasma concentration at a dose of 80 mg t.i.d. was 1.2  $\mu$ M and thus in the range of the  $IC_{50}$  values

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determined *in vitro*. Inhibition of bosentan hepatic uptake by sildenafil might therefore 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 in order to estimate the potential for drug-drug interactions with co-administered 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<sub>50</sub> values were estimated in the range of 100  $\mu$ M. Peak plasma concentrations of bosentan at the recommended maintenance dose of 125 mg b.i.d. are about 2  $\mu$ M, i.e. at least 50-fold lower than required for efficient uptake inhibition. It is therefore unlikely that bosentan will change the pharmacokinetics 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 man is mediated by OATP1B1 and OATP1B3, while 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, 2006) and fexofenadine (Shimizu et al., 2005). While the latter two drugs are eliminated mostly in unchanged form, bosentan is extensively metabolized prior to excretion. The described interactions with rifampicin, cyclosporin A and, to a minor extent, sildenafil, clearly demonstrate that inhibition of hepatic uptake might become the rate-limiting step in the

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overall elimination process even for drugs whose elimination is mainly dependent on metabolism.

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### **FOOTNOTES**

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### FIGURE LEGENDS

- Figure 1 The metabolism of bosentan in man
- Figure 2 Cellular uptake of  $^{14}\text{C}$ -labeled bosentan in OATP1B3-expressing and wild-type CHO cells
- Figure 3 Michaelis-Menten plots for the uptake of bosentan and Ro 48-5033 into OATP-expressing CHO cells
- Figure 4 Inhibition of bosentan uptake into OATP1B1-expressing CHO cells by rifampicin, cyclosporin A and sildenafil
- Figure 5 Inhibition of bosentan and Ro 48-5033 uptake into OATP1B3-expressing CHO cells by rifampicin, cyclosporin A and sildenafil

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### TABLES

Table 1 Uptake ratios of estrone-3-sulfate (E3S), estradiol-17 $\beta$ -glucuronide (E17 $\beta$ G) and dehydroepiandrosterone sulfate (DHEAS) into OATP-expressing CHO cells <sup>(1)</sup>

| substrate               | transporter | uptake rate OATP<br>(pmol/min * $\mu$ g) | uptake rate wild-type<br>(pmol/min * $\mu$ g) | ratio <sup>(2)</sup> |
|-------------------------|-------------|--|---|----------------------|
| DHEAS                   | OATP1B3     | 1.158                                    | 0.272   | 4.3 (7.0)            |
| E17 $\beta$ G           | OATP1B1     | 0.417                                    | 0.097   | 4.3 (6.2)            |
| E3S                     | OATP2B1     | 0.334                                    | 0.205   | 1.7 (2.3)            |
| warfarin <sup>(3)</sup> | OATP1B3     | 0.116                                    | 0.135   | 0.9                  |

(1) determined at substrate concentrations of 10  $\mu$ M; (2) literature values in parentheses (Kullak-Ublick et al., 2001); (3) negative control.

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Table 2 Michaelis-Menten parameters for the cellular uptake of bosentan and Ro 48-5033 into human OATP-expressing CHO cells

| substrate  | transporter | $K_m$             | $V_{max}$                   | $V_{max}/K_m$             |
|------------|-------------|-------------------|-----------------------------|---------------------------|
|            |             | ( $\mu M$ )       | (pmol/min* $\mu g$ protein) | ( $\mu l$ /min* $\mu g$ ) |
| bosentan   | OATP1B3     | $141 \pm 17$      | $3.21 \pm 0.40$             | 0.023                     |
|            | OATP1B1     | $44 \pm 5$        | $1.32 \pm 0.05$             | 0.030                     |
|            | OATP2B1     | $202 \pm 107$     | $2.27 \pm 0.69$             | 0.011                     |
| Ro 48-5033 | OATP1B3     | $166 \pm 16$      | $2.14 \pm 0.32$             | 0.013                     |
|            | OATP1B1     | $60 \pm 17$       | $0.89 \pm 0.08$             | 0.015                     |
|            | OATP2B1     | -- <sup>(1)</sup> | --                          | --                        |

(1) no transport detectable for Ro 48-5033.

