Differential Inhibition of Rat and Human Na\(^{+}\)-Dependent Taurocholate Cotransporting Polypeptide (NTCP/SLC10A1) by Bosentan: A Mechanism for Species Differences in Hepatotoxicity


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ABSTRACT

Bile acid accumulation in hepatocytes due to inhibition of the canalicular bile salt export pump (BSEP/ABC8B11) has been proposed as a mechanism for bosentan-induced hepatotoxicity. The observation that bosentan does not induce hepatotoxicity in rats, although bosentan has been reported to inhibit rat Bsep and cause elevated serum bile acids, challenges this mechanism. The lack of hepatotoxicity could be explained if bosentan inhibited hepatocyte uptake as well as canalicular efflux of bile acids. In the current study, bosentan was found to be a more potent inhibitor of Na\(^{+}\)-dependent taurocholate uptake in rat (IC\(_{50}\) 5.4 \(\mu\)M) than human (IC\(_{50}\) 30 \(\mu\)M) suspended hepatocytes. In addition, bosentan was a more potent inhibitor of taurocholate uptake by rat Na\(^{+}\)-dependent taurocholate cotransporting polypeptide (Ntcp/Slc10a1) (IC\(_{50}\) 0.71 \(\mu\)M) than human NTCP (SLC10A1) (IC\(_{50}\) 24 \(\mu\)M) expressed in HEK293 cells. Thus, bosentan is a more potent inhibitor of Ntcp than NTCP, and this should result in less intrahepatocyte accumulation of bile acids in rats during bosentan treatment. To begin characterization of this species difference, two chimeric molecules were generated and expressed in HEK293 cells; Ntcp\(_{1–140}$/NTCP\(_{141–362}\) and Ntcp\(_{1–140}$/NTCP\(_{141–349}\). The mode of bosentan inhibition was noncompetitive for Ntcp, and competitive for NTCP (K\(_{i}\) 18 \(\mu\)M) and NTCP\(_{1–140}$/NTCP\(_{141–362}\) (K\(_{i}\) 1.7 \(\mu\)M); bosentan affected both the K\(_{m}\) and V\(_{max}\) of Ntcp\(_{1–140}$/NTCP\(_{141–349}\) (K\(_{i}\) 7.0 \(\mu\)M). The carboxyl portions of NTCP and Ntcp were found to confer species differences in basal taurocholate transport V\(_{max}\). In conclusion, differential inhibition of Ntcp and NTCP may represent a novel mechanism for species differences in bosentan-induced hepatotoxicity.

Bosentan, a dual antagonist of endothelin (ET)\(_{A}\) and ET\(_{B}\) receptors, was the first ET receptor antagonist approved for the management of pulmonary arterial hypertension, a rare, debilitating, and fatal lung disease (Cohen et al., 2004). In the preapproval clinical trials, ~11% of patients treated with bosentan developed hepatotoxicity, as reflected by elevations in serum transaminases exceeding three times the upper limits of normal (Cohen et al., 2004; Williams et al., 2006). In a few patients with multiple comorbidities and multiple drug therapies, cases of severe hepatotoxicity were reported, in which the contribution of bosentan could not be excluded (Sitbon et al., 2005). As a result, it is recommended that all bosentan-treated patients undergo frequent biochemical monitoring of liver enzymes. Moreover, the potential for hepatotoxicity needs to be taken into consideration in patients with predisposing factors, such as liver fibrosis. Nevertheless, with monitoring, the risk/benefit ratio remains clearly
favorable for bosentan, since first-line bosentan treatment is associated with increased survival of patients with pulmonary arterial hypertension (Sitbon et al., 2005; Williams et al., 2006). Overall, there has been considerable interest in determining the mechanisms that underlie bosentan-induced hepatotoxicity.

Although most postulated mechanisms for drug-induced liver injury involve production of reactive metabolites (Park et al., 2005), it has been proposed that bosentan-induced liver injury is due to accumulation of bile acids in the hepatocyte resulting from inhibition of the canalicular bile salt export pump (BSEP/ABCB11) (Fattinger et al., 2001). This mechanism is consistent with the observation that human BSEP is inhibited by bosentan in vitro, that the incidence of liver injury is related to dose, and that mild liver injury frequently reverses with a reduction in daily dosing (Noé et al., 2002; Segal et al., 2005; Mano et al., 2007). Inhibition of BSEP as a mechanism for hepatotoxicity also is consistent with the reported observations that serum bile acids were increased before development of hepatotoxicity in patients in the bosentan clinical trials (Fattinger et al., 2001) and that bosentan hepatotoxicity was more common in patients also treated with glyburide, a known BSEP inhibitor (Stieger et al., 2000; Fattinger et al., 2001).

