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Structural requirements of bile acid transporters: C-3 and C-7 modifications of steroidal hydroxyl groups

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Abstract

The apical sodium dependent bile acid transporter (ASBT) and sodium-taurocholate cotransporting polypeptide (NTCP) are potential prodrug targets, but the structural requirements for these transporters are incompletely defined. The objective of this study was to evaluate the effect of C-3 and C-7 substitution on bile acid interaction with these bile acid transporters. Nineteen bile acid analogues were tested against ASBT and NTCP for binding, as well as translocation. Results indicated that ASBT and NTCP accommodated a wide range of substituents for binding, but all major C-7 modifications resulted in analogues that did not demonstrate active uptake by either ASBT or NTCP. A C-3 modification that was not tolerated at C-7 still afforded translocation via ASBT and NTCP, confirming the relative unacceptability of C-7 modification. Both ASBT and NTCP demonstrated a generally similar binding potency. Results suggest that drug conjugation to the C-3 hydroxyl group, rather than C-7, has potential to lead to a successful prodrug targeting ASBT and NTCP.

Keywords

ASBT; NTCP; substrate; inhibitor; bile acid; prodrug

1. INTRODUCTION

The human apical sodium dependent bile acid transporter (ASBT, SLC10A2), expressed at high levels in the terminal ileum, is one of the important transporters in the enterohepatic circulation. It is responsible for the active absorption of bile acids from the gut. Overall, a 3–5g bile acid pool results in the daily turnover of 12–18g and less than 0.5g is lost each day, reflecting the high capacity of this bile acid transporter. ASBT is thus an ideal prodrug target with its high efficiency and capacity (Balakrishnan and Polli, 2006).

Despite being a prodrug target, the design of prodrugs is hampered by absence of the high resolution ASBT crystal structure and the lack of understanding of interactions at the molecular level. Our laboratory has taken a systematic approach to understand the structure-activity relationship of ASBT. Previous reports evaluated interaction of native bile acids with ASBT in the stably transfected ASBT-MDCK cell line (Balakrishnan et al., 2006b). The evaluation of the C-24 chemical space has indicated that ASBT accommodates a range

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of substituents and charge conditions for binding. However, a single negative charge in the C-24 region promotes translocation across the transporter (Balakrishnan et al., 2006a). ASBT also accommodates various drug-like single ring scaffolds with different substituents attached to the bile acid (Gonzalez et al., 2009; Rais et al., 2010a; Rais et al., 2010b; Zheng et al., 2010). Using C-24 bile acid linkage chemistry, bile acid-based prodrugs of gabapentin, ketoprofen, and niacin have been shown to be ASBT substrates (Rais et al., 2011; Zheng and Polli, 2010). In addition to this C-24 chemical space, the C-3 bile acid chemical space has also been explored, where various linkers and drugs have been conjugated to a bile acid at C-3, resulting in some prodrugs (Kramer et al., 1994; Swaan et al., 1997).

After absorption by ASBT, bile acids are mostly extracted into hepatocytes by sodium-taurocholate cotransporting polypeptide (NTCP) at the basolateral surface of hepatocytes (Alrefai and Gill, 2007). Liver specific expression of this transporter can be exploited for hepatocyte selective drug action. Some studies have evaluated the effect of conjugation at the C-3 and C-24 region of bile acid on interaction with NTCP (Kramer et al., 1992; Kramer et al., 1997; Kullak-Ublick et al., 1997). However, this strategy is impeded by limited understanding of structure activity relationships.

In addition to C-3 and C-24 positions, the C-7 location is often hydroxylated and represents a potential conjugation site in bile acid prodrug design (Kramer, 2011; Sievanen, 2007; Zhang et al., 2002). Cholic acid and chenodeoxycholic acid, the two most abundant bile acids in humans, contain a C-7 hydroxyl group (Kullak-Ublick et al., 2004). Interestingly, there are no reports of C-7 substitution on bile acid interaction with the bile acid transporters.

The objective of this study was to evaluate the effect of C-3 and C-7 substitution on bile acid interaction with bile acid transporters. Nineteen bile acid analogues were tested against ASBT and NTCP, using stably transfected ASBT-MDCK and NTCP-HEK monolayers. Analogue conjugates were composed of bile acid (CDCA or UDCA) conjugated to substituted aromatic rings such as benzene and pyridine, the most common scaffolds among existing drugs (Bemis and Murcko, 1996). Results indicated that although ASBT and NTCP accommodated a wide range of substituents for binding, all major C-7 modifications resulted in analogues that were not translocated by either ASBT or NTCP. Interestingly, a C-3 modification that was not tolerated at C-7 still afforded translocation via ASBT and NTCP, further confirming the relative unacceptability of C-7 modification. Results also characterize the relative promiscuity of ASBT and NTCP. Compared to ASBT, NTCP demonstrated a generally similar binding potency across compounds, although showed less binding affinity toward unconjugated bile acids with aromatic substitution at C-7. Results suggest that drug conjugation to the C-3 hydroxyl group, rather than C-7, has potential to serve as a bile acid transporter prodrug strategy.

2. MATERIALS AND METHODS

2.1 Materials

Ursodeoxycholic acid was obtained from TCI America (Portland, OR). Chenodeoxycholic acid was purchased from AK Scientific (Mountain View, CA). Geneticin, fetal bovine serum (FBS), trypsin, and DMEM were obtained from Invitrogen (Rockville, MD). [³H]-Taurocholic acid was purchased from PerkinElmer Inc (Waltham, MA). Taurocholic acid, dehydrocholic acid (compound **13**), pyridine, and triethyl amine were obtained from Sigma Aldrich (St. Louis, MO). 7-oxo cholanic acid (compound **11**) was purchased from TCI America (Portland, OR). All other reagents and chemicals were of the highest purity commercially available.

2.2 Synthetic methods

2.2.1 Synthetic procedure overview—The carboxylic acid in the C-24 region was protected by methyl, methyl glycinate, or benzyl glycinate to yield C-24 protected bile acid (**Procedure A**). Selective monoacylation and diacylation of the free steroidal hydroxyl groups was then carried out by controlling the stoichiometry of the acyl chloride used during the reaction to obtain 3, 24 disubstituted or 3, 7, 24 trisubstituted bile acids. (**Procedure B**). Tri-substituted bile acids were then subjected to basic hydrolysis. Ease of ester hydrolysis was in following order C-3 > C-24 > C-7 (**Procedure C**). Thus compound **17** was obtained by selective hydrolysis only at C-3 while keeping esters at C-24 and C-7 intact while compounds **1–9** were obtained by selective hydrolysis at C-3 and C-24 position while keeping ester at C-7 intact. Compounds **16, 18, 19** were not subjected to basic hydrolysis to understand the effect of C-24 and C-3 substitution on transporter interaction. TLC, ¹H NMR and mass spectroscopy were performed for each compound to confirm identity and satisfactory purity, along with ensuring that no bile acid (i.e. a potent inhibitor and substrate of both ASBT and NTCP) was present in the final product (Gonzalez and Polli, 2008).

