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A Substrate Pharmacophore for the Human Sodium Taurocholate Co-transporting Polypeptide

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Abstract

Human Sodium Taurocholate Co-transporting Polypeptide (NTCP) is the main bile acid uptake transporter in the liver with the capability to translocate xenobiotics. While its inhibitor requirements have been recently characterized, its substrate requirements have not. The objectives of this study were a) to elucidate NTCP substrate requirements using native bile acids and bile acid analogs, b) to develop the first pharmacophore for NTCP substrates and compare it with the inhibitor pharmacophores, and c) to identify additional NTCP novel substrates. Thus, 18 native bile acids and two bile acid conjugates were initially assessed for NTCP inhibition and/or uptake, which suggested a role of hydroxyl pattern and steric interaction in NTCP binding and translocation. A common feature pharmacophore for NTCP substrate uptake was developed, using 14 native bile acids and bile acid conjugates, yielding a model which featured three hydrophobes, one hydrogen bond donor, one negative ionizable feature and three excluded volumes. This model was used to search a database of FDA approved drugs and retrieved the majority of the known NTCP substrates. Among the retrieved drugs, irbesartan and losartan were identified as novel NTCP substrates, suggesting a potential role of NTCP in drug disposition.

Keywords

Bile acids; sodium taurocholate co-transporting polypeptide (NTCP); pharmacophore; transporters; irbesartan; losartan

1. Introduction

The sodium taurocholate cotransporting polypeptide (NTCP, *SLC10A1*) is predominantly expressed at the basolateral membrane of hepatocytes and accounts for the efficient extraction of bile acids into the liver. Human NTCP has been the subject of computational modeling to determine the inhibitor pharmacophore (Dong et al., 2013; Greupink et al.,

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2012). A common feature pharmacophore possessed two hydrophobes, one hydrogen bond acceptor and excluded volume. As yet, there is no NTCP substrate pharmacophore available, nor analysis of what drugs may be substrates. Studies showed that NTCP is a target for hepatitis B virus (HBV) entry and infection (Fu et al., 2014; Yan et al., 2012). Recent results suggest that NTCP is one of the HBV receptors that contribute to human infection and therefore inhibition of uptake may also prevent HBV infection (Seeger and Mason, 2013).

In addition, this transporter translocates drugs such as rosuvastatin, pitavastatin, fluvastatin and micafungin (Choi et al., 2011; Ho et al., 2006; Yanni et al., 2010). NTCP has also been employed as a prodrug target for liver-specific drug delivery, where parent drug is conjugated to bile acid for NTCP uptake (Briz et al., 2002; Macias et al., 1998; Kramer et al., 1992; Kullak-Ublick et al., 1997). Thus understanding the substrate requirements of human NTCP, including a substrate pharmacophore, will help identify substrates, including drugs and prodrugs that target NTCP.

Previous published studies used native bile acids or bile acid analogs to probe the substrate requirements of rabbit and rat NTCP (Hata et al., 2003; Kramer et al., 1999; Mita et al., 2005). However, the structure-activity relationship of NTCP may be species specific, such that results from rabbit and rat may not apply to human NTCP. For example, rosuvastatin was found to be a substrate for human NTCP but not rat Ntcp (Ho et al., 2006). Also, bosentan is a much more potent inhibitor of rat Ntcp than human NTCP (Leslie et al., 2007).

The substrate requirements of human NTCP have not been systematically evaluated and no computational models for human NTCP substrates have been developed. Limited efforts have been put forth to assess drugs as NTCP substrates, including bile acids. Thus the objectives of this study were a) to elucidate NTCP substrate requirements using native bile acids and bile acid analogs b) to develop the first pharmacophore for NTCP substrates and compare it with the inhibitor pharmacophores, and c) to identify additional NTCP novel substrates.

Briefly, both native unconjugated bile acids and C-24 conjugates were initially assessed for human NTCP inhibition and uptake. Our results indicate the involvement of the steroidal hydroxyl groups and C-24 steric interaction in NTCP binding and translocation. Based on these native bile acids and bile acid analogs, a common feature pharmacophore was developed which included three hydrophobes, one hydrogen bond donor, one negative ionizable feature and three excluded volumes. The model was subsequently applied to identify the NTCP substrates from a database of FDA approved drugs. The angiotensin II receptor antagonist irbesartan and losartan were found to be novel NTCP substrates.

2. Material and methods

2.1 Materials

Figure 1 illustrates the general structure of native bile acids which vary in steroid hydroxyl pattern and C-24 conjugation pattern. For the first objective, 18 native bile acids (Table 1) were purchased. CDCA and HBTU were purchased from AK Scientific, Inc (Union City, CA). UDCA was purchased from Spectrum Chemical (New Brunswick, NJ). TCA, HDCA,

GLCA and TUDCA were purchased from EMD Millipore (Billerica, MA). HDCA was obtained from MP Biomedicals (Solon, OH). All other native bile acids and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Geneticin, fetal bovine serum (FBS), trypsin, and DMEM were obtained from Invitrogen (Rockville, MD).