Bosentan administered intravenously to rats increased serum bile acids presumably through inhibition of Bsep which has been observed in vitro (Fattinger et al., 2001), suggesting that the rat might be a good model to study bosentan hepatotoxicity. However, bosentan-treated rats do not develop hepatotoxicity (Fattinger et al., 2001). This finding suggests that either BSEP inhibition is not a mechanism underlying bosentan hepatotoxicity or that factors mitigate the impact of BSEP inhibition in the rat but not in human. Fattinger et al. (2001) proposed that rats may be less susceptible to bosentan-induced hepatotoxicity because rat bile acids are inherently less toxic than human bile acids. Indeed, rat bile acid composition is more hydrophilic and therefore, generally less toxic than the human bile acid pool to both hepatocytes and erythrocytes (Coleman et al., 1979); however, taurocholic acid and taurochenodeoxycholic acid, which make up ~81% of the rat bile acid pool (Alvaro et al., 1986), are capable of eliciting hepatotoxicity (Crocezîni et al., 1997; Sokol et al., 1998). Thus, another potential explanation was provided by the recent observation that bosentan inhibits Na⁺-dependent taurocholate uptake in rat hepatocytes (Kemp et al., 2005). The Na⁺-dependent taurocholate cotransporting polypeptide (Ntcp/Scl10a1) is the only Na⁺-dependent bile acid transporter in hepatocytes and is responsible for the majority of bile acid uptake from sinusoidal blood (Geyer et al., 2006). Bosentan inhibition of Ntcp-mediated bile acid uptake could counterbalance Bsep inhibition, reduce intrahepatocyte bile acid accumulation, and thereby prevent hepatotoxicity in the rat. Preferential inhibition by bosentan of rat Ntcp versus human NTCP would therefore provide a potential explanation for the rat resistance to bosentan-induced hepatotoxicity.

Species differences in inhibition of Ntcp/NTCP by bosentan is plausible because disparities between rat and human Ntcp/NTCP substrate specificities have been observed, including a difference in affinity for taurocholate uptake by Ntcp (Kₘ 25 μM) and NTCP (Kₘ 6 μM) (Hagenbuch et al., 1991; Hagenbuch and Meier, 1994). Moreover, Ho et al. (2006) recently reported that rosuvastatin is a substrate for NTCP but not for Ntcp.

In the current study, the hypothesis that bosentan is a more potent inhibitor of rat Ntcp than human NTCP was tested using suspended hepatocytes and expressed recombinant native and chimeric transporters. Our data support this hypothesis and suggest a novel mechanism for species differences in hepatotoxicity.

**Materials and Methods**

**Chemicals and Reagents.** [¹H]Taurocholate (1.19 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [³⁵S]Insulin (37 MBq/g) was purchased from American Radiolabeled Chemicals (St. Louis, MO). ITS⁺ (insulin, transferrin, and selenium), Biocoat culture plates, and Matrigel were purchased from BD Biosciences (Bedford, MA). Dulbecco’s modified Eagle’s medium (DMEM), penicillin, streptomycin, human recombinant insulin, and fetal bovine serum were purchased from Invitrogen (Grand Island, NY). Collagenase (type I, class I) was obtained from Worthington Biochemicals (Freehold, NJ). Collagenase (type IV), qualified fetal bovine serum, EGTA, taurocholate, Hanks’ balanced salt solution (HBSS), dexamethasone, HEPES, choline, glucose, silicone oil, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Sulfo-N-hydrosuccinimide-SS-biotin, streptavidin-agarose beads, and the BCA protein assay kit were purchased from Pierce Chemical (Rockford, IL). The pEF6/V5-His TOPO TA expression kit was purchased from Invitrogen (Carlsbad, CA). The protease inhibitor mixture tablets (Complete, mini) and FuGENE 6 were purchased from Roche Diagnostics (Indianapolis, IN). Bosentan was kindly provided by Dr. Alexander Treiber (Aetelion Pharmaceuticals Ltd., Allschwil, Switzerland).

**Antibodies and Cell Lines.** Rabbit pAb anti-calreticulin antibody was purchased from Novus Biologicals, Inc. (Littleton, CO), mouse anti-V5 antibody was purchased from Invitrogen (Carlsbad, CA). The protease inhibitor mixture tablets (Complete, mini) and FuGENE 6 were purchased from Roche Diagnostics (Indianapolis, IN). Bosentan was kindly provided by Dr. Alexander Treiber (Aetelion Pharmaceuticals Ltd., Allschwil, Switzerland).

