Inhibition of Oatp-Mediated Hepatic Uptake is The Major Determinant in the Pharmacokinetic Interaction between Bosentan and Cyclosporin A in the Rat

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Running Title:

Inhibition of Bosentan Hepatic Transport by Cyclosporin A

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ABBREVIATIONS:

AUC, area under the plasma concentration-time curve; AUMC, area under the first moment-time curve; b.i.d., bis in diem (twice daily); bsep, bile salt export pump; CL, systemic plasma clearance; C_{max}, peak plasma concentration; CCK-8, cholecystokinin; CYP, cytochrome P450; D, dose; EDTA, ethylenediamine tetra-acetate (sodium salt); F, absolute bioavailability; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; IC_{50}, half-inhibition constant; i.p., intraperitoneal; i.v., intravenous; Ki, inhibition

constant; LC-MS/MS, triple stage mass spectrometry coupled to liquid chromatography; λ_z, terminal elimination rate constant; mdr1, multi-drug resistance protein 1; mrp2, multi-drug resistance-associated protein 2; NaF, sodium fluoride; NCS, newborn calf serum; oatp/OATP, organic anion transporting polypeptide (in rat and human); QWBA, quantitative whole-body autoradiography; $T_{1/2}$, half-life; T_{max}, time to reach C_{max}; V_{ss}, volume of distribution at steady state.

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 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 which 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 appeared 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 man.

Bosentan (Tracleer[TM]) 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.) with the immunosuppressant cyclosporin A (300 mg b.i.d.) has been investigated in healthy male volunteers. After the first day of concomitant dosing, a 30-fold increase of bosentan trough plasma concentrations was observed when compared to volunteers receiving bosentan alone. In humans, bosentan is extensively metabolized by the cytochrome P450 (CYP) isoforms 2C9 and 3A4 prior to 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 paper, 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 while 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 appear to play only a

minor role.

Material and Methods

Materials

Bosentan, sodium salt (lot no. 71102B1758) used for intravenous dosing and in vitro experiments, bosentan, micronized free sulfonamide (lot no. 704004) for oral pharmacokinetic experiments, and ^{14}C-radiolabeled bosentan, free sulfonamide (specific activity: 40.9 µCi/mg, lot. no. 12352B65) were obtained from Actelion-internal sources. Cyclosporin A, digoxin, quinidine, cholecystokinin (CCK-8), and Triton X-100 were purchased from Fluka (Buchs, Switzerland), while verapamil hydrochloride was from Aldrich (Steinheim, Germany). Leibowitz L-15 medium, William's E medium, FBS, NCS, and HBSS were obtained from Invitrogen Ltd. (Basle, Switzerland). 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. Male Wistar rats used for pharmacokinetic experiments and tissue preparations were from RCC Biotechnology and Breeding Division (Füllinsdorf, Switzerland). FVB wild-type and *mdr-1a* knock-out mice were obtained from Taconic Farms Inc. (Germantown, NY) and imported via Bomholtgard Breeding and Research Center (Ry, Denmark).

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 250-300 g ($n = 5$-6). Two days prior to the experiments, animals used for oral administration had a catheter implanted into the left carotid artery under anesthesia with Brietal^{TM} (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 prior to the administration of bosentan. Animals receiving bosentan alone were injected an equivalent volume of vehicle. Verapamil was co-formulated 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 pre-defined timepoints 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 $^{\circ}$C. Samples were stored at -20 $^{\circ}$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 no. 169, Kantonales Veterinäramt Liestal, Switzerland).

Pharmacokinetic Experiments in Wild-Type and *Mdr-1a* Knock-Out Mice

Experiments in wild-type (FVB) and *mdr-1a* knock-out mice were performed at intravenous and oral doses of 2.5 and 5.0 mg/kg, respectively, with 13-15 animals per experiment. For intravenous dosing, bosentan (sodium salt) was dissolved in

physiological saline and administered either via the tail vein. For oral dosing, bosentan (free sulfonamide) was formulated as a microsuspension in 7.5% modified gelatin and administered by gavage. Blood samples of about 1 mL volume (n = 2-3 per timepoint) were collected by heart puncture under anesthesia at pre-defined timepoints over a period of 6 and 7 h after intravenous and oral dosing, respectively. Blood samples were collected in Eppendorf tubes containing EDTA and NaF. Plasma was generated by centrifugation at 4000 rpm for 10 min at 4 ^{o}C and stored at -20 ^{o}C pending analysis.

