Inhibition of Organic Anion Transporting Polypeptide-Mediated Hepatic Uptake Is the Major Determinant in the Pharmacokinetic Interaction between Bosentan and Cyclosporin A in the Rat

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Received October 14, 2003; accepted November 10, 2003

ABSTRACT

In clinical trials, a significant interaction between the endothelin receptor antagonist bosentan and the immunosuppressant cyclosporin A was observed, which could not be rationalized in terms of inhibition of drug-metabolizing enzymes. We present here a study performed in rats investigating the mechanisms underlying this interaction, including the inhibition of active drug transport processes as part of the gastrointestinal absorption and disposition into the liver. In vitro, the majority of bosentan uptake into liver cells was shown to depend on active transport and to be efficiently inhibited by cyclosporin A. All known members of the organic anion transporting polypeptide (oatp) transport protein family expressed in rat liver, i.e., oatp1, oatp2, and oatp4, were shown to be involved in the uptake of bosentan. Results from both series of experiments point to inhibition of active bosentan uptake into the liver by cyclosporin A as the major underlying mechanism for this pharmacokinetic interaction that is in line with reports on other oatp-transported drugs. Significant contributions of other mechanisms such as inhibition of mdr1-mediated drug efflux during gastrointestinal absorption, inhibition of bosentan metabolism, or inhibition of hepatobiliary excretion seemed to be unlikely. The interaction between bosentan and cyclosporin A is a rare example of a pharmacokinetic interaction, which can mostly be attributed to the inhibition of transport processes in the liver. It also demonstrates that inhibition of uptake into the liver might become rate-limiting in the overall elimination process even for compounds whose clearance is dependent on metabolism. The relevance of these findings in the rat for clinical use remains to be explored. It is, however, clear that inhibition of CYP3A4-mediated metabolism by cyclosporin A alone is insufficient to explain the increased bosentan concentrations and that inhibition of hepatocellular uptake offers an attractive mechanistic alternative also in human.

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 (Rubin et al., 2002). During the clinical development of bosentan, the potential interaction between bosentan (500 mg b.i.d.) and the immunosuppressant cyclosporin A (300 mg b.i.d.) has been investigated in healthy male volunteers. After the 1st day of concomitant dosing, a 30-fold increase of bosentan trough plasma concentrations was observed compared with volunteers receiving bosentan alone. In humans, bosentan is extensively metabolized by the cytochrome P450 isoforms 2C9 and 3A4 before excretion into bile, the latter process accounting for more than 90% of total drug elimination. Cyclosporin A is a known inhibitor of CYP3A4 (Wacher et al., 1998) and the observed pharmacokinetic interaction was initially assigned to the inhibition of CYP3A4-mediated clearance in the liver. However, in another interaction study with the potent CYP3A4 inhibitor ketoconazole, only a 2-fold increase in the exposure to bosentan was observed (van Giersbergen et al., 2002). This was in good agreement with in vitro data predicting a maximum 3-fold increase of bosentan.
plasma concentrations upon complete inhibition of CYP3A4 and pointed to the presence of additional factors governing this pharmacokinetic interaction. In the present article, we describe the work conducted in rats to elaborate on the mechanisms underlying the interaction between bosentan and cyclosporin A. Based on in vivo and in vitro data, it is proposed that the major determinant of this interaction constitutes inhibition of oatp-mediated active transport into the liver, whereas alternative mechanisms such as the inhibition of active drug efflux during gastrointestinal absorption, inhibition of bosentan metabolism in the liver, or inhibition of hepatobiliary excretion seem to play only a minor role.

**Materials and Methods**

*Materials.* Bosentan, sodium salt (lot 71102B1758) used for intravenous dosing and in vitro experiments, bosentan, micronized free sulfonamide (lot 704004) for oral pharmacokinetic experiments, and 14C-radioabeled bosentan, free sulfonamide (specific activity 40.9 μCi/mg, lot 12352B65) were obtained from Actelion Pharmaceuticals Ltd. (Allschwil, Switzerland) internal sources. Cyclosporin A, digoxin, quinidine, cholestyramin (CCK-8), and Triton X-100 were purchased from Fluka (Buchs, Switzerland), whereas verapamil hydrochloride was from Aldrich (Steinheim, Germany). Leibowitz L-15 medium, William’s E medium, FBS, newborn calf serum, and Hank’s balanced salt solution were obtained from Invitrogen (Basel, Switzerland).