**Human Hepatocyte Isolation and Culture.** The demographics of liver tissue donors are summarized in Table 1. Hu274 and Hu299...
were obtained from CellzDirect (Pittsboro, NC). Livers 1 to 3 were isolated from human liver tissue procured through the Department of Surgery, University of North Carolina at Chapel Hill School of Medicine (Chapel Hill, NC) by a modification of the two-step collagenase digestion method (Hamilton et al., 2001). All human liver tissue was obtained from fresh resections by qualified medical staff, with donor consent and with the approval of the University of North Carolina Hospitals Ethics Committee. Hepatocytes were cultured according to the methods described by Hoffmaster et al. (2004) with modifications. Hepatocytes were seeded at 1.5 × 10⁶ cells/well on six-well Biocoat culture plates in 1.5 ml of phenol red-DMEM supplemented with 5% fetal bovine serum, 0.1 mM modified essential medium nonessential amino acids, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 μg/ml human recombinant insulin, and 1 μM dexamethasone and allowed to attach for 2 to 6 h at 37°C in a humidified incubator with 95% air/5% CO₂. After cell attachment, culture dishes were gently swirled, and medium was replaced with phenol red-DMEM supplemented as above except with 0.1 μM dexamethasone, 6.25 μg/ml insulin, 6.25 μg/ml transferrin, and 6.25 mg/ml selenium (ITS¹). Cells were overlaid 6 to 12 h postseeding with 0.25 mg/ml Matrigel in 1.5 ml of ice-cold medium. Cultures were maintained with daily media changes for 6 days before use in experiments.

**Sandwich-Cultured Human Hepatocyte Transport Studies.** Methods to determine the accumulation and the in vitro biliary clearance (C₅₀₅₀,h,m) in sandwich-cultured (SC) hepatocytes have been described previously (Liu et al., 1999; Annaert and Brouwer, 2005). Briefly, human hepatocytes were rinsed twice with 2 ml of warm standard HBSS or Ca²⁺-free HBSS with EGTA (1 mM) and preincubated with 2 ml of the same buffer for 10 min at 37°C. The medium was removed, and SC hepatocytes were incubated for 10 min with [³H]taurocholate (1 μM; 100 nCi) in 1 ml of standard HBSS in the presence of boasant (0–100 μM). After incubation, dishes were washed vigorously 3 times with 2 ml of ice-cold HBSS. Hepatocytes were lysed with 1 ml of 0.1% Triton X-100, and 500 μl was analyzed by liquid scintillation counting. Data were normalized to the protein content of the hepatocytes using a BCA protein assay.

The in vitro biliary clearance (in vitro C₅₀₅₀,h,m) in microliters per minute per milligram of protein) of taurocholate was calculated using B-CLEAR technology (Qualyst, Inc., Raleigh, NC) based on the following equation:

\[
C_{50_{50},h,m} = \frac{\text{Accumulation}_{\text{HBSS}} - \text{Accumulation}_{\text{Ca}^{2+}-\text{free HBSS}} \times 100}{\text{Time}_{\text{incubation}} \times C}
\]

where C represents the initial substrate concentration in the incubation medium (1 μM).

**Animals.** Male Wistar rats (220–300 g) (Charles River Laboratories, Inc., Raleigh, NC) were used as liver donors for hepatocyte isolation. Rats were maintained on a 12-h light/12-h dark cycle with free access to water and rodent chow. Rats were allowed to acclimate for at least 5 days before experimentation. The Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill approved all procedures.

**Transport Studies Using Suspended Primary Rat and Human Hepatocytes.** Rat hepatocytes were isolated from male Wistar rats by a two-step collagenase perfusion method as described previously (Liu et al., 1999). Human hepatocytes were isolated in a similar manner, as described above. Isolated hepatocytes were washed twice with cold HBSS modified with 10 mM Tris and 5 mM glucose (pH 7.4) or Na⁺-free choline buffer (10 mM Tris, 5 mM glucose, 5.4 mM KCl, 1.8 mM CaCl₂, 0.9 mM MgSO₄, 10 mM HEPES, and 137 mM choline). Cells were suspended at 1 × 10⁶ cells/ml in the same buffer, placed on ice, and used immediately in experiments. Hepatocyte suspensions (4 ml for rat; 1 ml for human) were preincubated at 37°C for 5 min in bottom inverted Erlenmeyer flasks (rat) or for 3 min in 16 × 100 mm test tubes (human); vehicle or boasant (0.1–100 μM) was added followed by [³H]taurocholate (1 μM; 60 nCi/ml). At 30 s (rat) and 45 s (human), 200-μl samples were removed, placed in a 0.4-mL polyethylene tube containing a top layer of silicone oil/mineral oil (82:18, v/v) (100 μl) and a bottom layer of 3 M KOH (50 μl) and centrifuged. Radioactivity in the cell pellet and supernatant was quantified by liquid scintillation counting. The adherent fluid volume was determined by incubation of cells with [¹⁴C]ulinulin. Protein concentrations in the incubation mixtures were quantitated at the end of each experiment.