Bioanalytical Method for Bosentan and Metabolites

The analysis of rat plasma samples and samples from in vitro experiments was done with LC-MS/MS using a modified method published earlier (Lausecker et al., 2000). Plasma and microsomal proteins were precipitated with ice-cold acetonitrile containing known quantities of the tetra-deuterated analogues of bosentan and its metabolites, Ro 48-5033 and Ro 47-8634, as internal standards, vortexing for 20 min, and centrifugation at 4000 rpm for 10 min at 4 ^{o}C. Analytes were quantified using a Sciex API 2000 mass spectrometer (PE Applied Biosystems, Foster City, CA, USA) operated in positive ion mode using the following selective transitions: 552 to 202 (bosentan), 568 to 202 (Ro 48-5033), and 538 to 189 (Ro 47-8634). The limit of quantification for bosentan and its metabolites from rat plasma was 1.0 ng/mL, and 5.0 ng/mL for samples from in vitro experiments.

Pharmacokinetic Analysis

Pharmacokinetic parameters were estimated using the WinNonLin software package (Professional version 3.1, Pharsight Corporation, Mountain View, CA, USA). Total plasma clearance (CL) was calculated as $D/AUC_{0\text{-inf.}}$, with D equals dose and $AUC_{0\text{-inf.}}$ equals the area under the plasma concentration-time curve calculated by the linear/logarithmic trapezoidal rule and extrapolated to infinity using the apparent terminal rate constant λ_z. The apparent terminal half-life ($T_{1/2}$) was estimated from the terminal rate constant λ_z with $T_{1/2} = \ln2/\lambda_z$ (λ_z was determined from the linear terminal portion of the log plasma concentration-time curve). The volume of distribution at steady state (V_{ss}) was calculated as $V_{ss} = MRT \times CL$ with the mean residence time (MRT) being defined as the area under the first moment curve $AUMC_{0\text{-inf.}}$ divided by $AUC_{0\text{-inf.}}$. Peak plasma concentrations (C_{max}) and the time to reach peak plasma concentrations (T_{max}) were directly taken from the respective plasma concentration-time curves. Oral bioavailabilities (F) were calculated by the ratios of dose-normalized $AUC_{0\text{-inf.}}$ after oral and intravenous dosing. All parameters are given as mean $\pm$ SEM. Statistical analysis was performed by analysis of variance (ANOVA) using the Statistica software package (Statsoft) 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 260-290 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 of sodium dihydrogenphosphate. After the operation, animals were allowed to recover for 2 days with free access to food and water. ^{14}C-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 µCi/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 HPLC 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 Spectraflow 773 UV detector (Kratos Analytical, Manchester, UK) set at a wavelength of 260 nm, and a LB506 C-1 radioactivity monitor (Berthold Technologies, Regensdorf, 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 35-70% of mobile phase B within 35 min on a Superspher 100 column (RP-18 endcapped, 250×4 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 300-320 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 90-95%. 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, 2001). Immediately before the start of the uptake experiment, the cell monolayer was washed three times with pre-warmed (37°C) or ice-cold (4°C) uptake buffer containing 116 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2HPO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM D-glucose, and 20 mM HEPES. Linearity of ^{14}C-bosentan uptake with time and cell concentration was checked beforehand. The different inhibitors, dissolved in DMSO, were added to the uptake medium about one minute prior to the addition of ^{14}C-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 lysate 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 ± SD from 4-5 independent determinations. Statistical analysis was performed by analysis of variance (ANOVA) 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 auto-inhibition of bosentan metabolism by its metabolites were performed using the tetra-deuterated analogues as inhibitors. Experiments with D_4-Ro 47-8634 were performed at inhibitor concentrations of 10, 30, and 100 µM, while D_4-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 DMSO, 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 analogues 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

INSERT TABLE 1

INSERT FIGURE 1

concentration-time curves are depicted in Figure 1. After oral administration, mean peak plasma concentrations and systemic exposure in terms of $AUC_{0-inf.}$ reached 744 ng/mL and 3228 ng*h/mL, respectively. When the experiment was repeated in the presence of cyclosporin A, C_{max} and $AUC_{0-inf.}$ were 4.4- and 17-fold increased compared to bosentan alone. Plasma levels of bosentan declined to the quantification limit of the analytical method within 24 h post-administration when given alone but were still detectable 48 h after dosing in the presence of cyclosporin A. The increase in C_{max} was accompanied by a delay in T_{max} 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 $AUC_{0-inf.}$ was 38% higher compared to bosentan alone. T_{max} was reached after 2.5 h and oral bioavailability was similar with 36%.