2.2 Synthesis of CDCA-L-Val-OH and CDCA-L-Glu- γ -Benzyl Ester

Two bile acid C-24 conjugates (Table 1) were synthesized to assess the impact C-24 side chain on NTCP interaction and transport. CDCA-L-Val-OH and CDCA-L-Glu- γ -Benzyl Ester were synthesized as previously described with minor modification (Rais et al., 2010). Briefly, CDCA (5 g, 12.7 mmol) was added to 15mL dimethylformamide(DMF) along with N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU, 5.07 g, 13.37 mmol), 1-Hydroxybenzotriazole hydrate (HOBt, 0.86 g, 6.37 mmol), and triethylamine (TEA, 1.86 mL, 13.37 mmol). After 4 hours in room temperature, the reaction was quenched with 50mL water and extracted with ethyl acetate (EtOAc) (3x). The combined organic layer was washed with water (3x), brine (1x) and dried over the anhydrous sodium sulfate. The solvent was evaporated to yield HOBt ester of CDCA.

HOBt ester of CDCA (500 mg, 0.98 mmol) was added to 5mL DMF along with TEA (0.273 mL, 1.96 mmol) and either L-Glutamic acid γ -benzyl ester (212 mg, 0.89 mmol) or L-Valine benzyl ester hydrochloride (217 mg, 0.89 mmol). The reaction was stirred overnight and extracted with EtOAc and washed with water and brine. After dried over the anhydrous sodium sulfate, the solvent was evaporated to yield crude product which was subjected to silica gel column chromatography for further purification. CDCA-L-Val-benzyl ester was purified with mobile phase as 80% EtOAc and 20% dichloromethane (DCM), followed by catalytic hydrogenation to remove benzyl ester group using 10% Pd/charcoal in methanol for 4 hours. Filtration and evaporation of solvent yielded final product CDCA-L-Val-OH. CDCA-L-Glu- γ -Benzyl Ester was purified by silica gel column chromatography with mobile phase as DCM and methanol (v/v=12.5:1) with 0.5% acetic acid yielding final product. Both final compounds were assessed via NMR and MS (Table S1).

2.3 Cell culture

HEK293 cells were stably transfected with human NTCP and were grown in 37 °C, 5% CO₂ atmosphere with 90% relative humidity and fed every 2 days, as previously described (Dong et al., 2013). The medium is DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μ g/mL streptomycin and geneticin (1 mg/mL). Cells were passaged when they reach 80% confluence.

2.4 Inhibition study

After reaching 80% confluence, cells were seeded in 24 well Biocoated plates at the density of 300,000 cells/well for two days. Inhibition study was conducted in either Hank's Balance Salts Solution (HBSS, pH=6.8) with 137mM sodium chloride or sodium free buffer (SFB, pH=6.8) where sodium chloride was replaced with 137mM tetraethylammonium chloride. Cells were exposed to donor solution containing cold 10 μ M taurocholate as NTCP typical substrate, 0.5 μ Ci/ml [³H]-taurocholate and test compound. After incubation for 5 min, buffer was removed, followed by cell wash with ice cold SFB. Cell was lysed by

acetonitrile. After acetonitrile evaporated, the lysate was dissolved in phosphate buffered saline (PBS) and subject to liquid scintillation counting of associated radioactivity. As the positive control, 200 μM taurocholate uptake was measured in the presence of sodium while 200 μM taurocholate uptake in the absence of sodium was measured as the negative control since NTCP is not functional in the absence of sodium.

To determine inhibition potency K_i of NTCP inhibitors, seven drug concentrations were employed. Inhibition data was fitted to Equation 1 and calculated through non-linear regression using WinNonlin (Pharsight; Sunnyvale, CA).

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

where J is the taurocholate flux, $J_{\max}$ is maximal flux of taurocholate without inhibitor. $J_{\max}$ was estimated from taurocholate uptake studies at high taurocholate concentrations (i.e. 200 μM) where transporter was saturated. I is the inhibitor concentration, S is the taurocholate concentration, P_p is the taurocholate passive permeability which was determined by 200 μM taurocholate uptake in the absence of sodium, K_i is the NTCP inhibition constant, and K_t is the NTCP taurocholate Michaelis-Menten constant which is 22.7(+3.4) μM (Dong et al., 2013).

2.5 Uptake study

Uptake study was conducted on NTCP-HEK293 cells in 24 well Biocoated plates. On day 2 after seeding, cells were exposed for 5 min to various concentrations of compound (0-200 μM in HBSS buffer). For CA, CDCA, UDCA and TCA, 0.5 $\mu\text{Ci/ml}$ corresponding tritium labeled bile acids was also added. After the buffer was removed, cells were immediately washed with ice cold SFB followed by cell lysis by acetonitrile. The lysate was then dissolved in the acetonitrile/ H_2O (1:1) with 500nM internal standard. Non-NTCP mediated passive uptake was assessed by measuring uptake in the absence of sodium. The samples were stored at -80°C for further analysis with either LC/MS or liquid scintillation counter as describe above.