**Construction of Vectors Encoding Ntcp/NTCP Hybrid Proteins.** The full open reading frame of NTCP and Ntcp cDNAs were obtained by PCR, using AmpliTaq DNA polymerase (PerkinElmer Life and Analytical Sciences), from cDNA libraries synthesized from human and rat liver mRNA, respectively, as described previously (Tirona et al., 2003). NTCP and Ntcp cDNAs were cloned using TOPO TA cloning technology into pEFP/V5-His (pEFP-NTCP and pEF6-Ntcp, respectively) and Ntcp was also cloned into pcR2.1 (pcR2.1-Ntcp) with primers that removed the stop codon and left the flanking sequences of the gene intact. Ntcp1–140/NTCP141–349 was generated by PCR amplification of the first 414 nucleotides of Ntcp from pcR2.1-Ntcp using a 5′ primer complementary to the pcR2.1 vector, 38 nucleotides before the start of the Ntcp ORF 5′…ccgtgctaacagctgctgcatgcag…3′ with an engineered BamHI site (italics) and a 3′ primer complementary to nucleotides 392 to 411 in Ntcp, …tgtagtacaagaggtaggtcatgcagcc…3′ with an engineered BsrGI site (italics). The PCR product and the pEFP-NTCP vector (construction described previously (Ho et al., 2004)) were then digested with BamHI and BsrGI and then ligated together replacing the first 414 nucleotides of Ntcp with the first 414 nucleotides of Ntcp.

The NTCP₁–₁⁴₀/NTCP₁₄₁–₃₄⁹ was generated by PCR amplification of nucleotides 414 to 1086 of rat Ntcp from pEFP-Ntcp using a 5′ primer complementary to nucleotides 419 to 438 in Ntcp, 5′…atctgtagtcagctgctgcatgcag…3′, with an engineered BamHI site (italics) and a 3′ primer complementary to the BGI reverse priming site in the pEFP/V5-His vector, 5′…ccgtgctaacagctgctgcatgcag…3′. The PCR product and the pEFP-NTCP vector were digested with BsrGI and NotI and then ligated together, replacing the first 414 nucleotides of Ntcp with the first 414 nucleotides of NTCP.

**Expression and Functional Characterization of NTCP, Ntcp, NTCP₁₄₁–₃₄⁹, and Ntcp₁–₁⁴₀/NTCP₁₄₁–₃₄⁹ in HEK293 Cells.** The wild-type and hybrid NTCP and Ntcp constructs were transiently transfected into HEK293 cells using FuGENE6 according to the manufacturer’s instructions. Briefly, cells were seeded at 3 × 10⁶ cells in a T-75 flask, and 24 h later, 7.5 μg of DNA and 23 μl of FuGENE 6 (1.3 DNA-FuGENE 6) were combined in serum-free media and added to each flask. When confluent (60–72 h later), cells were trypsinized, washed twice with HBSS, and suspended at 4 × 10⁶ cells/ml of HBSS. Uptake experiments were conducted as described above for suspended primary human hepatocytes. In addition, at the end of each experiment approximately 4 × 10⁶ cells were retained for immunoblot analysis. Immunoblots of NTCP, Ntcp, NTCP₁₄₁–₃₄⁹, Ntcp₁–₁⁴₀/NTCP₁₄₁–₃₄⁹, and Ntcp₁–₁⁴₀/NTCP₁₄₁–₃₄⁹ were performed using the mouse anti-V5 antibody diluted at 1:5,000. Membranes were stripped and blotted with the mouse anti-β-actin antibody diluted at 1:5,000, and protein loading was normalized by densitometry. The functional activities of NTCP, Ntcp, NTCP₁₄₁–₃₄⁹, Ntcp₁–₁⁴₀/NTCP₁₄₁–₃₄⁹, and Ntcp₁–₁⁴₀/NTCP₁₄₁–₃₄⁹ reported throughout the manuscript were normalized for protein expression levels.

**Total and Cell Surface Expression of NTCP, Ntcp, NTCP₁₄₁–₃₄⁹, and Ntcp₁–₁⁴₀/NTCP₁₄₁–₃₄⁹ in HEK293 Cells.** HEK293 cells were grown on six-well plates and transfected as described above. Biotinylation experiments were conducted 48 to 72 h post-transfection as described previously (Ho et al., 2004), with modifications. Cells were washed with ice-cold phosphate-buffered saline (PBS) and then treated with sulfo-N-Hydroxysuccinimide-SS-biotin (1.5 mg/ml in PBS) for 1 h at 4°C. Cells were washed three times with PBS containing 100 mM glycine and incubated for 20 min at 4°C with the same buffer. Cells were washed with PBS, scraped into 1 ml of phosphate-buffered saline, and pelleted by centrifugation at 800g for 2 min. The pellet was treated with 700 μl
of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 1% Triton X-100, pH 7.4, with protease inhibitors) for 1 h at 4°C with shaking. Cell lysates were spun at 16,000g for 10 min, the supernatant was retained, and protein concentrations were determined using a BCA kit. Lysates were diluted to equivalent protein concentrations (−0.1 μg/μl) by adding additional lysis buffer, and 600 μl was incubated with 140 μl of streptavidin-agarose beads for 1 h at 25°C with constant agitation. Beads were pelleted at 5000g and washed 4 times with ice-cold lysis buffer. The beads were resuspended in 50 μl of Laemmli buffer and incubated at 70°C for 20 min, and 25 μl was subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis along with 12.5 μl (1.25 μg of protein) of total cell lysate. NTCP, Ntcp, and chimeric proteins were detected with the mouse anti-V5 antibody diluted at 1:1000. Blots were stripped and to ensure that the biotinylation was specific to cell surface proteins, the endoplasmic reticulum-specific protein calreticulin was detected using rabbit pAb anti-calreticulin diluted at 1:1000. Protein loading was normalized using the mouse anti-β-actin antibody diluted at 1:5000.