2.2.2 Procedure A: Protection of C-24 carboxylic acid—Three different protecting groups were used for the synthesis of three groups of compounds. To synthesize compounds **1–6, 16, 18** and **19**, carboxylic acid was protected as methyl ester to get common intermediate (II) (Figure 1). To synthesize compounds **7–9**, and **17** methyl glycinate was used as protecting group to get common intermediate (V) (Figure 2) while for synthesis of compounds **10, 12, 14** and **15**, benzyl glycinate was used as protecting group to get common intermediate (X) (Figure 3). Below are the typical procedures used for the synthesis of intermediates II, V and X.

2.2.2.1 Synthesis of methyl ester of bile acid (II): Thionyl chloride (415 mg, 3.5 mmole) was added into a cold solution of ursodeoxycholic acid (UDCA) or chenodeoxycholic acid (CDCA) (500 mg, 1.3 mmole) in methanol. The reaction was allowed to warm to RT and stirred overnight. Methanol was then evaporated under vacuum to obtain the methyl ester of the respective bile acid (II) in quantitative yield. (MS, M+1 407.3).

2.2.2.2 Synthesis of glycine esters for bile acid (V and X): The glycine conjugate of the bile acid was synthesized, as described previously (Tserng et al., 1977). Briefly, methyl glycinate hydrochloride (878 mg, 7 mmol) was stirred with triethylamine (1 ml) in ethyl acetate at RT for 30 min. Bile acid (1.96 g, 5 mmol) and EEDQ (1.73 g, 7 mmol) were added to the suspension. After 10 min, the reaction mixture was heated at 65 °C and allowed to stir overnight. The ethyl acetate layer was then washed successively with water (25 ml), 0.5N NaOH (50 ml), water (25 ml), 0.5N HCl (2 X 50 ml), water (25 ml) and brine. The resulting ethyl acetate layer was evaporated under vacuum to yield methyl glyco ursodeoxycholate or methyl glyco chenodeoxycholate. (MS M+1 464.3). Use of benzyl glycinate instead of methyl glycinate yielded common intermediate (X) (MS, M+1 540.2) which was used for the synthesis of compounds **10, 14** and **15**.

2.2.3 Procedure B: Acylation of C-24 protected bile acid—Monoacylation at C-3 position of intermediate II and X with 2 equivalents of acid chloride (nicotinic acid chloride) yielded compound **18** and intermediate XI respectively (Figure 2 and 3) while reaction of intermediates II, V with excess (4 equivalents) of appropriate acyl chlorides yielded C-3, C-7-diacyl products III, VI respectively. Typical procedure used for diacylation is as follows: Acyl chloride (4 eq.) was added to a solution of C-24 protected bile acid (1eq.) in pyridine. The reaction mixture was stirred at 40–60 °C for 2–8 hr. After cooling, the reaction mixture was poured over ice and acidified with concentrated HCl to pH 2. The suspension was extracted with dichloromethane. The organic layer was dried over sodium sulfate, and

evaporated under vacuum to yield a crude mixture which was purified by flash column chromatography over silica gel using an appropriate mixture of ethyl acetate and hexane as eluent.

2.2.4 Procedure C: Selective hydrolysis of 3,7,24-trisubstituted bile acid derivatives—Intermediates III, VI were subjected to basic conditions to achieve selective hydrolysis (Kritchevsky et al., 1986). Selective hydrolysis at C-3 and C-24 positions of III yielded compounds **1–6**. Selective hydrolysis at C-3 and C-24 positions of intermediate VI yielded compounds **7–9** while selective hydrolysis of intermediate VI at C-3 position yielded compound **17**. A typical procedure for hydrolysis is as follows: The 3, 7, 24-trisubstituted bile acid (1 eq.) was dissolved in methanol. A 50% aqueous KOH solution (0.5 ml) was then added to it. The reaction was heated at 40–60 °C for 2–10 hr. After the reaction was complete as indicated by TLC and MS, methanol was evaporated under vacuum to yield a white residue which was dissolved in water and acidified with concentrated HCl to pH 2. The crude product was extracted with dichloromethane. The organic layer was dried over anhydrous sodium sulfate and evaporated under vacuum to obtain final compounds which were purified by column chromatography over silica gel column using an appropriate mixture of ethyl acetate and hexane.

2.2.5 Procedure D: Formation of hemisuccinate (compound 14)—C-24 protected CDCA (6.3 mmol) was suspended in 25 ml ethyl acetate with succinic anhydride (3 g, 30 mmol), triethylamine (0.63 ml, 6.3 mmol), and DMAP (70 mg, 0.6 mmol). The reaction was heated at 65 °C for 48 hr. It was then extracted with water, followed by wash with acidic water (pH 1) (Bortolini et al., 2006). Ethyl acetate was then dried over sodium sulfate, filtered and evaporated under vacuum to get a crude product which was purified by column chromatography over silica gel, using 20% hexane in ethyl acetate as an eluent to get compound **14**.

2.2.6 Procedure E: Deprotection of benzyl group—Intermediates X, XI and compound **14** were subjected to catalytic hydrogenation to obtain compounds **12**, **10** and **15** respectively. A typical procedure used for catalytic hydrogenation is as follows: Benzyl protected compound was dissolved in ethanol and subjected to catalytic hydrogenation in a Parr hydrogenator using Pd/C as catalyst. Hydrogenation was performed overnight at 45 psi and at RT. The suspension was filtered using celite and organic solvents evaporated under vacuum to obtain final products.

2.3 Cell culture

Stably transfected ASBT-MDCK and NTCP-HEK cells were cultured as previously described (Balakrishnan et al., 2005; Han et al., 2010). Briefly, cells were grown at 37 °C, 90% relative humidity, 5% CO₂ atmosphere, and fed every two days. Culture media consisted of DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 µg/ml streptomycin. Geneticin (1 mg/ml) and hygromycin (100 µg/ml) were added to maintain selection pressure for ASBT-MDCK and NTCP-HEK cells, respectively. Cells were passaged after four days or after reaching 90% confluency.