**Pharmacokinetic Experiments in Rats.** Pharmacokinetic experiments with bosentan, either alone or in combination with cyclosporin A or verapamil, were performed in male Wistar rats weighing 250 to 300 g (n = 5–6). Two days before the experiments, animals used for oral administration had a catheter implanted into the left carotid artery under anesthesia with Breital (70 mg/kg). For intravenous dosing, an additional catheter was implanted into the jugular vein for drug administration. During the recovery period, animals were housed in individual cages with free access to food and water. Access to water was maintained during the experiment, but food was deprived the night before the experiment and until 4 h after drug administration. Rats received oral bosentan (free sulfonamide) formulated as a microsuspension in 7.5% modified gelatin at a dose of 10 mg/kg by gavage. For intravenous application at a dose of 1.0 mg/kg, bosentan (sodium salt) was dissolved in physiological saline. Cyclosporin A was formulated as a microsuspension in 7.5% modified gelatin and injected intraperitoneally at a dose of 50 mg/kg, 30 min before the administration of bosentan. Animals receiving bosentan alone were injected an equivalent volume of vehicle. Verapamil was coformulated with bosentan for oral administration at a dose of 10 mg/kg and as an aqueous solution in physiological saline for intravenous dosing at 1.0 mg/kg. Blood samples of about 300-μl volume were drawn at predefined time points over a period of 48 h and fortified with EDTA and NaF. Plasma was generated by centrifugation at 4000 rpm for 10 min at 4°C. Samples were stored at –20°C pending analysis. All experiments with rats and mice were performed in accordance with the NIH guidelines and approved by the Cantonal Veterinary Office (permit 169; Kantonales Veterinäramt Liestal, Switzerland).

**Pharmacokinetic Experiments in Wild-Type and Mdr-1a Knockout Mice.** Experiments in wild-type (FVB) and mdr-1a knockout mice were performed at steady state (Vss) was calculated as V ss = MRT x CL with the mean residence time (MRT) being defined as the area under the first moment curve AUMC0-inf. divided by AUC0-inf. Peak plasma concentrations (Cmax) and the time to reach peak plasma concentrations (Tmax) were directly taken from the respective plasma concentration-time curves. Oral bioavailabilities (F) were calculated by the ratios of dose-normalized AUC0-inf. after oral and intravenous dosing. All parameters are given as mean ± S.E.M. Statistical analysis was performed by analysis of variance using the Statistica software package (StatSoft, Tulsa, OK) and the Student-Newman-Keuls test for multiple comparisons. The null hypothesis was rejected with P < 0.05.

**Determination of in Vivo Metabolic Patterns.** Male Wistar rats weighing 260 to 290 g had a bile fistula implanted in the upper bile duct under pentobarbital anesthesia. The cannula was externalized via the neck and its extracorporeal part fortified against biting. A second catheter was implanted into the lower common bile duct for intraduodenal infusion of artificial bile containing 25 mM taurocholate, 12.5 mM lecithin, and 0.3 mM cholesterol in physiological saline fortified with 5.0 g/l sodium dihydrogen phosphate. After the operation, animals were allowed to recover for 2 days with free access to food and water. 14C-Radiolabeled bosentan was formulated as a microsuspension in 7.5% modified gelatin and administered orally by gavage at a dose of 5.0 mg/kg containing 17.7 Ci/kg of the radiolabel. Animals were placed individually in metabolic cages and bile, urine, and plasma were collected for up to 72 h. The analysis of metabolic patterns from all three matrices was done with an high-performance liquid chromatography device (Shimadzu Scientific Instruments, Kyoto, Japan) consisting of two LC-6A pumps, a SIL-6B auto-injector, and a SCL-6B system controller connected to a Spectraflor 773 UV detector (Kratos Analytical, Manchester, UK) set at a wavelength of 260 nm, and a LB506 C-1 radioactivity monitor (Berthold Technologies, Regensburg, Switzerland) equipped with a
150-μl solid flow cell YG150. Chromatographic separations were achieved with a flow rate of 1.0 ml/min and a linear gradient from 35 to 70% of mobile phase B within 35 min on a Superspher 100 column (RP-18, 4.6 × 250 mm i.d.; Merck, Germany) with 50 mM aqueous ammonium acetate containing 1.3% tetrahydrofuran (v/v) (phase A) and a 97:3 mixture of acetonitrile and tetrahydrofuran (v/v) (phase B).