Intravenous administration of bosentan resulted in an $AUC_{0-inf.}$ 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 co-administered with cyclosporin A, while 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. V_{ss} decreased as well by a factor of about 2 to from 2.5 L/kg to 1.1 L/kg in the presence of cyclosporin A. When bosentan was 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 co-administration experiment with cyclosporin A while 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 Figure 6 for chemical structures) never exceeded 1-2% 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 co-administration of cyclosporin A or verapamil.

Pharmacokinetic co-administration experiments with bosentan and verapamil in the rat as well as experiments with bosentan in *mdr-1a* knock-out mice were performed in order to investigate the potential involvement of mdr1-mediated drug efflux during gastrointestinal absorption. Bosentan was administered orally and intravenously to *mdr-1a* knock-out mice and pharmacokinetic parameters were compared to those of wild-type (FVB) mice (Table 2).

INSERT 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 analogues 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
Figure 2. The respective apparent K_i values were estimated by re-plotting the slopes of
the reciprocal plots against the inhibitor concentrations.

INSERT FIGURE 2!

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 μM, respectively. The effect of D_4-Ro 48-5033 was equally weak with apparent K_i
values above 200 μM 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 μM on the
formation of Ro 48-5033, and 15 μM 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 μM which closely resembled the plasma concentrations observed in

the pharmacokinetic experiments (C_{max} = 1.3 μM). Figure 3 shows the effect of

INSERT FIGURE 3!

cyclosporin A at concentrations up to 100 μM on the active transport of bosentan. The

apparent IC_{50} value for the inhibition of bosentan uptake was determined as 0.16 μM. In

addition, inhibition of bosentan uptake has been investigated in the presence of digoxin,

quinidine and CCK-8 as fairly specific inhibitors of 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) (Figure 4).

INSERT FIGURE 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 while only about 1% was detected in urine. As depicted in Figure 5, the major

INSERT FIGURE 5!

component detected in rat bile was metabolite Ro 48-5033, representing 62-64% of drug-

related material, accompanied by 9-10% of Ro 47-8634 and 4-5% of the secondary

metabolite Ro 64-1056. Unchanged bosentan accounted for only 6-8% of the radioactivity. The major components detected in rat urine were metabolites Ro 48-5033 and Ro 64-1056 while 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, CYP-dependent metabolism to three phase I metabolites that subsequently undergo biliary excretion (Figure 6). Hydroxylation at the t-butyl group

INSERT FIGURE 6!

yields metabolite Ro 48-5033, while 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. Co-administration of both drugs led to a 17- and 4.4-fold increase in $AUC_{0\text{-inf.}}$ and C_{max}, respectively, after oral dosing of bosentan. The 10-fold increase in $AUC_{0\text{-inf.}}$ 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. a) inhibition of mdr1-mediated efflux during gastrointestinal absorption; b) inhibition of bosentan metabolism; c) inhibition of hepatic uptake; d) inhibition of hepatobiliary excretion.

The effect of cyclosporin A on the absorption of co-administered drugs through inhibition of mdr1-mediated drug efflux is well described (Augustijns et al., 1993; Sikic et al., 1997). However, based on the co-administration 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 knock-out and wild-type mice, a major role of the mdr1 efflux pump in the gastrointestinal absorption process of bosentan appeared unlikely.