Results

Bosentan Inhibited the in Vitro Biliary Clearance of Taurocholate in Sandwich-Cultured Human Hepatocytes. SC hepatocytes acquire and maintain normal cell polarity and develop functional and extensive bile canicular networks. In Ca2+-containing buffer, the canalicular tight junctions are sealed, allowing quantitation of substrate accumulation in hepatocyte and canicular networks; incubation of SC hepatocytes in Ca2+-free buffer disrupts the canalicular tight junctions and substrate accumulates only in hepatocytes (Liu et al., 1999). Thus, SC hepatocytes are a valid and increasingly popular model to study transport processes responsible for basolateral uptake and biliary excretion (Liu et al., 1999; Ghibellini et al., 2006). The effect of bosentan on taurocholate accumulation in SC human hepatocytes was determined at a 10-min time point (Fig. 1). The accumulation of taurocholate in cells + canicular networks (Ca2+-containing condition) was inhibited in a concentration-dependent manner by bosentan and was reduced significantly by ≥30 μM bosentan (Fig. 1A). Inhibition of taurocholate accumulation in cells alone (Ca2+-free conditions) also occurred in a concentration-dependent manner and was significantly reduced at bosentan concentrations of ≥10 μM (Fig. 1A). Calculation of the mean in vitro CLba (Fig. 1B) showed a concentration-dependent inhibition of taurocholate biliary clearance at ≥30 μM bosentan (Fig. 1B).

Bosentan Inhibited Na+-Dependent Taurocholate Uptake in Suspended Rat Hepatocytes More Potently Than in Human Hepatocytes. To compare the effect of bosentan on inhibition of Na+-dependent taurocholate uptake, experiments were conducted in freshly isolated suspended primary rat and human hepatocytes. Suspended hepatocytes are preferred over SC hepatocytes for analysis of basolateral uptake because of the technical ease of determining initial rates of uptake. Time courses for Na+-dependent and Na+-independent taurocholate uptake were measured for each hepatocyte preparation. Consistent with previous publications, uptake was linear for at least 60 s (data not shown) (Torchia et al., 1996; Shitara et al., 2003), and Na+-dependent uptake was the predominant mechanism of taurocholate uptake by both rat and human hepatocytes (Shitara et al., 2003). Na+-dependent uptake accounted for ~68 and 97% of taurocholate uptake by rat and human hepatocytes, respectively. Bosentan was 6-fold more potent as an inhibitor of initial rates of Na+-dependent taurocholate uptake in rat than in human hepatocytes (IC50 5.4 μM versus 30 μM, respectively) (Fig. 2, Table 2).

Bosentan Was a More Potent Inhibitor of Ntcp Than NTCP Transiently Expressed in HEK293 Cells. To confirm the finding that bosentan inhibits Na+-dependent taurocholate uptake more potently in rat than in human hepatocytes (IC50 0.7 μM versus 30 μM; 100 nCi) in the presence of bosentan for 10 min at 37°C in hepatocytes preincubated with Ca2+-containing cells + canicular networks, □ or Ca2+-free cells alone, □ buffer. Bars represent the means ± S.D. of three experiments (hepatocytes were from human livers 1–3 described in Table 1). *, statistically different from Ca2+-containing control (0 μM bosentan); †, statistically different from Ca2+-free control (analysis of variance followed by Newman-Keuls post hoc test, P < 0.01). B, the in vitro biliary clearance (CLba) of taurocholate was calculated using data from A. Bars represent the means ± S.D. of three experiments (#, statistically different from 0 μM bosentan control; analysis of variance followed by Newman-Keuls post hoc test, P < 0.05).

Protein Expression of NTCP/Ntcp Chimeras. NTCP and Ntcp are 77% identical and 88% similar in amino acid sequence. To characterize the species differences in both taurocholate transport and bosentan inhibition of taurocholate uptake, two chimeric Ntcp/NTCP molecules were generated, one containing the first 140 amino acids of NTCP and amino acids 141 to 362 of Ntcp (NTCP1-140/Ntcp141-362).