2.3.1 Inhibition study in ASBT-MDCK monolayers—To characterize ASBT binding affinities, taurocholate uptake inhibition studies were conducted. Taurocholate is a potent ASBT substrate. Stably-transfected ASBT-MDCK cells were grown on 12-well plates (3.8 cm², Corning, Corning, NY) under conditions described above. Cells were seeded at a density of 1.5 million/well and induced with 10 mM sodium butyrate 12–15 hr at 37 °C prior to study on day 4. Cells were washed three times with Hank's balanced salt solution (HBSS) prior to assay. Studies were conducted at 37 °C, 50 rpm for 10 min in an orbital

shaker. Uptake buffer consisted of HBSS, which contained 137 mM NaCl (pH 6.8) and was supplemented with 1 g/L of glucose. Cells were exposed to the donor solution containing 0.5 $\mu\text{Ci/ml}$ [^3H]-taurocholate (TCA), 2.5 μM cold TCA, with varying concentrations of the analogues (from 0 μM to up to 500 μM) for 10 min at 37°C at 50 rpm. After this time, donor solution was removed and cells were washed three times with chilled sodium-free buffer (SFB) where NaCl was replaced by 137 mM tetraethylammonium chloride. Cells were lysed using 250 μl of 1N NaOH and allowed to stand for at least 2 hr. After that time, cell lysate was neutralized with 250 μl of 1N HCl. Lysate was then counted for associated radioactivity (i.e. [^3H]-taurocholate) using an LS6500 liquid scintillation counter (Beckmann Instruments, Inc., Fullerton, CA). Inhibition data was regressed using a modified Michaelis-Menten equation (eqn 1) to determine the inhibition constant K_i , as previously described (Gonzalez et al., 2009).

$$J = \frac{P_{ABL} \cdot \left(\frac{J_{\max}}{K_t \left(1 + \frac{I}{K_i} \right) + S} + P_p \right)}{P_{ABL} + \frac{J_{\max}}{K_t \left(1 + \frac{I}{K_i} \right) + S} + P_p} \cdot S \quad \text{eqn 1}$$

where I is inhibitor concentration, K_i is inhibition constant, J is taurocholate flux, $J_{\max}$ and K_t are the Michaelis-Menten constants, S is taurocholate concentration (i.e. 2.5 μM), P_p is passive taurocholate permeability, and P_{ABL} is aqueous boundary layer permeability. K_t was set to 5.03 μM , as obtained from pooled kinetic analysis of historical taurocholate uptake studies. P_{ABL} was set to 1.5×10^{-4} cm/s (Balakrishnan et al., 2007). Parallel studies were performed on each occasion to estimate $J_{\max}$ and P_p for taurocholic acid. P_p was estimated from taurocholate uptake studies in absence of sodium. K_i was estimated by using nonlinear regression fitting performed by WinNonlin Professional (Pharsight; Mountain View, CA). Compounds with K_i up to 25 μM are considered strong inhibitors. K_i between 25 μM - 100 μM are denoted moderate inhibitors. K_i above 100 μM are weak inhibitors.

2.3.2 Uptake study in ASBT-MDCK monolayers—Analogue uptake by ASBT was evaluated. Cells were seeded as above for inhibition studies. Uptake studies employed HBSS buffer or modified HBSS buffer (i.e., sodium-free buffer). Since ASBT is a sodium-dependent transporter, studies using sodium-free buffer enabled passive permeability measurement of analogue (Rais et al., 2010a; Weinman et al., 1998). Cells were exposed to donor solution in HBSS or SFB containing a range of analogue concentrations (from 1 μM to up to 250 μM) for 10 min. Subsequently, cells were lysed by adding 300 μl acetonitrile to each well. After two hr, acetonitrile was evaporated at RT. A solution (1 ml) containing 50% water and 50% acetonitrile was added to each well for 15 min (Rais et al., 2008; Rais et al., 2010a). Samples were stored in -80°C until analysis by LC/MS. Additionally, taurocholate $J_{\max}$ was measured on each occasion, as taurocholate is a reference ASBT substrate. For each analogue, $J_{\max}$ was normalized to the observed taurocholate $J_{\max}$ to yield substrate normalized $J_{\max}$, denoted norm $J_{\max}$. norm $J_{\max}$ measures transporter capacity for analogue, while accommodating variable ASBT protein expression across occasions. Uptake data was fitted to the modified Michaelis-Menten equation (eqn 2) to yield estimates for K_m and $J_{\max}$. Data from sodium free studies were fitted to eqn 3, which represents the passive transport.

$$J = \frac{P_{ABL} \left(\frac{J_{\max}}{K_t + S} + P_p \right)}{P_{ABL} + \frac{J_{\max}}{K_t + S} + P_p} \cdot S \quad \text{eqn 2}$$

$$J = \frac{P_{ABL} P_p S}{P_{ABL} + P_p} \quad \text{eqn 3}$$

Kinetic parameters were estimated via nonlinear regression by WinNonlin Professional.

2.3.3 Inhibition study in NTCP-HEK monolayer—The taurocholate uptake inhibition studies were performed in NTCP-HEK cells to characterize compound binding affinity with NTCP, as described above for ASBT. Stably-transfected NTCP-HEK cells were grown on BioCoat poly-D-lysine-coated 24-well plates (2 cm², BD, Franklin Lakes, NJ) under conditions described above. Cells were seeded at a density of 2.5×10^5 cells/well at 37°C. After culturing for two days, cells were washed three times with HBSS prior to assay. Cells were exposed to the donor solution containing 0.5 µCi/ml [³H]-taurocholate, 2.5 µM cold taurocholate, with varying concentrations of the analogues (from 0 µM to up to 500 µM) for five min at 37°C at 50 rpm. After this time, donor solution was removed and cells were washed three times with chilled sodium-free buffer. Cells were lysed by adding 150 µl acetonitrile to each well. Following evaporation at RT for about 2 hr, 250 µl of DPBS was added and 125 µl was then counted for associated radioactivity using an LS6500 liquid scintillation counter. Inhibition data was regressed using a modified Michaelis-Menten equation (eqn 1) to determine the inhibition constant Ki. Kt was set to 5.31 µM. Parallel studies were performed on each occasion to estimate Jmax and Pp for taurocholic acid.

2.3.4 Uptake study in NTCP-HEK monolayers—The uptake of analogues by NTCP was performed, as described above for ASBT. Since NTCP is a sodium-dependent transporter, uptake studies employed HBSS buffer or sodium free buffer. Cells were exposed to donor solution containing a range of analogue concentrations (from 1 µM to up to 250 µM) in HBSS or sodium free buffer. Subsequently, cells were lysed by adding 150 µl acetonitrile to each well. After two hr, acetonitrile was evaporated at RT. A solution (500 µl) containing 50% water and 50% acetonitrile was added to each well for 15 min. Samples were stored in −80 °C until analysis by LC/MS. Additionally, taurocholate Jmax was measured on each occasion. For each analogue, Jmax was normalized to observed taurocholate Jmax to yield substrate normalized Jmax, denoted normJmax. Kinetic data was subjected to eqn 2 and 3.