Inhibition of CYP-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 µM, respectively. Cyclosporin A concentrations in plasma under the experimental conditions were estimated as about 6 µM (Luke et al., 1990) and a significant increase of bosentan plasma concentrations due to inhibition of metabolism thus appears 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 in the pharmacokinetic experiments was demonstrated by the fact that uptake rates were 7-10 times higher at 37 ^{o}C compared to 4 ^{o}C. As depicted in Figure 3, the uptake was inhibited by cyclosporin A in a concentration-dependent

manner with an apparent IC_{50} value of 0.16 µM. The plasma concentrations of

cyclosporin A present in the pharmacokinetic experiments are therefore largely sufficient

to significantly inhibit the active bosentan transport of from blood into the liver. Bosentan

is a sulfonamide derivative and exists predominantly as a mono-anion under

physiological conditions. Organic anion transporters (oatp) were therefore considered as

the primary candidates responsible for the active uptake of bosentan. Digoxin, quinidine

(Shitara et al., 2002) and cholecystokinin (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 (Figure 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 µM. 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 HMG-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 bsep-mediated excretion of bile salts (Fattinger et al., 2001). Moreover, the stimulation of mrp2-modulated excretion of bilirubin was reported after intravenous dosing (Fouassier et al., 2002). Cyclosporin A is a known inhibitor of both transporters (Stieger et al., 2000; Bramow et al., 2001; Morrow et al., 2000). Since, 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 (Figure 2). Ro 47-8634, however, inhibited both processes with apparent K_i values of 6.6 and 15 μM, respectively. In order 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 (QWBA) data (Actelion Pharmaceuticals, 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 (Figure 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 appeared to constitute the major determinant in this interaction. Cyclosporin A was able to efficiently block in vitro 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 appeared 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.

Acknowledgements

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Footnotes

Legends for Figures

Fig. 1. Plasma concentration-time profiles in rats of bosentan alone and in combination with cyclosporin A or verapamil. Rats (n = 5-6) received bosentan at oral and i.v. doses of 1.0 and 10 mg/kg, respectively. Cyclosporin A was given i.p. at a dose of 50 mg/kg, 30 min prior to bosentan. Verapamil was co-administered with bosentan at intravenous and oral doses of 1 and 10 mg/kg, respectively.

Fig. 2. Lineweaver-Burk plots of the inhibition of bosentan metabolite formation by cyclosporin A or the deuterated bosentan metabolites, D$_4$-Ro 48-5033 and D$_4$-Ro 47-8634. Bosentan as a substrate was used in a concentration range up to 300 µM. Final concentrations of cyclosporin A as inhibitor were 30 and 100 µM, while 20, 60 and 200 µM of D$_4$-Ro 48-5033, and 10, 30 and 100 µM of D$_4$-Ro 47-8634 were used, respectively. Incubations without inhibitors were performed with an equivalent volume of organic solvent. Rates of metabolite formation versus bosentan concentrations were plotted in a doubly reciprocal manner.

Fig. 3. Inhibition of active bosentan uptake into freshly prepared rat hepatocytes by cyclosporin A. Uptake into hepatocytes was determined using ^{14}C-radiolabeled bosentan at a concentration of 1 μM while cyclosporin A was used at concentrations up to 100 μM. Active uptake rates were calculated as the difference of total radioactivity incorporated into hepatocytes at 37 $^{\circ}$C and 4 $^{\circ}$C divided by cell number and time. Data are means ± SD from 4-5 independent experiments.

Fig. 4. Inhibition of bosentan uptake into rat hepatocytes by various specific inhibitors of rat oatp transporters. Active uptake was determined using ^{14}C-radiolabeled bosentan at a concentration of 1 µM and rates were calculated as the differences of total radioactivity incorporated into hepatocytes at 37 ^{o}C and 4 ^{o}C divided by cell number of and time. Data are means ± SD from 4-5 independent experiments (** $p < 0.01$; *** $p < 0.001$).

Fig. 5. In vivo metabolic profiles of bosentan in rat bile, urine and plasma. Rats received ^{14}C-radiolabeled bosentan at a total dose of 5.0 mg/kg containing 17.7 µCi/kg of the radiolabel. Bile, urine and plasma were collected over a time period of 72 h with replacement of the collected volume of bile with artificial bile via a catheter implanted into the common bile duct.