Fig. 1. Effect of bosentan on taurocholate accumulation and in vitro biliary clearance in SC human hepatocytes. A, SC human hepatocytes were incubated with [3H]taurocholate (1 μM; 100 nCi) in the presence of bosentan for 10 min at 37°C in hepatocytes preincubated with Ca2+-containing cells + canicular networks, □ or Ca2+-free cells alone, □ buffer. Bars represent the means ± S.D. of three experiments (hepatocytes were from human livers 1–3 described in Table 1). *, statistically different from Ca2+-containing control (0 μM bosentan); †, statistically different from Ca2+-free control (analysis of variance followed by Newman-Keuls post hoc test, P < 0.01). B, the in vitro biliary clearance (CLba) of taurocholate was calculated using data from A. Bars represent the means ± S.D. of three experiments (#, statistically different from 0 μM bosentan control; analysis of variance followed by Newman-Keuls post hoc test, P < 0.05).
and one containing the first 140 amino acids of Ntcp and amino acids 141 to 349 of NTCP (Ntcp1–140/NTCP141–349) (Fig. 4). NTCP, Ntcp, NTCP1–140/Ntcp141–362, and Ntcp1–140/NTCP141–349 were expressed in HEK293 cells, and total versus cell surface protein expression levels were compared using biotinylation and immunoblot analysis (Fig. 5A). Because of the lack of availability of a high-affinity antibody for NTCP, chimeric and wild-type proteins were generated with a V5 tag. This epitope tag, which also allowed for the direct comparison of protein expression levels, did not affect the kinetics of taurocholate transport (data not shown). The V5 antibody detected a band at ~45 kDa for rat Ntcp and NTCP1–140/Ntcp141–362 and ~43 kDa for human NTCP and Ntcp1–140/NTCP141–349. Expression levels of NTCP and Ntcp1–140/NTCP141–349 were consistently lower than those for Ntcp and NTCP1–140/Ntcp141–362 for both total and cell surface protein (Fig. 5, A, top, and B). Cell surface biotinylation experiments indicated that all four proteins were expressed at the cell membrane, and levels at the cell surface were proportional to total protein levels (Fig. 5, A and B). Calreticulin, an endoplasmic reticulum-specific protein, was detected in the total protein but not the cell surface fraction (Fig. 5A, middle), indicating that the biotinylation method successfully enriched the fraction for proteins expressed at the cell surface.

Uptake of Taurocholate by NTCP, Ntcp, NTCP1–140/Ntcp141–362, and Ntcp1–140/NTCP141–349. As a preliminary characterization of chimera function, a time course of taurocholate (1 μM; 60 nCi/ml) uptake by HEK293 cells expressing NTCP, Ntcp, NTCP1–140/Ntcp141–362, and Ntcp1–140/NTCP141–349 was measured (Fig. 6A). Compared with the untransfected control, significant taurocholate uptake in the linear range was observed for all proteins from 30 s to 180 s. Activities at 180 s for untransfected HEK293 cells and cells expressing NTCP, Ntcp, NTCP1–140/Ntcp141–362, and Ntcp1–140/NTCP141–349 were 2.95, 514, 211, 207, and 522 pmol/mg/min, respectively. Interestingly, the activity of taurocholate uptake appeared to be dictated by the carboxyl half of the protein with the Ntcp1–140/NTCP141–349 chimera having activity similar to that of NTCP and the NTCP1–140/Ntcp141–362 chimera having activity similar to that of Ntcp.

Kinetics of Taurocholate Uptake by NTCP, Ntcp, NTCP1–140/Ntcp141–362, and Ntcp1–140/NTCP141–349. Uptake of taurocholate by NTCP, Ntcp, NTCP1–140/Ntcp141–362, and Ntcp1–140/NTCP141–349 was characterized further by determining the initial rates of uptake over several concentrations of taurocholate (Fig. 6B; Table 2). Kinetic parameters determined for NTCP (apparent \( K_m \) 2.1 μM and \( V_{max} \) 814 pmol/mg/min) and Ntcp (apparent \( K_m \) 2.6 μM and \( V_{max} \) 357 pmol/mg/min) suggested that the difference in taurocholate transport activity for NTCP and Ntcp was due to a difference in \( V_{max} \). This result contrasted with previous reports suggesting that the apparent \( K_m \) was ~4-fold higher for rat Ntcp compared with human NTCP (Hagenbuch et al., 1991; Hagenbuch and Meier, 1994).
Consistent with the time course (Fig. 6A), the kinetic parameters determined for NTCP1–140/Ntcp141–362 (apparent $K_m$ 3.5 μM and $V_{max}$ 420 pmol/mg/min) were similar to those for Ntcp. In addition, kinetic parameters determined for Ntcp1–140/NTCP141–349 (apparent $K_m$ 4.1 μM and $V_{max}$ 713 pmol/mg/min) were similar to those for NTCP. No significant difference existed between the apparent $K_m$ values determined for the four proteins.