2.4 Analytical method

Analogues were quantified by LC/MS on a Thermo Finnigan Surveyor HPLC system, equipped with a Thermo Finnigan Surveyor autosampler and a Thermo TSQ quantum mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). The column was a Phenomenex Luna C8 (50 × 2.0 mm, 3 µm; Phenomenex; Torrance, CA, USA). The mobile phase was water and acetonitrile. Formic acid (0.1%) was used as modifier in each mobile phase. A flow rate of 0.4 ml/min was used with an injection volume of 10 µl. Detection was achieved under positive or negative ion electrospray, depending on the approach that provided maximum sensitivity. The limit of quantification for all analogues was between 1 and 10 nM.

2.5 Assessment of inhibition type

7-nicotinoyl glycocholate (Compound 8) inhibited both ASBT and NTCP. In order to assess type of inhibition (i.e. competitive, noncompetitive, or uncompetitive), an additional ASBT inhibition study and an additional NTCP inhibition study was performed for 7-nicotinoyl glycocholate, using ASBT-MDCK and NTCP-HEK monolayers. Inhibition studies were performed as described above, except inhibitor concentration was fixed at one level (i.e. ten times Ki) and substrate concentration was varied. This approach is

denoted the nonconventional inhibition study, since it differs from the conventional approach where inhibitor concentration is varied and substrate concentration is fixed (Kolhatkar and Polli, 2010). Cells were exposed to the donor solution containing 152 μM (for ASBT-MDCK) and 118 μM (for NTCP-HEK study) inhibitor and 1–200 μM taurocholate with 0.5 $\mu\text{Ci/ml}$ [^3H]-taurocholate. The inhibition data were fitted to the competitive, noncompetitive, and uncompetitive models per eqn 4, 5, and 6 using WinNonlin Professional (Kolhatkar and Polli, 2010).

$$J = \frac{J_{\max} S}{K_t (1 + I/K_i) + S} + P_p S \quad \text{eqn 4}$$

$$J = \frac{J_{\max} S}{(1 + I/K_i) (K_t + S)} + P_p S \quad \text{eqn 5}$$

$$J = \frac{J_{\max} S}{K_t + S (1 + I/K_i)} + P_p S \quad \text{eqn 6}$$

3. RESULTS

3.1 Synthesis

Seventeen conjugates were successfully synthesized in moderate to high yield. Compounds **11** and **13** were purchased. Table 1 identifies the 19 compounds. A majority (i.e. 12; compounds **1–9**, **11**, **12**, **17**) were C-7 analogues. Compounds **1–9** were C-7 conjugates that differ in their substitution pattern on the C-7 aromatic ring. Four compounds were C-3 conjugates (compounds **10**, **14**, **15**, and **18**). Compounds **13** and **19** were both C-3 and C-7 analogues. Compound **16** lacked conjugation at C-3 and C-17. Of the 19 compounds, 11 employed UDCA as the bile acid and five employed CDCA as the bile acid. UDCA is the 7- β epimer of the primary bile acid CDCA.

3.2 Inhibition study

Table 1 lists compound K_i values against ASBT and NTCP. Binding affinity ranged from strong inhibition with K_i in the single micromolar range, to non-inhibitor. Figure 4 illustrates the inhibition of taurocholate by 7-(4-fluorobenzoyloxy) ursodeoxycholate [compound **3**] in ASBT-MDCK monolayers. Taurocholate uptake was inhibited by relatively low concentrations of 7-(4-fluorobenzoyloxy) ursodeoxycholate, which is a C-7 conjugate of UDCA.

3.2.1 Conjugation at C-7—C-7 conjugation in CDCA had negative impact on binding. K_i of CDCA against ASBT was 1.94 (± 0.17) μM (Balakrishnan et al., 2006b) and against NTCP was measured here to be 0.939 (± 0.057) μM , which represent a very potent inhibition of each transporter. From compound **1**, conjugation at C-7 in CDCA minimally reduced ASBT inhibition potency to $K_i = 5.79$ (± 0.08) μM , but reduced NTCP inhibition potency to a greater extent with $K_i = 15.4$ (± 2.7) μM .

Meanwhile, C-7 conjugation in UDCA generally promoted inhibition potency in ASBT, but hindered NTCP binding affinity, as assessed using compounds **2**, **3**, and **4**. K_i of UDCA against ASBT was 22.6 (± 3.0) μM (Balakrishnan et al., 2006b) and against NTCP was measured here to be 4.90 (± 0.47) μM , which represent strong inhibition of each transporter. Compounds **2**, **3**, and **4** modestly improved ASBT inhibition. Compounds **3** and **4** were

weak NTCP inhibitors. Compound **6** with the C-7 nicotinoyl substituent did not practically differ from UDCA in terms of binding affinity for ASBT and NTCP.

Glycine was attached at C-24 to lower passive permeability, since high passive permeability can cause inability to differentiate between the active and passive components, making it difficult to differentiate a substrate from a nonsubstrate. Conjugation with glycine at C-24 dominated changes in C-7 steroidal substitution effects, for both ASBT and NTCP. Comparing compound **7** ($K_i = 8.39 \mu\text{M}$ for ASBT and $K_i = 2.55 \mu\text{M}$ for NTCP) to compound **3**, and comparing compound **8** ($K_i = 15.2 \mu\text{M}$ for ASBT and $K_i = 11.8 \mu\text{M}$ for NTCP) to compound **6**, binding potency was generally increased. The effect was most significant in the fluorobenzene moiety in compound **3** for NTCP, where K_i changed from $183 (\pm 32) \mu\text{M}$ for C-24 unconjugated to $2.55 \mu\text{M}$ after glycine conjugation. This dramatic change in inhibition potency from weak to strong inhibitor highlights the favorable effect of C-24 conjugation on binding.

Of note, two pairs of C-7 conjugates differ in only the C-7 orientation. Compounds **1** and **2** are an epimer pair, derived from CDCA and UDCA respectively, and each being a C-7 benzoyl conjugate. Compound **1** is the C-7 α hydroxyl conjugate, where reactant CDCA possesses a C-7 α hydroxyl group (i.e. axially oriented) (Thomas et al., 2008). Compound **2** is the C-7 β hydroxyl conjugate (i.e. equatorial orientation of the C-7 benzoyl conjugate). Compounds **8** and **9** are the second epimer pair. In each case, there was no epimer effect on ASBT or NTCP binding, and all four compounds were potent inhibitors.

Figure 5 shows the ASBT and NTCP inhibition profiles by 7-nicotinoyl glycochenodeoxycholate (compound **8**), which is a C-7 conjugate. Conjugation at C-7 resulted in competitive inhibition of each ASBT and NTCP, as indicated by the inhibition data of compound **8**. Figure 5 panel A plots the results for ASBT-MDCK cells, while panel B plots the results for NTCP-HEK cells. The studies were performed at $I/K_i = 10$, which is a preferred condition in order to correctly identify the type of inhibition (Kolhatkar and Polli, 2010). The competitive model was the best fitting model in both cases, indicating this compound to be a competitive inhibitor of each ASBT and NTCP.