Fig.6. The metabolism of bosentan in the rat

Tables

TABLE 1

Single-dose pharmacokinetic parameters of bosentan in rats

Oral	$AUC_{0-inf.}$	C_{max}	T_{max}	F
	(ng*h/mL)	(ng/mL)	(h)	(%)
Bosentan alone	3228 ± 693	744 ± 168	1.7 ± 0.5	42 ± 8
+ cyclosporin A	53500 ± 12100 **	3310 ± 523 ***	4.3 ± 0.8	69 ± 15
+ verapamil	4453 ± 292	541 ± 63	2.5 ± 0.3	36 ± 2

| i.v.	$AUC_{0-inf.}$	CL	V_{ss}	$T_{1/2}$
	(ng*h/mL)	(mL/min*kg)	(L/kg)	(h)
Bosentan alone	765 ± 93	23 ± 3	2.5 ± 0.5	1.9 ± 0.1
+ cyclosporin A	7762 ± 1093 ***	2.2 ± 0.3 **	1.1 ± 0.2	7.2 ± 0.4 ***
+ verapamil	1275 ± 62	13 ± 1	1.4 ± 0.3	1.7 ± 0.1

** $p < 0.01$; *** $p < 0.001$

TABLE 2

Single-dose pharmacokinetic parameters of bosentan in *mdr-1a* knock-out and wild-type

(FVB) mice

Oral	$AUC_{0-inf.}$ (ng*h/mL)	C_{max} (ng/mL)	T_{max} (h)	F (%)
mdr-1a knock-out	1190	432	1.0	74
wild-type (FVB)	953	299	2.0	53
i.v.	$AUC_{0-inf.}$ (ng*h/mL)	CL (mL/min*kg)	V_{ss} (L/kg)	$T_{1/2}$ (h)
mdr-1a knock-out	807	53	2.5	0.8
wild-type (FVB)	893	47	2.1	0.8
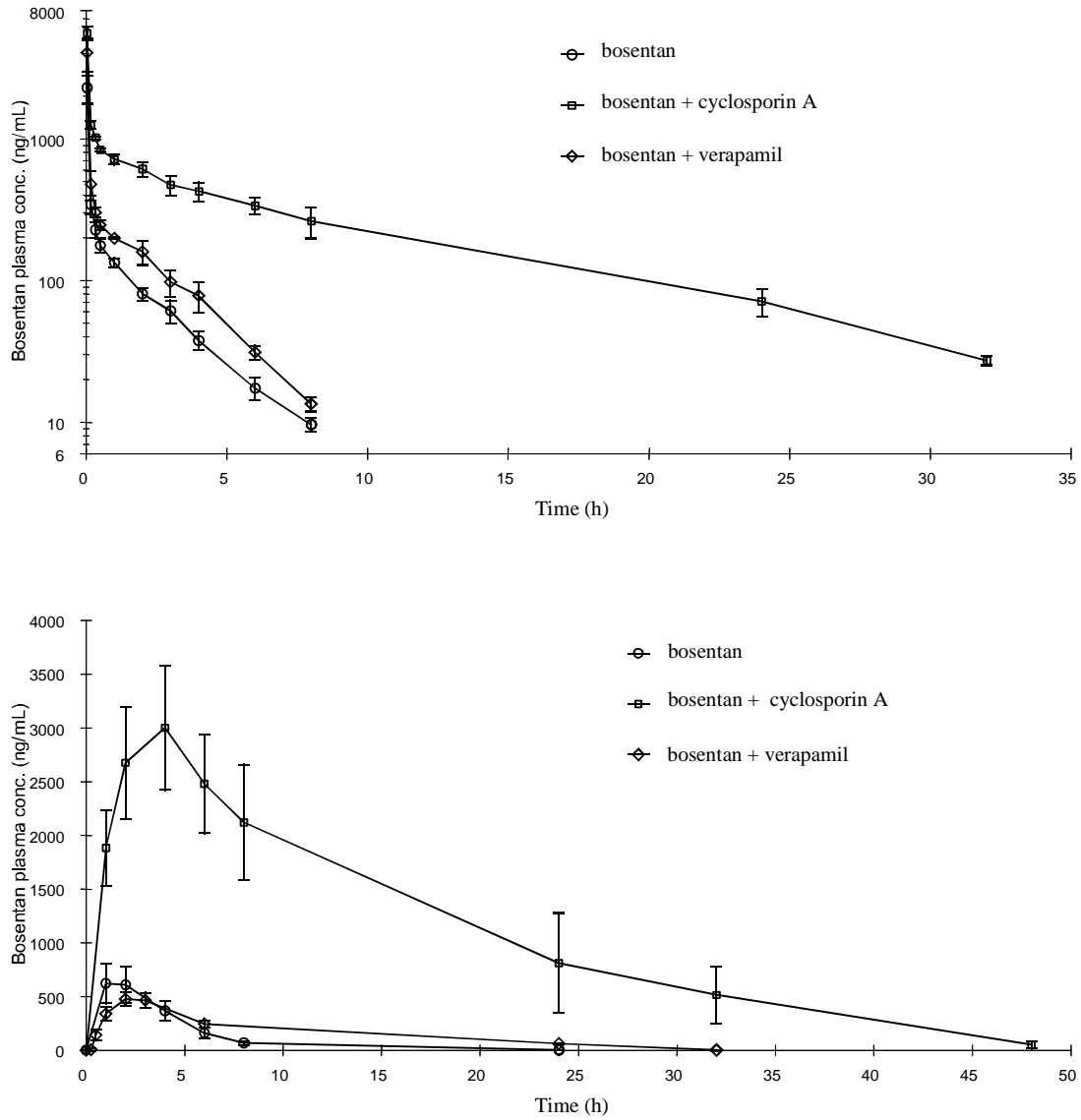
Figures