### Bosentan Inhibition of NTCP, Ntcp, NTCP1–140/Ntcp141–362, and Ntcp1–140/NTCP141–349.

The ability of bosentan to inhibit taurocholate uptake by HEK293 cells expressing NTCP1–140/Ntcp141–362 and Ntcp 1–140/NTCP141–349 was evaluated and compared with that for

| TABLE 2 | Summary of IC$_{50}$ and $K_i$ values for Na$^+$-dependent taurocholate transport inhibition by bosentan and kinetic parameters for taurocholate transport. |
|-----------------|-----------------|-----------------|-----------------|
| **Hepatocytes**| **NTCP** | **Ntcp** | **NTCP$_{1-140}$/Ntcp$_{141-362}$** | **NTCP$_{1-140}$/NTCP$_{141-349}$** |
| Human | Rat | Human | Rat | Human | Rat | Human | Rat |
| IC$_{50}$ (μM) | 30 (n = 2) | 5.4 ± 1.7 (n = 4) | 24 ± 3.8 (n = 3)$^b$ | 0.71 ± 0.36 (n = 3)$^b$ | 3.0 ± 2.4 (n = 3)$^b$ | 2.4 ± 0.5 (n = 3)$^b$ |
| $K_i$ (μM) | 18 ± 4.4 (n = 5)$^c,d$ | Noncompetitive | 1.7 ± 1.1 (n = 6)$^b,c$ | 7.0 ± 4.4 (n = 4)$^b,c$ |
| $K_m$ (μM) | 2.1 ± 1.4 (n = 8)$^c$ | 2.6 ± 1.6 (n = 8)$^c$ | 3.5 ± 0.67 (n = 5)$^c$ | 4.1 ± 2.2 (n = 5)$^c$ |
| $V_{max}$ (pmol/mg/min)$^e$ | 814 ± 278 (n = 8)$^b,c$ | 357 ± 134 (n = 8)$^b,c$ | 420 ± 209 (n = 5)$^b,d$ | 713 ± 112 (n = 5)$^b,c$ |

Analysis of variance followed by a Newman Keuls post hoc test; $P < 0.05$.

$^b$ Statistically different from Ntcp.

$^c$ Statistically different from NTCP$_{1-140}$/Ntcp$_{141-362}$.

$^d$ Statistically different from NTCP$_{1-140}$/NTCP$_{141-349}$.

$^e$ $V_{max}$ values were normalized for the expression level of each protein relative to NTCP, as determined by immunoblot analysis.
NTCP and Ntcp (Fig. 7). Consistent with previous experiments, taurocholate uptake by Ntcp- and NTCP1–140/Ntcp141–362 expressing cells was lower than that by NTCP and Ntcp1–140/NTCP141–349 (Fig. 7). Somewhat unexpectedly, the difference between bosentan inhibition of taurocholate uptake for NTCP1–140/Ntcp141–349 was a mixture of competitive and noncompetitive inhibition with a decrease in both the apparent $K_m$ and $V_{max}$, the apparent $K_m$ was 7 μM, and the $V_{max}$ was decreased from 615 to 395 pmol/min in the presence of 10 μM bosentan (Fig. 8C). Bosentan was a competitive inhibitor of taurocholate uptake by NTCP1–140/Ntcp141–362 with a $K_i$ value of 1.7 μM (Fig. 8D).
Discussion

The association between bosentan and hepatotoxicity has been attributed to inhibition of BSEP, which should result in accumulation of bile acids within the hepatocyte (Fattlinger et al., 2001; Noé et al., 2002; Mano et al., 2007). Bosentan also inhibits Na⁺-dependent taurocholate uptake by rat hepatocytes (Kemp et al., 2005). This result suggests that hepatotoxicity may reflect the balance between bosentan inhibition of bile acid efflux versus uptake. Thus, preferential inhibition of rat Ntcp versus human NTCP could explain why the rat is resistant to bosentan-induced hepatotoxicity.

In SC human hepatocytes, the biliary clearance of taurocholate was inhibited by bosentan in a concentration-dependent manner. This result is consistent with what has previously been reported in SC rat hepatocytes treated with bosentan (Kemp et al., 2005) and supports simultaneous inhibition of both NTCP and BSEP. We next tested the hypothesis that rat Ntcp would be more sensitive to inhibition than human NTCP. The initial rate of Na⁺-dependent taurocholate uptake was inhibited by bosentan 6-fold more potently in rat hepatocytes than in human hepatocytes. Bosentan also was a more potent inhibitor of rat Ntcp than human NTCP expressed in HEK293 cells.