3.2.2 Oxidation of C-7 hydroxyl to keto—Compounds **11–13** were evaluated to determine effect of conjugation of a smaller ketone functional group. 7-oxo cholanolic acid (compound **11**) was less potent with only moderate binding affinity ($K_i = 95.5 \mu\text{M}$) to ASBT, compared to its 7-hydroxyl counterparts (i.e. CDCA and UDCA). Although compound **11** was a strong inhibitor of NTCP ($K_i = 10.3 \mu\text{M}$), this K_i was higher than CDCA and UDCA. Conjugation of compound **11** with glycine (i.e. compound **12**) increased inhibition potency to $20.4 (\pm 2.6) \mu\text{M}$ and $4.21 (\pm 0.44) \mu\text{M}$ in ASBT and NTCP, respectively, in agreement with C-24 glyco-conjugation effect on C-7 analogues. In Table 1, dehydrocholic acid (compound **13**) did not inhibit ASBT and was a weak inhibitor of NTCP.

3.2.3 Structural changes in C-3 region—Compounds **10, 14, 15**, and **18** were C-3 conjugates. Conjugation of a nicotinoyl group at C-3 of glycochenodeoxycholate (i.e. compound **10**) yielded strong inhibition of ASBT and NTCP.

Compounds **14** and **15** were evaluated to study effect of charge on conjugation at C-3. The introduction of a hemisuccinate group at C-3 in compound **14** and **15** allowed for the study of the effect of a negative charge at C-3. For compound **14**, the carboxylic acid in the C-24 region was protected by a benzyl group, such that compound **14** was monoanionic by virtue of the negative charge at C-3. Compound **15** was dianionic, with one charge at C-3 and the other in the C-24 region. Compound **14** was a strong inhibitor of ASBT with $K_i = 6.73 \mu\text{M}$,

but did not inhibit NTCP. Interestingly, compound **15** was a moderate inhibitor of ASBT, and a strong inhibitor of NTCP.

Compound **18** was not an inhibitor of either ASBT or NTCP, even though compound **18** has similarity to compound **6** and compound **10**, which were each much stronger inhibitors. For example, compound **18** has the same nicotinoyl group conjugated at C-3 instead to C-7 in compound **6**. However, compound **18** differs from compounds **6** and **10** by it being a methyl ester (i.e. neutral).

One compound studied that was neither a C-3 nor C-7 conjugate was compound **16**. Compound **16** (methyl ursodeoxycholate) was studied to identify contribution of conjugation in C-24 region towards interactions observed with the transporters. Esterification of the C-24 carboxylate with methyl (compound **16**) did not change binding affinity, compared to compound UDCA. K_i of compound **16** was 29.8 μM and 1.22 μM in ASBT and NTCP, respectively, which are similar to that of UDCA for these transporters. This lack of effect on C-24 esterification on ASBT inhibition has been observed previously and is the general case (Balakrishnan et al., 2006a), which further affords the lack of inhibition by compound **18** even more surprising.

3.2.4 Effect of modification at both C-3 and C-7—Compounds **13** and **19** were both C-3 and C-7 analogues, although they substantially differed chemically. Compound **13** was a tri-keto analogue of cholic acid. It did not inhibit ASBT and only weakly inhibited NTCP. Compound **19**, which lacks steroidal hydroxyl groups inhibited neither, ASBT nor NTCP.

3.3 Uptake study

Uptake studies into ASBT and NTCP were performed for compounds **1**, **5**, **6**, **8**, **9**, **10**, **12**, and **14** (only ASBT for compound **14**). These compounds showed inhibition of ASBT and NTCP, except compound **14** did not inhibit NTCP. Table 2 lists uptake parameters K_t , normalized J_{max} , and P_p . Of these eight compounds, six are C-7 analogues (i.e. compounds **1**, **5**, **6**, **8**, **9**, and **12**); while two are C-3 analogues (i.e. compound **10** and **14**).

In spite of all being ASBT and NTCP inhibitors, none of the C-7 analogues were apparently translocated by ASBT or NTCP. In Table 2, kinetic parameters reflect that they did not demonstrate active transport. Figure 6 shows practically the same uptake of 7-nicotinoyl glycochenodeoxycholate (compound **8**) in presence and absence of sodium in ASBT-MDCK monolayers. Thus, compound **8** was not a substrate of ASBT. Qualitatively similar plots were obtained for uptake of this and other compounds (**1**, **5**, **6**, **8**, **9**, and **14**) in ASBT-MDCK and NTCP-HEK cells. P_p ranged from 0.254×10^{-6} to 21.3×10^{-6} cm/s in ASBT-MDCK, and ranged from 0.727×10^{-6} to 85.6×10^{-6} cm/s in NTCP-HEK.

Based on P_p of a compound, its highest K_t that was detectable was estimated (see appendix). Subsequently, analogues were classified as non-substrate (NS) or not determinable (ND). Non-substrates are denoted to demonstratively not to be substrates. Not determinable denotes that apparent uptake was not observed, but that being a substrate could not be excluded, since sufficiently high passive permeability may possibly obscure active uptake.

In Figure 7, the uptake study for 3-nicotinoyl glycochenodeoxycholate (compound **10**) showed higher uptake with sodium than without sodium in both ASBT-MDCK monolayers and NTCP-HEK monolayers. Hence, compound **10** was a substrate of both transporters. Kinetic parameters (Table 2) were in the range of native bile acids. K_t was 8.49 μM for ASBT and 16.3 μM for NTCP, compared to taurocholate K_t of 5.03 μM and 5.31 μM , respectively. J_{max} of compound **10** was 0.000242 nmol/s/cm² and 0.00298 nmol/s/cm² for ASBT and NTCP, respectively. As J_{max} varies with transporter expression level, the J_{max}

values were normalized to the measured $J_{\max}$ of taurocholate on the same study day, yielding normalized $J_{\max}$ of compound **10** to be 2.11 and 1.48 for ASBT and NTCP, respectively. Hence, its $J_{\max}$ was about twice than that of taurocholate for ASBT, and about 50% higher than that of taurocholate for NTCP. Of note, substitution with the same nicotinoyl group at position 7 (i.e. compound **9**) yielded a non-substrate.

Compound **12** was also a substrate of both ASBT and NTCP. Figure S1 shows the uptake profile in ASBT and NTCP. Its substrate parameters were comparable to native bile acids. Compound **14** was not an ASBT substrate. This finding was in accordance with previous observations that a single negative charge in C-24 region promotes transport by ASBT. This compound was not evaluated for uptake in NTCP-HEK cells since it did not show NTCP inhibition.