Figure 1

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Figure 2

Figure 3

IC50 = 0.16 ± 0.05 uM
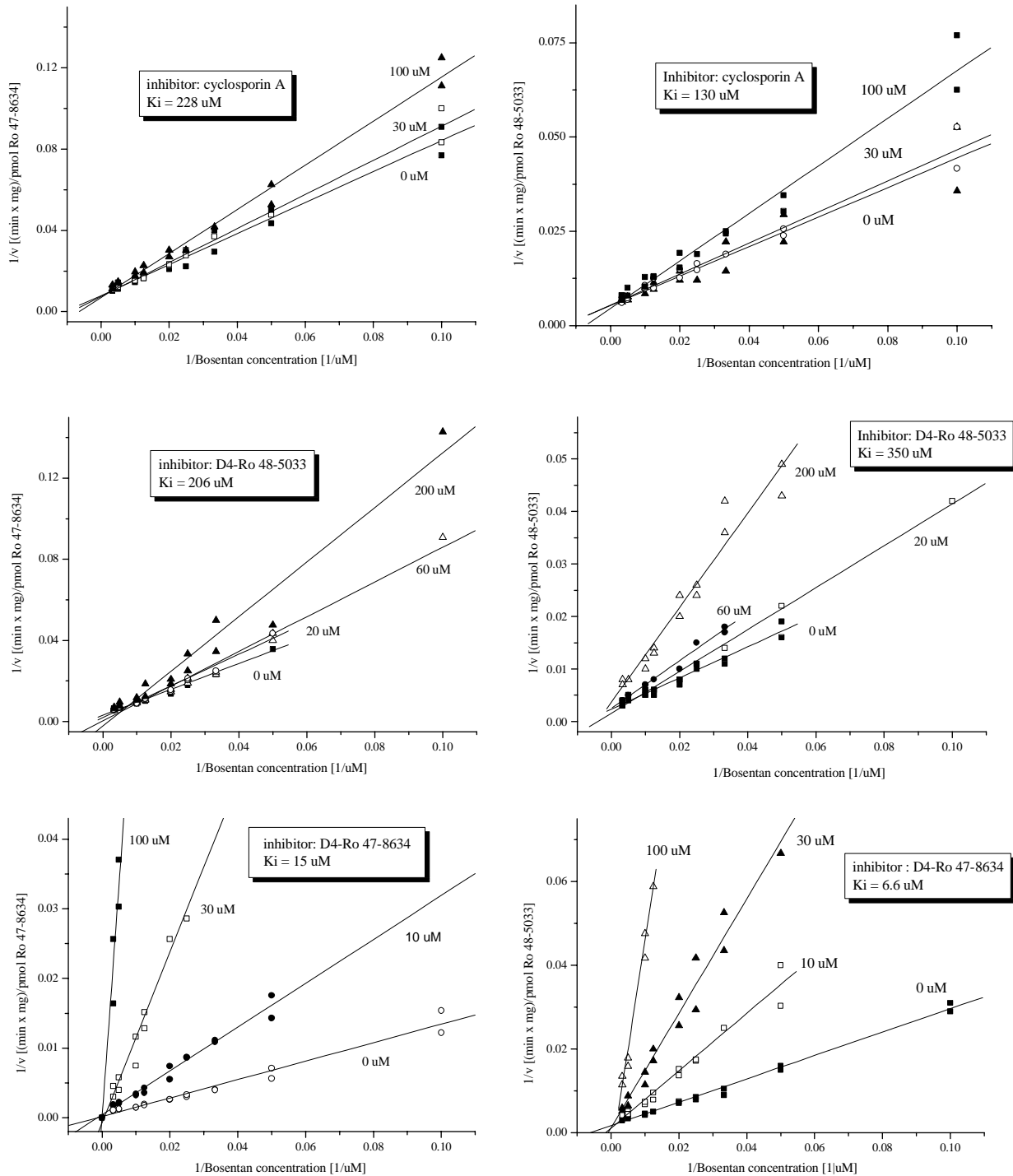