Uptake Experiments with Rat Hepatocytes. Primary rat hepatocytes were prepared from male Wistar rats weighing 300 to 320 g using a modified three-step collagenase perfusion (Seglen, 1993), purification by a Percoll treatment and resuspension in William’s E medium supplemented with 10% FBS. The cell viability in each experiment was checked using the erythrosine B exclusion test and found to be in the range of 90 to 95%. Cells were seeded, at a density of 4 × 10^5 cells/ml, onto 24-well culture plates, previously coated with collagen, in William’s medium E supplemented with 10% FBS (v/v), 200 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 μg/ml insulin. Hepatocytes were allowed to attach for 3 h at 37°C in a humidified atmosphere containing 5% CO_2. Uptake experiments were performed according to a slightly modified literature protocol (Ismair et al., 2001). Immediately before the start of the uptake experiment, the cell monolayer was washed three times with prewarmed (37°C) or ice-cold (4°C) uptake buffer containing 116 mM NaCl, 5.3 mM KCl, 1.1 mM KH₂PO₄, 0.8 mM MgSO₄, 1.8 mM CaCl₂, 11 mM d-glucose, and 20 mM HEPES. Linearity of 14C-bosentan uptake with time and cell concentration was checked beforehand. The different inhibitors, dissolved in dimethyl sulfoxide, were added to the uptake medium about 1 min before the addition of 14C-labeled bosentan at a final concentration of 1 μM. The uptake was stopped by aspiration of the uptake buffer followed by three rapid washing steps with ice-cold, substrate-free uptake buffer. Cells were lysed with 9% Triton X-100 (v/v), 0.4 ml of the lysis buffer was mixed with 4.5 ml of scintillation cocktail, and cell-associated radioactivity determined by liquid scintillation counting. All experiments were identically performed in parallel at 37°C and 4°C. Results represent means ± S.D. from four to five independent determinations. Statistical analysis was performed by analysis of variance using the Statistica software and the Student-Newman-Keuls test for multiple comparisons. The null hypothesis was rejected when P < 0.05.

Inhibition of Bosentan Metabolism in Liver Microsomes. Enzyme kinetic analysis on the formation of the two primary metabolites Ro 48-5033 and Ro 47-8634 were performed using rat liver microsomes and bosentan at concentrations up to 300 μM. Initial experiments checking for the linearity of the metabolite production rate with respect to microsomal protein concentration and time revealed 1.0 mg/ml of protein and an incubation time of 5 min as the most appropriate conditions. Cyclosporin A was used at final concentrations of 30 and 100 μM. Experiments on the autoinhibition of bosentan metabolism by its metabolites were performed using the tetra-deuterated analogs as inhibitors. Experiments with D₄-Ro 47-8634 were performed at inhibitor concentrations of 10, 30, and 100 μM, whereas D₄-Ro 48-5033 was used at concentrations of 20, 60, and 200 μM, respectively. For all inhibition experiments, 100-fold concentrated stock solutions of cyclosporin A and the bosentan metabolites were prepared in ethanol and dimethyl sulfoxide, respectively, thus keeping the final concentration of organic solvent below 1%. Experiments in the absence of inhibitor were performed by adding an equivalent volume of organic solvent. Inhibition experiments with cyclosporin A were terminated by the addition of one equivalent (v/v) of ice-cold acetonitrile containing known quantities of the deuterated analogs of Ro 48-5033 and Ro 47-8634, storage on ice for 30 min, and centrifugation at 10,000 rpm for 10 min at 4°C. Supernatants were directly subjected to LC-MS/MS analysis. Quantification was performed against a calibration curve using samples with the analytes spiked into a deactivated incubation matrix. In the experiments with the deuterated metabolites as inhibitors, formation of bosentan metabolites was quantified using external calibration.