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Table 3 Inhibition of bosentan and Ro 48-5033 uptake into OATP1B1 and OATP1B3 expressing CHO cells by rifampicin, cyclosporin A and sildenafil

| substrate  | inhibitor     | OATP1B1               | OATP1B3               |
|------------|---------------|-----------------------|-----------------------|
|            |               | IC <sub>50</sub> (μM) | IC <sub>50</sub> (μM) |
| bosentan   | rifampicin    | 3.2 ± 1.6             | 1.6 ± 0.8             |
|            | cyclosporin A | 0.3 ± 0.1             | 0.8 ± 0.2             |
|            | sildenafil    | 1.5 ± 0.5             | 0.8 ± 0.3             |
| Ro 48-5033 | rifampicin    | ~ 50                  | 0.8 ± 0.4             |
|            | cyclosporin A | > 100                 | 0.5 ± 0.1             |
|            | sildenafil    | > 100                 | 4.0 ± 1.3             |

Figure 1

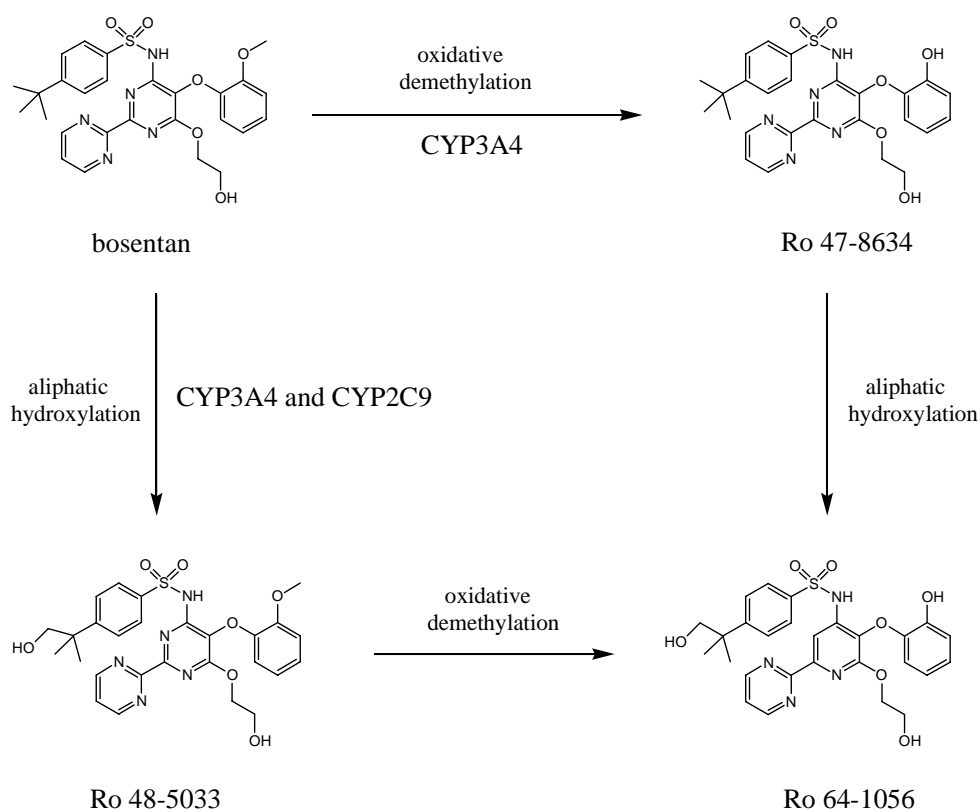


Figure 2

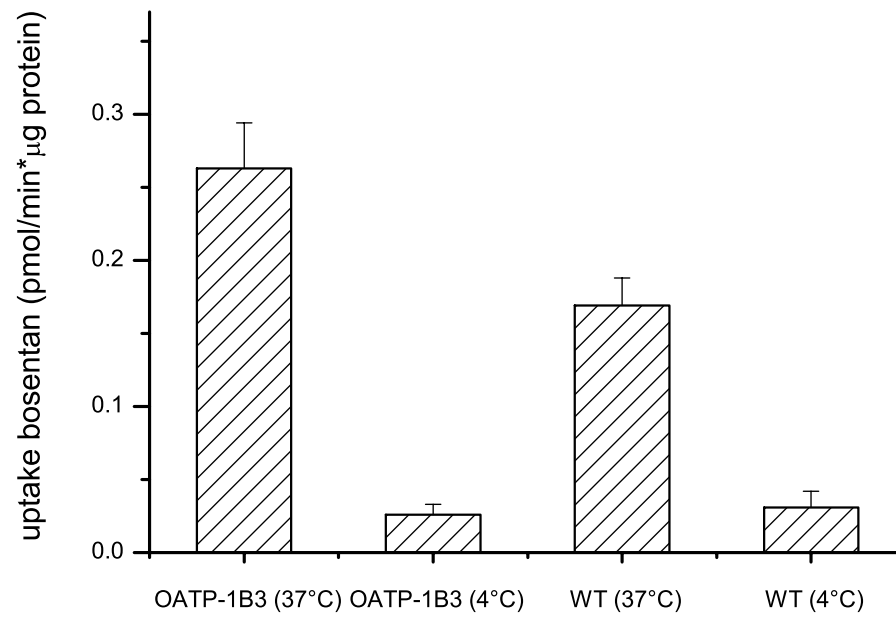


Figure 3

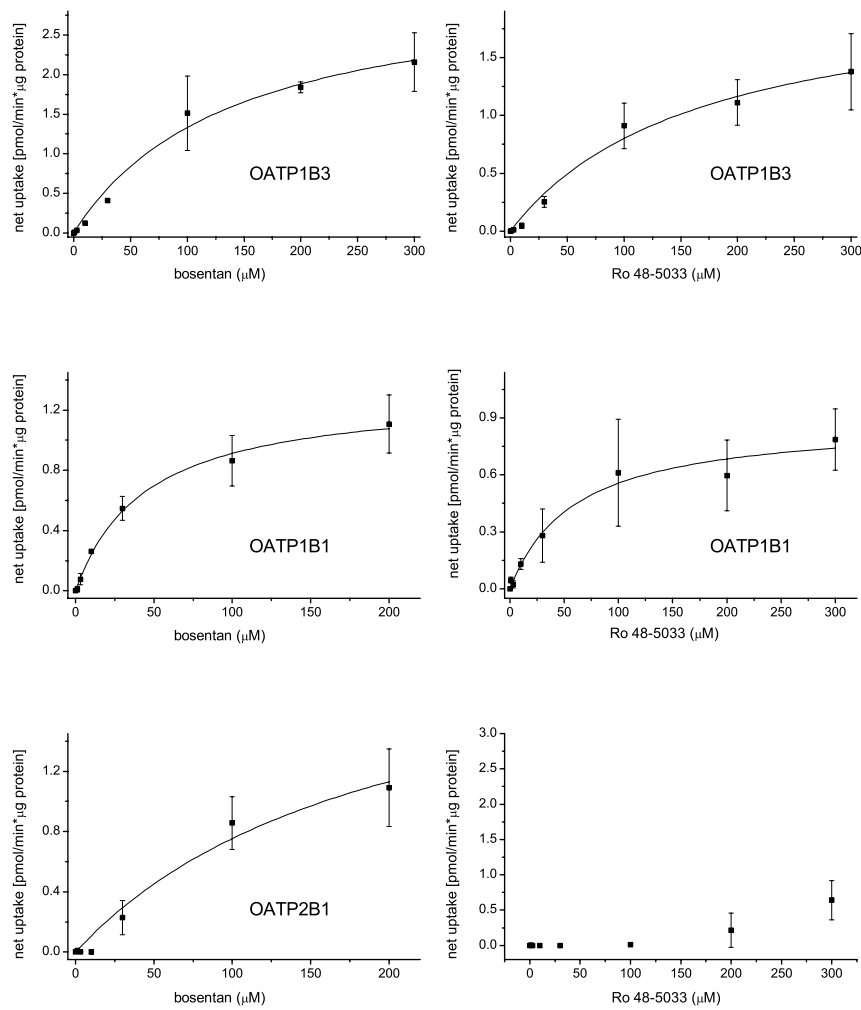


Figure 4

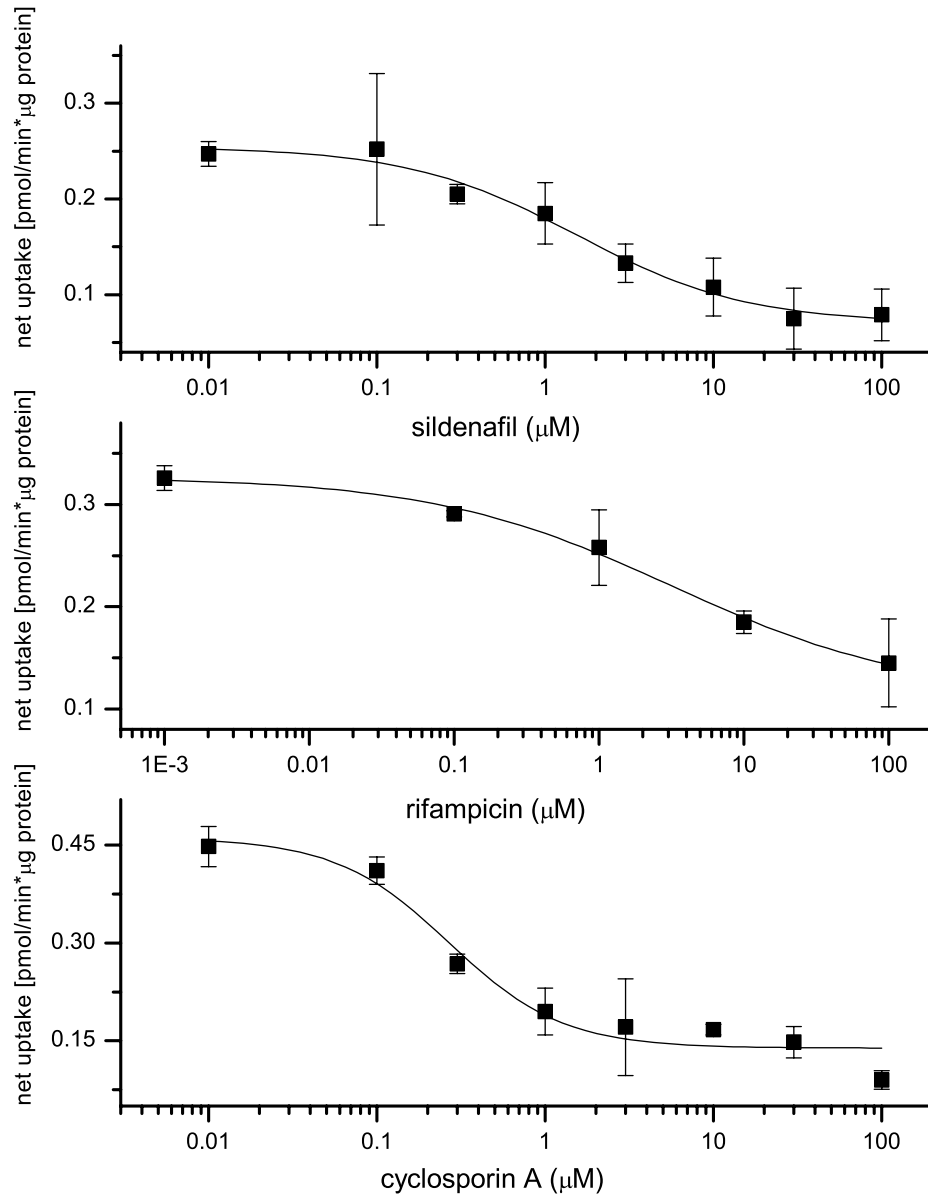


Figure 5