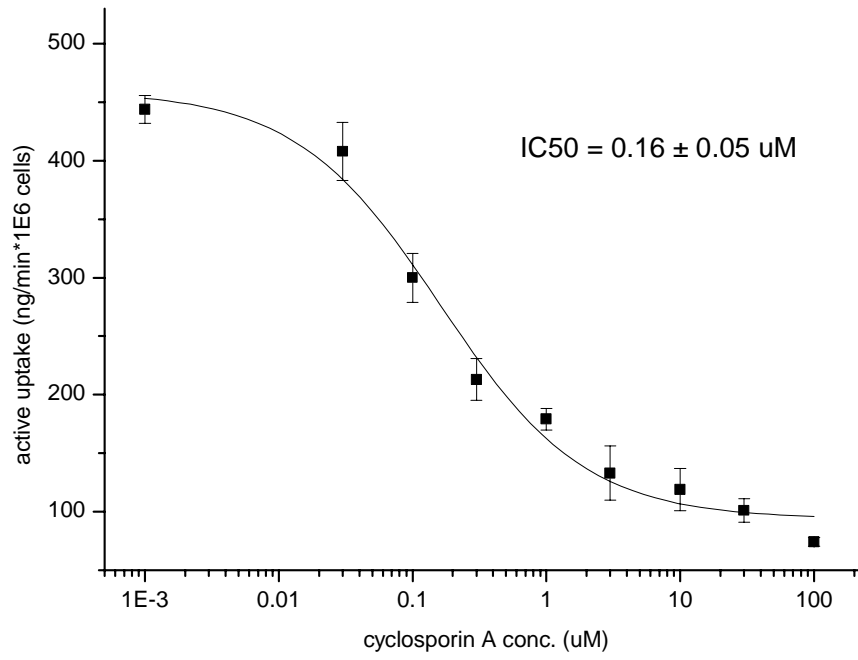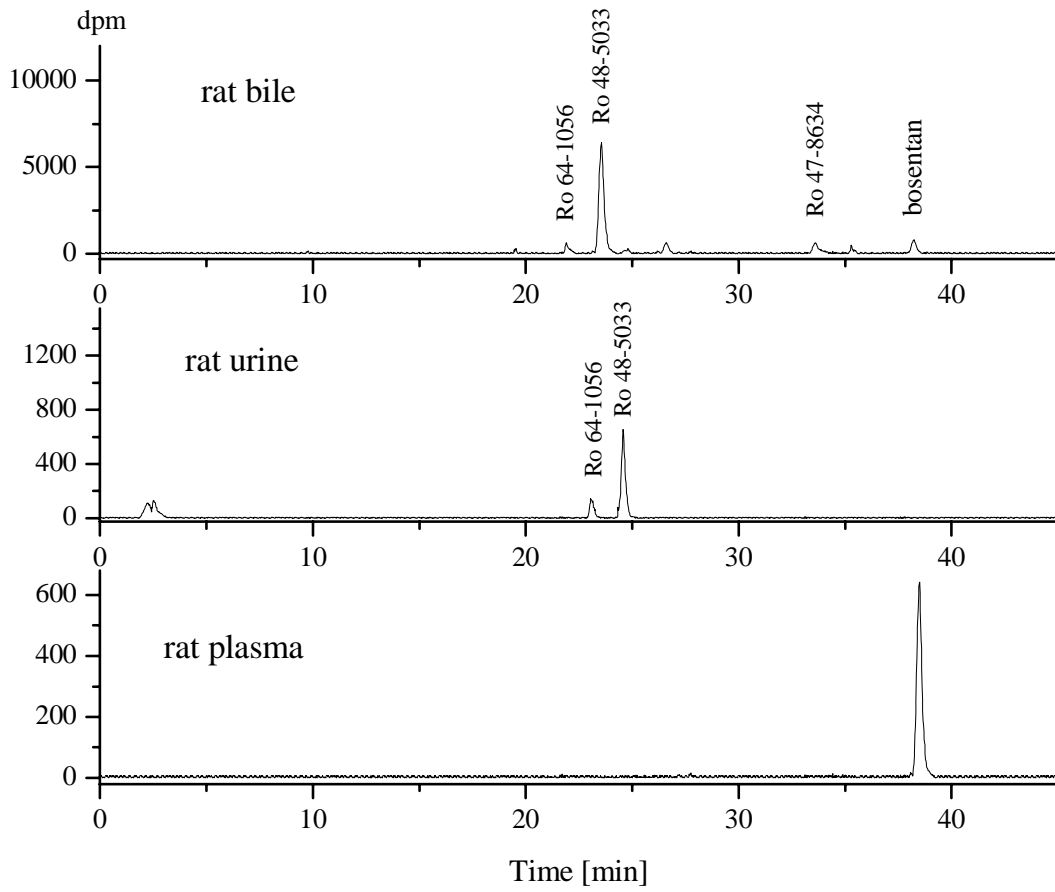
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Figure 4