Results

The results of the pharmacokinetic experiments with bosentan in rats either alone or in combination with cyclosporin A or verapamil are summarized in Table 1 and the plasma concentration-time curves are depicted in Fig. 1. After oral administration, mean peak plasma concentrations and systemic exposure in terms of AUC₀-inf. reached 744 ng/ml and 3228 ng · h/ml, respectively. When the experiment was repeated in the presence of cyclosporin A, Cₘₐₓ and AUC₀-inf. were 4.4- and 17-fold increased compared with bosentan alone. Plasma levels of bosentan declined to the quantification limit of the analytical method within 24 h postadministration when given alone but were still detectable 48 h after dosing in the presence of cyclosporin A. The increase in Cₘₐₓ was accompanied by a delay in Tₘₐₓ from 1.7 to 4.3 h, and F increased from 42 to 69%. When bosentan was administered together with the mdr1 inhibitor verapamil, the AUC₀-inf. was 38% higher compared with bosentan alone. Tₘₐₓ was reached after 2.5 h and oral bioavailability was similar with 36%.

Intravenous administration of bosentan resulted in an AUC₀-inf. of 765 ng · h/ml, which increased more than 10-fold in the presence of cyclosporin A. As with oral administration, bosentan was detectable in plasma 48 h after dosing when coadministered with cyclosporin A, whereas bosentan plasma concentrations were below the limit of quantification 24 h after dosing when given alone. CL was decreased from 23 ml/min · kg to 2.2 ml/min · kg in the presence of cyclosporin A. Vₐ decreased as well by a factor of about 2 from 2.5 to 1.1 l/kg in the presence of cyclosporin A. When bosentan was

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<th>Table 1</th>
<th>Single-dose pharmacokinetic parameters of bosentan in rats</th>
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**P < 0.01; ***P < 0.001.
given together with verapamil, AUC_{0-inf.} was 1275 ng·h/ml resulting in a CL of 13 ml/min·kg. V_{ss} was 1.4 l/kg, i.e., similar as in the coadministration experiment with cyclosporin A, whereas the apparent terminal half-life was close to that of bosentan alone.

Plasma concentrations of the two major bosentan metabolites Ro 48-5033 and Ro 47-8634 (see Fig. 6 for chemical structures) never exceeded 1 to 2% of parent bosentan. The normalized ratio between both metabolites in plasma ranged from 76:24 to 66:34 in favor of Ro 48-5033 and was virtually unaffected by the different routes of administration and coadministration of cyclosporin A or verapamil.

Pharmacokinetic coadministration experiments with bosentan and verapamil in the rat as well as experiments with bosentan in mdr-1a knockout mice were performed to investigate the potential involvement of mdr1-mediated drug efflux during gastrointestinal absorption. Bosentan was administered orally and intravenously to mdr-1a knockout mice and pharmacokinetic parameters were compared to those of wild-type (FVB) mice (Table 2).
The inhibition of bosentan metabolism by cyclosporin A or the bosentan metabolites Ro 48-5033 and Ro 47-8634 was investigated in vitro using rat liver microsomes. Deuterated analogs of both metabolites were used as inhibitors to allow for a determination of enzyme kinetic parameters. The upper limits of substrate and inhibitor concentrations were defined by the solubilities of the compounds under the experimental conditions. The Lineweaver-Burk plots of all inhibition experiments are depicted in Fig. 2. The respective apparent $K_i$ values were estimated by reploting the slopes of the reciprocal plots against the inhibitor concentrations.

Cyclosporin A showed only a weak inhibitory effect on the formation rates of both bosentan metabolites with apparent $K_i$ values for Ro 48-5033 and Ro 47-8634 of 130 and 228 $\mu$M, respectively. The effect of D$_4$-Ro 48-5033 was equally weak with apparent $K_i$ values above 200 $\mu$M for both metabolic pathways. Only D$_4$-Ro 47-8634 had a more pronounced effect on bosentan metabolism. Apparent $K_i$ values were 6.6 $\mu$M on the formation of Ro 48-5033, and 15 $\mu$M on Ro 47-8634 with competitive inhibition as the apparent mode of action.

The effect of cyclosporin A on the active uptake of bosentan into the liver was investigated using freshly prepared rat hepatocytes and radiolabeled bosentan at a single concentration of 1 $\mu$M, which closely resembled the plasma concentrations observed in the pharmacokinetic experiments ($C_{max} = 1.3$ $\mu$M). Figure 3 shows the effect of cyclosporin A at concentrations up to 100 $\mu$M on the active transport of bosentan. The apparent IC$_{50}$ value for the inhibition of bosentan uptake was determined as 0.16 $\mu$M. In addition, inhibition of bosentan uptake has been investigated in the presence of digoxin, quinidine, and CCK-8 as fairly specific inhibitors of the different oatp isoforms in the rat, at concentrations significantly exceeding the respective $K_i$ values of these inhibitors (Ismair et al., 2001; Shitara et al., 2002) (Fig. 4).