4. DISCUSSION

ASBT and NTCP represent the ileal and hepatic bile acid transporters that have a vital role in gut absorption and hepatic extraction of bile acids during enterohepatic recycling. These bile acid transporters have seven transmembrane domains with 35% sequence identity and 63% amino acid sequence similarity (Balakrishnan and Polli, 2006; Banerjee and Swaan, 2006; Hagenbuch and Meier, 1994). Both transport native bile acids with high capacity and efficiency. It has been suggested that the hepatic bile acid transporter exhibits a much broader specificity for interaction than the ileal transporter, although little data has compared the substrate requirements of ASBT and NTCP (Bhat et al., 2005; Kramer et al., 1999; Kramer et al., 1997). The present work was motivated in part to compare the ASBT and NTCP activities of a single set of compounds, in order to elucidate the similarities and differences between these transporters.

Studies here were also intended to evaluate structural modifications in C-7 chemical space. Prior reports investigating the ileal and hepatic bile acid transporters were limited to the structural changes in C-24 region or C-3 regions (Balakrishnan et al., 2006a; Bhat et al., 2005; Gonzalez et al., 2009; Kramer et al., 1992; Kramer et al., 1994; Rais et al., 2010a; Rais et al., 2010b; Swaan et al., 1997; Zheng et al., 2010). No reports examined the effect of structural modifications in the C-7 chemical space on compound interactions with either ASBT or NTCP. Analogue modifications in this region were hypothesized to be able to modulate interaction with these transporters, including providing a new perspective to rational prodrug design that targets these transporters and liver-specific delivery.

4.1 Conjugate synthesis

A total of 17 conjugates were synthesized. Substituted aromatic rings such as benzene and pyridine, the most common frames among existing drugs, were conjugated to bile acids CDCA or UDCA (Bemis and Murcko, 1996). Successful synthesis of C-7 acyl derivatives was based on the difference in C-3 and C-7 hydroxyl groups reactivities (Kritchevsky et al., 1986). Most analogues employed UDCA, since UDCA is less toxic than CDCA (Trauner and Graziadei, 1999).

4.2 Inhibition study

Figure 8 plots $\log K_i$ in NTCP against $\log K_i$ in ASBT for all 19 compounds. The compounds that did not show inhibition were assigned $K_i = 1000 \mu\text{M}$. In Figure 8, there was a weak correlation between ASBT $\log K_i$ and NTCP $\log K_i$ ($R^2 = 0.24$). If plotted using K_i values that are not log transformed, $R^2 = 0.46$ due to effect of non-inhibitors. Compounds can be segregated into three groups, depending on relative inhibition affinities against ASBT and NTCP.

4.2.1 Group A: Equi-potent inhibitors—Most compounds showed similar affinities for ASBT and NTCP: compounds **1**, **2**, **6–10**, **13**, and **17–19**. For this subset of compounds, there was a strong correlation between ASBT log K_i and NTCP log K_i ($R^2 = 0.88$; $R^2 = 0.88$ if K_i values are not log transformed). Compound **1**, **2**, and **6** include unsubstituted aromatic groups conjugated at C-7 that showed strong-to-moderate inhibition of the transporters. For compounds **7–10**, glycine conjugation dominated the steroidal substitution, resulting in potent inhibition. Compound **13** did not show any ASBT inhibition and was also a weak NTCP inhibitor; the distorted shape of its steroidal nucleus due to ketone substitution at 3, 7, and 12 may be responsible for its relative lack of affinity. For compounds **17–19**, which possess aromatic substitution at C-3 and/or C-7, steric hindrance at the possible binding sites may hamper activity.

4.2.2 Group B: ASBT-preferred inhibitors—Four compounds showed higher affinity for ASBT than NTCP: compound **3**, **4**, **5**, and **14**. Although previous literature reports suggested NTCP exhibits much broader substrate specificity, these compounds showed a strong affinity for ASBT and demonstrated weak or no inhibition of NTCP. Among these four compounds, compounds **3**, **4**, and **5** are similar compounds that displayed the pattern where increased substituent size reduced ASBT and NTCP affinity ($F < CH_3 < t\text{-butyl}$). The effect was more pronounced in NTCP, with NTCP K_i of about 200 μM or higher.

Compound **14** has a benzyl ester in the C-24 region and carboxylate group in C-3 region. This compound was a strong inhibitor of ASBT but lacked affinity for NTCP. The lack of affinity may not be due to the charge in the C-3 region, but rather due to the presence of the bulky benzyl group in C-24 region, which may hinder interaction with the transporter binding site. Removal of this benzyl group (i.e. compound **15**) afforded a strong NTCP inhibitor. Strong ASBT inhibition by compound **14** but lack of NTCP inhibition suggests differences in binding sites between these transporters that involves the C-24 bile acid region.

4.2.3. Group C: NTCP-preferred inhibitors—Five compounds showed higher affinity for NTCP than ASBT: compound **11**, **12**, **15** and **16**. Compound **11** was a strong NTCP inhibitor but a moderate ASBT inhibitor, indicating that oxidation of the 7-hydroxyl group to ketone reduced affinity for ASBT. This observation was similar to that of Kramer *et al.* where oxidation of the 12-hydroxyl group in cholic acid to keto reduced affinity for the rabbit ileal bile acid transporter (Kramer *et al.*, 1999). Removal of this ketone functional group at C-7 and C-12 yields lithocholic acid and chenodeoxycholic acid, respectively, which each have a strong ASBT affinity (Balakrishnan *et al.*, 2006b). A ketone group perhaps distorts the steroid nucleus structure, reducing affinity for ASBT but not NTCP affinity. Although compound **11** was a strong inhibitor of NTCP, its K_i was higher than that of UDCA and CDCA in NTCP. As expected, conjugation with glycine (compound **12**) improved binding affinity of compound **11** in ASBT and NTCP.

Compound **15** was a strong inhibitor of NTCP but only a moderate inhibitor of ASBT. Compared to compound **14**, compound **15** involves the removal of the benzyl group at C-24, which allowed more potent interaction with NTCP, but reduced binding with ASBT.

Compound **16** was a strong inhibitor for ASBT and NTCP, although it possessed higher affinity for NTCP. Its K_i values were similar to that of UDCA. Compared to UDCA, compound **16** involves methyl esterification of the C-24 carboxylic acid, which did not affect affinity, especially when 3- and 7- hydroxyl groups were not conjugated.

4.3 Uptake study

A C-3 analogue, compound **10** afforded active uptake by ASBT and NTCP, with substrate parameters comparable to that of native bile acids. Compound **12** also demonstrated active uptake by both transporters. None of the five C-7 conjugates (i.e. compounds **1**, **5**, **6**, **8**, and **9**) were a substrate of either ASBT or NTCP. The aromatic ring at C-7 in these five appears to disallow translocation. Meanwhile, the C-7 keto in compound **12** was translocated across both ASBT and NTCP, perhaps due to the smaller size of the ketone functional group versus the aromatic ring, or a ketone allows similar interactions as a native hydroxyl group.