Because inhibition of NTCP/Ntcp may offset the effect of BSEP inhibition by preventing the uptake of bile salts from blood and the subsequent accumulation within the hepatocyte, the more potent inhibition of Ntcp than NTCP by bosentan provides a plausible mechanism for protection of the rat from bosentan hepatotoxicity. At the therapeutically relevant unbound plasma concentrations of bosentan in patients (C_max 2 μM, 98% protein-bound) (Weber et al., 1999), no significant inhibition in bile acid uptake by human NTCP would be expected. Thus, differential inhibition of bile acid uptake and efflux could account for differences between rats and humans in susceptibility to bosentan-induced hepatotoxicity.

Kinetic parameters of taurocholate transport by NTCP and Ntcp were determined and revealed a 2.3-fold higher V_max for human protein than for rat protein but virtually identical apparent K_m values (Table 2). The addition of the V5 epitope tag on the proteins allowed the normalization of uptake activity for protein expression level, of clear importance for comparing V_max values. This finding is consistent with data generated recently in human and rat hepatocytes showing that differences in V_max are functionally more important than differences in K_m for determining species differences in taurocholate uptake (Shitara et al., 2003). However, this trend is in disagreement with several previous studies suggesting that Ntcp had a higher apparent K_m for taurocholate than NTCP. The apparent K_m value determined in the present study for human NTCP-mediated taurocholate transport was 2.1 ± 1.4 μM and is within the range of reported literature values of 1.6 to 7.9 μM (Hagenbuch and Meier, 1994; Kim et al., 1999; Shitara et al., 2003; Ho et al., 2004). In contrast, our rat Ntcp apparent K_m of 2.6 ± 1.6 μM was lower than published values for Ntcp [e.g., 10 ± 6.0 and 30 ± 17 (mean ± S.D. calculated from reported S.E. assuming n = 3)] (Hagenbuch et al., 1991; Torchia et al., 1996; Schroeder et al., 1998; Sun et al., 2001; Hata et al., 2003). To our knowledge the present study in HEK293 cells is the first report in which side-by-side kinetic parameters for taurocholate uptake have been determined for NTCP and Ntcp.

To characterize regions of NTCP and Ntcp that are important for species differences in taurocholate uptake and bosentan inhibition of taurocholate transport, two chimeric Ntcp/NTCP molecules were generated. These proteins were expressed, and cell surface biotinylation experiments confirmed that they were localized properly to the plasma membrane of HEK293 cells. Kinetic analysis of taurocholate transport by chimeric proteins provided evidence that the carboxyl halves of NTCP and Ntcp are important for the differences in V_max. Results from the characterization of boenteran inhibition of NTCP, Ntcp, NTCP1–140/Ntcp141–362, and Ntcp1–140/NTCP141–349 were more complicated because of the different modes of inhibition.

The mode of bosentan inhibition of taurocholate uptake by NTCP and NTCP1–140/Ntcp141–362 was found to be competitive with apparent K_i values of 18 and 1.7 μM, respectively. These data suggested that the first 140 amino acids of NTCP were important for competitive inhibition by bosentan. In contrast, bosentan was a noncompetitive inhibitor of Ntcp and a mixed competitive and noncompetitive inhibitor of Ntcp1–140/NTCP141–349, which is not consistent with the first 140 amino acids being important for competitive inhibition. The apparent K_i values demonstrated that, as for Ntcp, bosentan was a more potent inhibitor of NTCP1–140/Ntcp141–362 than NTCP, suggesting that the carboxyl portion of Ntcp was important for the species difference in bosentan inhibitory potency. However, this theory was not confirmed using the Ntcp1–140/NTCP141–349 chimera, which had an apparent K_i value of 7 μM, which falls midway between the K_i values for NTCP and Ntcp. Thus, the mechanism of bosentan inhibition of NTCP, Ntcp, NTCP1–140/Ntcp141–362, and Ntcp1–140/NTCP141–349 was found to be complex. Random chimeragenesis and site-directed mutagenesis are currently underway to further define the regions of rat Ntcp and human NTCP important for function and interaction with bosentan and other drugs of hepatotoxic relevance.

NTCP and BSEP are known to be polymorphic, and genetic mutations that alter the interaction of bosentan with either of these proteins could shift the balance of bile acid uptake and efflux (Ho et al., 2004; Meier et al., 2006). In addition, bile acids are also substrates for multidrug resistance-associated proteins (MRP) 3/ABCC3 and MRP4/ABCC4 (Hirohashi et al., 1999; Rius et al., 2003; Zelcer et al., 2003), efflux pumps present on the basolateral surface of hepatocytes; polymorphisms in these genes (Saito et al., 2002; Lang et al., 2004; Lee et al., 2004) also could contribute to interindividual susceptibility to toxicity. We speculate that such polymorphisms could be related to the interindividual differences in susceptibility to bosentan-induced hepatotoxicity observed clinically.

Overall, our observation that Ntcp is more sensitive than NTCP to inhibition by bosentan may represent a novel mechanism to account for the apparent species differences in bosentan-induced hepatotoxicity and should be considered in other cases of drug-induced liver injury.

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