The metabolic profiles in rat bile, urine, and plasma were determined after oral administration of $^{14}$C-radiolabeled bosentan to bile duct-cannulated male Wistar rats. During a sampling period of 72 h, more than 98% of the bosentan dose was recovered in rat bile, whereas only about 1% was detected in urine. As depicted in Fig. 5, the major component detected in rat bile was metabolite Ro 48-5033, representing 62 to 64% of drug-related material, accompanied by 9 to 10% of Ro 47-8634 and 4 to 5% of the secondary metabolite Ro 64-1056. Unchanged bosentan accounted for only 6 to 8% of the radioactivity. The major components detected in rat urine were metabolites Ro 48-5033 and Ro 64-1056, whereas Ro 47-8634 and unchanged bosentan were not present in detectable amounts. The only component detected in rat plasma was unchanged bosentan. This was in agreement with the results of the plasma analysis using LC-MS/MS in which the contribution of metabolites Ro 48-5033 and Ro 47-8634 to the total plasma pattern did not exceed 2% and 1%, respectively.

### Discussion

Bosentan is an endothelin receptor antagonist with a sulfonamide substructure, which exists predominantly as a mono-anion under physiological conditions. In the rat, it is eliminated by hepatic P450-dependent metabolism to three phase I metabolites that subsequently undergo biliary excretion (Fig. 6). Hydroxylation at the t-butyl group yields metabolite Ro 48-5033, whereas Ro 47-8634 is formed by oxidative demethylation of the guajacol ether to the corresponding phenol. Metabolite Ro 64-1056 is formed as a minor product from both primary metabolites (Hopfgartner et al., 1996; Weber et al., 1999). Renal excretion of bosentan is negligible.

The marked pharmacokinetic interaction between bosentan and cyclosporin A observed in clinical trials was shown to be also present in the rat. Coadministration of both drugs led to a 17- and 4.4-fold increase in $AUC_{0\text{-}\infty}$ and $C_{max}$, respectively, after oral dosing of bosentan. The 10-fold increase in $AUC_{0\text{-}\infty}$ observed after intravenous dosing pointed to a systemic effect as the origin for the interaction. We have investigated several mechanisms that we hypothesized as potential causes for this interaction, i.e., 1) inhibition of mdr1-mediated efflux during gastrointestinal absorption, 2) inhibition of bosentan metabolism, 3) inhibition of hepatic uptake, and 4) inhibition of hepatobiliary excretion.

The effect of cyclosporin A on the absorption of coadministered drugs through inhibition of mdr1-mediated drug efflux is well described (Augustijns et al., 1993; Sikic et al., 1997). However, based on the coadministration experiments with bosentan and the known mdr-1 inhibitor verapamil (Sonneveld, 1999) in the rat together with the observation that bosentan pharmacokinetics were not significantly different in mdr-1a knockout and wild-type mice, a major role of the mdr1 efflux pump in the gastrointestinal absorption process of bosentan seems unlikely.

Inhibition of P450-dependent metabolism by cyclosporin A was investigated as another possibility. Cyclosporin A is a known inhibitor of human CYP3A4 activity (Jacobsen et al., 1999), and inhibition of bosentan metabolism might, as least in part, serve as an explanation for the decreased clearance in humans. Enzyme kinetic data in rat, however, indicated that cyclosporin A had only a weak inhibitory effect on the metabolism of bosentan in vitro with apparent $K_i$ values of 130 and 228 $\mu$M, respectively. Cyclosporin A concentrations in plasma under the experimental conditions were estimated as about 6 $\mu$M (Luke et al., 1990), and a significant increase