Interestingly, uptake results across ASBT and NTCP were similar, in spite of greater difference in inhibition data. Hence, results from combined inhibition and uptake studies suggest differences in initial binding sites between ASBT and NTCP, whereas solute translocation involves similar interaction in ASBT and NTCP. An prodrug that targets hASBT and hNTCP would ideally be a strong substrate for both transporters.

NTCP has previously been shown to transport non-steroidal molecules, such as rosuvastatin (Ho et al., 2006). Substrate specificity of ASBT was though to be limited to bile acids. However, we recently identified non-bile acid small molecules as substrates of ASBT (Kolhatkar et al., 2011). Whether rosuvastatin is a substrate of ASBT remains to be examined. Nevertheless, such studies that compare inhibition, as well as uptake, in both transporters have the potential to provide insights into the solute interaction with these transporters.

4.4 Comparison to previous studies

Baringhaus *et al.* developed a pharmacophore model based on a training set of 17 diverse rabbit Asbt inhibitors (Baringhaus et al., 1999). This model had five features: one hydrogen bond donor, one hydrogen bond acceptor, and three hydrophobic features. Native bile acids, including taurocholate, taurochenodeoxycholate and taurodeoxycholate, mapped only four of the five features. The model mapped the 12 α -hydroxy group in taurocholate as a hydrogen bond donor. The high affinity and uptake rate for CDCA (i.e. 3 α - and 7 α -hydroxyl bile acid) was explained by the 7 α -hydroxyl group as the hydrogen bond donor in absence of 12 α -hydroxy group. The 3 α -hydroxy group which is present in all native bile acids did not map any specific feature in the model. However, lithocholic acid, which lacks 12 α - and 7 α -hydroxyls and only possesses a 3 α -hydroxyl group, had high affinity and uptake rates for ASBT (Balakrishnan et al., 2006b). In addition to not mapping the 3 α -hydroxyl group, this model of Baringhaus *et al.* did not map any interaction with steroidal ring A and suggested that steroidal ring A or position 3 may serve as attachment for drug moieties for drug delivery.

However, Bhat *et al.* attached a substituted pyrazole to glycocholic acid (i.e. 7 α - and 12 α -hydroxyl bile acid) via steroidal ring A (Bhat et al., 2005). The conjugate showed only weak interaction with ASBT. Another compound with substituted pyrazole attached to ring A but with no steroidal hydroxyl groups was a strong inhibitor of ASBT and NTCP, and was translocated by ASBT (Bhat et al., 2005). No pharmacophore model has explained these results.

In conclusion, results show that ASBT and NTCP accommodate a wide range of substituents for binding, but that all major C-7 modifications resulted in analogues that were not translocated by either ASBT or NTCP. Interestingly, a C-3 modification that was not tolerated at C-7 still afforded translocation via ASBT and NTCP, further confirming the relative unacceptability of C-7 modification. Results also characterize the relative promiscuity of ASBT and NTCP. Compared to ASBT, NTCP demonstrated a generally similar binding potency across compounds, although showed less binding affinity toward

C-24 unconjugated bile acids with aromatic substitution at C-7. Results suggest that drug conjugation of drug to the C-3 hydroxyl group, rather than C-7, has potential to provide as a bile acid transporter prodrug strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LIST OF ABBREVIATIONS

ASBT	apical sodium dependent bile acid transporter
NTCP	sodium-taurocholate cotransporting polypeptide
HBSS	Hank's balanced salt solution
SFB	sodium-free buffer
UDCA	ursodeoxycholic acid
CDCA	chenodeoxycholic acid
TCA	taurocholic acid

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APPENDIX

The objective was to identify a decision tool to conclude when a compound is a non-substrate.

Decision tool derivation

Transport of a substrate across a transporter-expressing monolayer involves active and passive flux, as represented by eqn A1.

$$J_t = \frac{J_{\max} S}{K_t + S} + P_p S \quad \text{eqn A1}$$

where J_t is total flux.

Distinguishing a substrate from a non-substrate depends on the ability to differentiate between total and passive flux. Passive flux depends on P_p and substrate concentration. P_p is generally independent of experimental conditions. Flux due to transporter-mediated translocation depends on K_t , transporter expression level, and substrate concentration. However, high P_p and/or high substrate concentration can cause inability to differentiate between the active and passive components, since passive flux can dominate. This inability to differentiate is most problematic for substrates with high K_t (i.e. low affinity substrates).

when $S = K_t$, eqn A1 yields

$$J_t = \frac{J_{\max}}{2} + J_p \quad \text{eqn A3}$$

where J_p is the passive flux under this condition (i.e. $J_p = P_p \cdot K_t$).

Assuming $J_t = 2J_p$ and substituting into eqn A3,

$$2J_p = \frac{J_{\max}}{2} + J_p \quad \text{eqn A4}$$

$$J_p = \frac{J_{\max}}{2} \quad \text{eqn A5}$$

Substituting $J_p = P_p \cdot K_t$ into eqn A5

$$P_p K_t = \frac{J_{\max}}{2} \quad \text{eqn A6}$$

$$K_t = \frac{J_{\max}/2}{P_p} \quad \text{eqn A7}$$

Eqn A7 delineates the requisite condition when total flux is twice the passive flux when $S = K_t$. Since it is assumed that substrates cannot be differentiated from non-substrates when $J_t < 2 \cdot P_p S$, eqn A7 identifies the highest K_t value that allows for substrates to be differentiated from non-substrates. Hence, eqn A7 is re-written

$$K_t^{\max} = \frac{J_{\max}/2}{P_p} \quad \text{eqn A8}$$

where $K_t^{\max}$ is not the actual K_t of the substrate, but the maximum allowable K_t that will allow $J_t \geq 2 \cdot P_p S$, when $S = K_t$.

Eqn A8 indicates that two factors promote the differentiation of substrates from non-substrates when $S = K_t$: high $J_{\max}$ and low P . That is, compounds with low affinity (i.e. higher K_t) can be discerned as substrates or non-substrates, provided they have sufficiently high $J_{\max}$ or low P_p .

Implications of eqn A8 were confirmed by simulations performed under varying P_p , $J_{\max}$

values, and K_t values. Calculations confirmed that if $K_t > \frac{J_{\max}/2}{P_p}$, then $J_t < 2 P_p S$, which presumably affords an experiment to not be able to differentiate a substrate from a non-substrate.

It should be noted that this above approach assumes that an experiment employs $S = K_t$. However, uptake studies often use substrate concentrations above K_t , which only makes differentiating between substrates and non-substrates more difficult, due to an increasing relative contribution of passive flux.