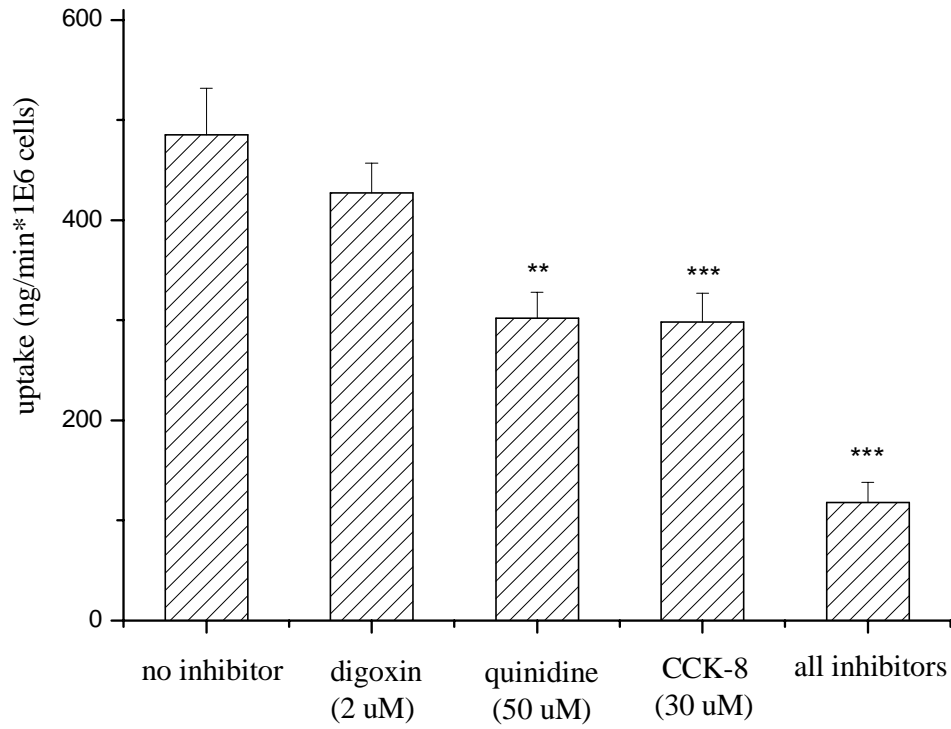
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Figure 5

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Figure 6