### Table 2

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<th>Intravenous</th>
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<tr>
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<td>$AUC_{0\text{-}\infty}$</td>
<td>$C_{max}$</td>
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<tr>
<td>$mdr$-1a knockout</td>
<td>ng · h/ml</td>
<td>ng/ml</td>
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<tr>
<td>Wild-type (FVB)</td>
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<td>432</td>
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<td></td>
<td>953</td>
<td>299</td>
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<tr>
<th></th>
<th>Intravenous</th>
<th>$AUC_{0\text{-}\infty}$</th>
<th>$CL$</th>
<th>$V_{ss}$</th>
<th>$T_{1/2}$</th>
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<tbody>
<tr>
<td>$mdr$-1a knockout</td>
<td>ng · h/ml</td>
<td>ml/min · kg</td>
<td>l/kg</td>
<td>h</td>
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<tr>
<td>Wild-type (FVB)</td>
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<tr>
<td></td>
<td>893</td>
<td>47</td>
<td>2.1</td>
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The inhibition of bosentan pharmacokinetics were not significantly different from both primary metabolites (Hopfgartner et al., 1996; Weber et al., 1999). Renal excretion of bosentan is negligible.
of bosentan plasma concentrations due to inhibition of metabolism thus seems unlikely.

Inhibition of hepatocellular uptake was studied in vitro as another potential mechanism underlying this interaction. Active transport of bosentan into rat hepatocytes at a concentration similar to those observed in the pharmacokinetic
Inhibition of Bosentan Hepatic Transport by Cyclosporin A

Inhibition of active bosentan uptake into rat hepatocytes by cyclosporin A. Uptake into hepatocytes was determined using \(^{14}\text{C}\)-radiolabeled bosentan at a concentration of 1 \(\mu\text{M}\), whereas cyclosporin A was used at concentrations up to 100 \(\mu\text{M}\). Active uptake rates were calculated as the difference of total radioactivity incorporated into hepatocytes at 37°C and 4°C divided by cell number and time. Data are means \(\pm\) S.D. from four to five independent experiments.

Fig. 3.

Inhibition of active bosentan uptake into freshly prepared rat hepatocytes by cyclosporin A. Uptake into hepatocytes was determined using \(^{14}\text{C}\)-radiolabeled bosentan at a concentration of 1 \(\mu\text{M}\), whereas cyclosporin A was used at concentrations up to 100 \(\mu\text{M}\). Active uptake rates were calculated as the difference of total radioactivity incorporated into hepatocytes at 37°C and 4°C divided by cell number and time. Data are means \(\pm\) S.D. from four to five independent experiments.

Experiments were demonstrated by the fact that uptake rates were 7 to 10 times higher at 37°C compared with 4°C. As depicted in Fig. 3, the uptake was inhibited by cyclosporin A in a concentration-dependent manner with an apparent IC\(_{50}\) value of 0.16 \(\mu\text{M}\). The plasma concentrations of cyclosporin A present in the pharmacokinetic experiments are therefore largely sufficient to significantly inhibit the active bosentan transport from blood into the liver. Bosentan is a sulfonamide derivative and exists predominantly as a mono-anion under physiological conditions. Organic anion transporters were therefore considered as the primary candidates responsible for the active uptake of bosentan. Digoxin, quinidine (Shitara et al., 2002), and CCK-8 (Ismair et al., 2001) were recently described as fairly selective inhibitors for the three major oatp isoforms, i.e., oatp1, oatp2, and oatp4, expressed on the sinusoidal membrane of rat hepatocytes. All three compounds inhibited the uptake of bosentan into hepatocytes (Fig. 4). Digoxin had the smallest effect with a 12% reduction of incorporated radioactivity indicating a minor contribution of oatp1 to the total uptake of bosentan under these experimental conditions. Quinidine and CCK-8 exhibited more pronounced effects with reductions of 38 and 39%, respectively. The combination of all three inhibitors, at concentrations equal to the individual experiments, led to a decrease of 76%, which was about as strong as the effect of cyclosporin A at a concentration of 100 \(\mu\text{M}\). Cyclosporin A has recently been described as an inhibitor of both oatp1- and oatp2-mediated transport in the rat (Shitara et al., 2002). Moreover, inhibition of different human OATP isoforms has been demonstrated as the, at least in part, underlying mechanism behind the interactions of cyclosporin A with the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors cerivastatin (Shitara et al., 2003) and atorvastatin (Lennernas, 2003) as well as with the bicyclic peptide phalloidin (Fehrenbach et al., 2003). The combination of all these indirect lines of evidence strongly suggests that inhibition of oatp-mediated active bosentan transport into the liver by cyclosporin A also represents the major mechanism in the present case. Finally, it should be noted that neither by the highest cyclosporin A concentration nor by the combination of all oatp inhibitors a complete suppression of active bosentan uptake could be achieved. This indicates that, apart from the possibility of incomplete inhibition of the oatp-mediated transport under the experimental conditions, there might be other transport proteins involved in the hepatocellular uptake of bosentan whose function is neither modulated by cyclosporin A nor by typical oatp inhibitors.