Application

Table 2 shows several compounds were concluded to not be substrates. From eqn A8, their estimated $K_t^{\max}$ ranged from about 10 – 1000 μM . Experiments below the estimated $K_t^{\max}$ found total flux was the same, either with or without sodium, such that non-substrate was concluded.

In Table 2, one compound (compound **1**) was not determinable – i.e. it was not possible to differentiate whether it was a non-substrate or that its active uptake was not measurable. The compound's high permeability ($P_p = 85.6 \times 10^{-6}$ cm/s) and assumed $J_{\max}$ equal to taurocholate resulted in $K_t^{\max}$ equal to about 1 μ M. An analytical method could not be developed to measure drug uptake from a donor concentration of 1 μ M. Experiments using $S = 10$ μ M found total flux was the same, either with or without sodium. However, since the S was above the estimated $K_t^{\max}$, it was conservatively concluded that active uptake was not determinable, although perhaps others would conclude compound **1** not to be a substrate.

Figure 1.
Synthetic approach for compounds **1–6**, **16**, **18** and **19**.

Figure 2.
Synthetic approach for compounds 7–9, 12 and 17.

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Figure 4. Inhibition profile for 7-(4-fluorobenzoyloxy) ursodeoxycholate [i.e. compound **3**] in ASBT-MDCK monolayers. K_i was 10.3 μM .

Panel A

Panel B

Figure 5. Nonconventional inhibition data for 7-nicotinoyl glycocholate [Compound 8] in (A) ASBT-MDCK monolayers and (B) NTCP-HEK monolayers. Inhibitor concentration was 152 μM and 118 μM in ASBT and NTCP studies, whereas taurocholate concentration varied from 1–200 μM. The competitive model was the best fitting model in both cases.

Figure 6.

Concentration-dependent uptake profile for 7-nicotinoyl glyoursodeoxycholate [i.e. compound **8**] into ASBT-MDCK monolayers. Compound **8** was not an ASBT substrate since it exhibited similar uptakes in the presence (●) and in the absence (○) of sodium.

Panel A

Panel B

Figure 7. Concentration-dependent uptake profile for 3-nicotinoyl glycochenodeoxycholate [i.e. compound **9**] into (A) ASBT-MDCK monolayers and (B) NTCP-HEK monolayers. For each transporter, uptake was greater in the presence of sodium ($\bullet$) than in the absence ($\circ$) of sodium, indicating this compound to be both ASBT and NTCP substrate.

Figure 8. Plot of $\log K_i$ against NTCP versus $\log K_i$ against ASBT. Inhibition data can be classified in three groups: equi-potent inhibitors, ASBT-preferred inhibitors, NTCP-preferred inhibitors.

Table 1

Structures and Ki values of bile acid conjugates. Inhibitory Ki value was measured against ASBT and NTCP. Compounds varied in terms of structural features at C-3, C-7, and the C-24 region. Compounds **1** – **9** are C-7 conjugates with a negative change in the C-24 region. Ki ranged from single μM to non-inhibitor.

No.	Structure					Ki (μM)	
	R1	R2	R3	X	ASBT	NTCP	
1	OH		H	OH	5.79±0.80	15.4±2.7	
2	OH		H	OH	8.19±1.56	24.4±4.5	
3	OH		H	OH	10.3±1.1	183±32	
4	OH		H	OH	13.4±2.6	198±72

No.	Structure					Ki (μM)		
	R1	R2	R3	X	ASBT	NTCP		
5	OH		H	OH	30.8±6.8	264±82		
6	OH		H	OH	34.2±4.0	11.7±2.5		
7	OH		H		8.39±2.52	2.55±0.17		
8	OH		H		15.2±0.8	11.8±2.1		
9	OH		H		15.6±2.2	9.29±1.80

No.	Structure				Ki (μM)	
	R1	R2	R3	X	ASBT	NTCP
10		HO	H		6.45±0.66	5.38±0.44
11	OH		H	OH	95.5±14.9	10.3±2.30
12	OH		H		20.4±2.6	4.21±0.44
13				OH	No inhibition	355±59
14		HO	H		6.73±0.60	No inhibition
15		HO	H		73.9±5.6	10.7±0.7
16	OH		H	OCH ₃	29.8±5.1	1.22±0.10

No.	Structure				Ki (μM)	
	R1	R2	R3	X	ASBT	NTCP
17	OH		H		140±31.2	136±15
18		HO	H	OCH ₃	No inhibition	No inhibition
19			H	OCH ₃	No inhibition	No inhibition
CDCA	OH	HO	H	OH	1.94±0.17 ^a	0.939±0.057
UDCA	OH	HO	H	OH	22.6±3.0 ^a	4.90±0.47

^aBalakrishnan et al., 2006b

Table 2

Structures and uptake kinetic parameters of bile acid conjugates. Uptake was measured across ASBT and NTCP. Kt, norm Jmax, and Pp are the transporter Michaelis-Menten parameters, transporter capacity parameter, and compound passive permeability. Compound **10** was the most potent ASBT substrate. Compound **10** and **12** were about equi-potent as NTCP substrates. C-7 conjugates were either non-substrates or could not be determined to be substrates, due to high passive permeability.

No	Structure			ASBT			NTCP		
	R1	R2	X	Kt (μM)	Norm Jmax	Pp*10 ⁶ (cm/s)	Kt (μM)	Norm Jmax	Pp*10 ⁶ (cm/s)
1	OH		OH	NS	NS	21.3±1.8	ND	ND	85.6±14.3
5	OH		OH	NS	NS	2.41±0.27	NS	NS	2.17±0.09
6	OH		OH	NS	NS	21.2±0.9	NS	NS	7.11±0.23
8	OH			NS	NS	0.95±0.05	NS	NS	7.26±0.45

No	Structure		ASBT				NTCP			
	R1	R1	X	Kt (μM)	Norm Jmax	Pp*10 ⁶ (cm/s)	Kt (μM)	Norm Jmax	Pp*10 ⁶ (cm/s)	
9	OH			NS	NS	0.88±0.01	NS	NS	0.73±0.04	
10		HO		8.49±1.34	2.1	5.62±0.13	16.3±3.0	1.48	12.7±0.9	
12	OH			34.0±7.64	0.8	0.25±0.06	20.7±3.9	1.57	1.75±0.49	
14		HO		NS	NS	24.9±1.1	-	-	-	
CDCA	OH	HO	OH	ND	ND	20.7±0.7 ^a	ND	ND	60.7±0.5	
UDCA	OH	HO	OH	ND	ND	21.2±0.9 ^a	ND	ND	27.2±0.8	

ND = not determinable due to high passive permeability

NS= not substrate

^aBalakrishnan et al., 2006b