Finally, we followed the hypothesis that cyclosporin A might intervene into the hepatobiliary excretion of bosentan. Little is known so far about the processes governing its biliary secretion. In the rat, bosentan and Ro 47-8634 were shown to interfere with the bile salt export pump-mediated excretion of bile salts (Fattinger et al., 2001). Moreover, the stimulation of multidrug resistance protein 2-modulated excretion of bilirubin was reported after intravenous dosing (Fouassier et al., 2002). Cyclosporin A is a known inhibitor of both transporters (Morrow et al., 2000; Stieger et al., 2000; Bramow et al., 2001). Because, however, bosentan is excreted into bile only after extensive metabolism, a direct effect of cyclosporin A on the pharmacokinetics of parent bosentan was not expected. We explored the possibility that inhibition of biliary excretion of the bosentan metabolites by cyclosporin A might result in their accumulation in hepatocytes eventually leading to an inhibition of bosentan metabolism by its own biotransformation products. The major metabolite Ro 48-5033 had virtually no inhibitory effect on the two metabolic pathways of bosentan (Fig. 2). Ro 47-8634, however, inhibited both processes with apparent \(K_i\) values of 6.6 and 15 \(\mu\text{M}\), respectively. To estimate the magnitude of inhibition by Ro 47-8634, its free concentration in hepatocytes was estimated using the following assumptions: 1) an 100-fold accumulation of total radioactivity in liver as deduced from quantitative tissue distribution data (Actelion Pharmaceuticals Ltd., on file); 2) a free fraction of Ro 47-8634 in liver cells equal to that in plasma, i.e., \(f_u = 0.006\); and 3) a product ratio
of bosentan and metabolites somewhere in between the composition observed in plasma or bile (Fig. 5). Based on these assumptions, the range of free concentrations in the hepatocytes was calculated between 0.006 μM (from plasma data) and 0.08 μM (from bile data). As a result, no relevant inhibition of bosentan metabolism by its metabolite Ro 47-8634 was expected.

In conclusion, we have shown that the interaction observed between bosentan and cyclosporin A in clinical trials is also present in the rat. Among several possible mechanisms investigated as potential causes for this interaction, inhibition of active uptake of bosentan from blood into the liver seemed to constitute the major determinant in this interaction. In vitro, cyclosporin A was able to efficiently block the active transport of radiolabeled bosentan into liver cells in a concentration-dependent manner. As indicated by the use of
selective inhibitors, bosentan uptake was to a large extent mediated by three members of the oatp transporter family, i.e., oatp1, oatp2, and oatp4. Significant contributions of alternative mechanisms as inhibition of mdr1-mediated drug efflux during gastrointestinal absorption, inhibition of bosentan metabolism in the liver, or inhibition of hepatobiliary excretion seemed to be unlikely. The interaction between bosentan and cyclosporin A represents one of the few examples in which a significant drug-drug interaction can, at least to a large extent, be attributed to the inhibition of liver transport processes, which is in contrast to many literature reports (Spoendlin et al., 1998; Mullins et al., 1998) in which inhibition of drug metabolism has been demonstrated as the major mechanism of action. It also demonstrates that inhibition of liver uptake might become the rate-limiting step in the overall elimination process even for compounds whose clearance is mainly dependent on metabolism. The relevance of these findings in the rat for the clinical situation still remains to be explored. It is, however, clear that inhibition of CYP3A4-mediated metabolism by cyclosporin A is by far insufficient to explain the observed increases in bosentan plasma concentrations and that inhibition of hepatocellular uptake offers an attractive mechanistic alternative.

Acknowledgments

We thank Prof. Y. Sugiyama (University of Tokyo, Tokyo, Japan) for fruitful scientific discussions. We also thank A. Chougnet (Department of Chemistry, University of Basel, Basel, Switzerland) and P. Coassolo (F. Hoffmann-La Roche, Basel, Switzerland) for experimental contribution and P. Hess (Actelion Pharmaceuticals Ltd.) for the statistical analysis.

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