In order to determine the K_t and $J_{\max}$ of the native bile acids and bile acid conjugates, eight concentrations of the compound were employed. The uptake data in HBSS buffer was fit to equation 2 and calculated through non-linear regression using WinNonlin.

$$J = \frac{J_{\max} S}{K_t + S} + P_p S \quad \text{Equation 2}$$

where J is compound flux, $J_{\max}$ is maximal flux of the compound; S is the compound concentration; P_p is the compound passive permeability which is estimated from non-NTCP mediated passive uptake. As a positive control, $J_{\max}$ of taurocholate was estimated from taurocholate uptake studies at high taurocholate concentrations where transporter was saturated (i.e. 200 μM) in each study. The $J_{\max}$ of the compound was normalized against

$J_{\max}$ of taurocholate yielding normalized $J_{\max}$ to correct the variation in NTCP expression level across studies.

2.6 Analytical method

Flux of native bile acids, bile acid conjugates, and drugs into cells was quantified using Waters ACQUITY UPLC/TQD system (Waters, Milford, MA). For bile acid or bile acid conjugates analysis, an XBridge C18 column (3.5 μm , 2.1 $\times$ 50 mm; Waters, Milford, MA) was used. For drug analysis, a UPLC Ethylene Bridged Hybrid C8 column (1.7 μm , 2.1 $\times$ 50 mm; Waters, Milford, MA) was used. The mobile phase was composed of a gradient with acetonitrile and water with 0.1% formic acid respectively. The flow rate was 0.7mL/min and 0.5mL/min for bile acids and drugs respectively. The limit of quantification for all compounds was between 1nM and 50nM. Mass transitions and internal standard performance are detailed in Table S2.

2.7 Common feature pharmacophore development

A common feature pharmacophore was developed as previously described (Dong et al., 2013). Briefly, the common feature pharmacophore was developed using Discovery Studio 4.1 (BIOVIA, San Diego, CA). CAESAR was used for conformation generation, with a maximum of 255 conformations, energy threshold of 20 kcal/mol, and up to 10 excluded volume. 14 native bile acids or bile acid analogs were used as the training set where chenodeoxycholic acid and lithocholic acid were assigned as non-substrates (based on $J_{\max}/K_t$). Hydrogen bond acceptors, hydrogen bond donors, hydrophobic, positive ionizable and negative ionizable features were selected. The model was subsequently used to screen the Clinician's Pocket Drug Reference (SCUT) database using a maximum of one omitted feature with rigid fitting which resulted in 295 drugs retrieved with the ligand pharmacophore mapping protocol. Pharmacophore alignments were performed using the pharmacophore comparison protocol.

3. Results

3.1 Inhibition study by bile acids

Eighteen native bile acids and two bile acid conjugates were initially assessed for NTCP inhibition potency. Among native bile acids, CA has three hydroxyl groups (Figure 1 and Table 1). HDCA, CDCA, UDCA and DCA possess two hydroxyl groups. LCA has one hydroxyl group. All hydroxyl groups are oxidized to ketone in DHCA. All native bile acids but DHCA inhibit taurocholate uptake in a concentration dependent manner with a similar inhibition profile as is shown for GUDCA (Figure 2). DHCA is not an inhibitor of NTCP indicating the importance of a hydroxyl group in bile acid-transporter interaction.

Table 2 lists the human NTCP K_i values for the 17 native bile acids that inhibited human NTCP. NTCP inhibition potency was generally the inverse to the number of the hydroxyl groups. Meanwhile, glyco- and tauro-conjugation had no apparent impact on K_i . CA and its corresponding glycine or taurine conjugates had higher K_i (e.g. about 30 μM) than other bile acids. Meanwhile, K_i of LCA, GLCA and TLCA were generally lower (e.g. about 5 μM) than other unconjugated, glyco-conjugates, and tauro-conjugates, respectively. Among the

di-hydroxy bile acids (i.e. HDCA, UDCA, CDCA and DCA), the position of hydroxyl groups had no apparent impact of K_i .

The K_i for CDCA-L-Val-OH and CDCA-L-Glu- γ -Benzyl Ester were 6.10 (+0.45) μM and 3.28 (+0.26) μM respectively, which are slightly lower than either K_i of GCDCA or TCDCA. Hence, the C-24 side chain on CDCA-L-Val-OH and CDCA-L-Glu- γ -Benzyl Ester may exert some favorable steric interaction with NTCP.

3.2 Uptake study of bile acids

CA, CDCA, UDCA, LCA and their corresponding glycine and taurine conjugates, along with two C-24 conjugates, were assessed for NTCP uptake. Table 3 lists their uptake parameter K_t , normalized $J_{\max}$, transport efficacy (normalized $J_{\max}/K_t$), and passive permeability. All conjugated bile acids were translocated into cells in a sodium dependent way with a similar profile as shown for GUDCA (Figure 3). Also, two unconjugated bile acids (i.e. CA and UDCA, but not CDCA and LCA) were substrates. CDCA and LCA showed indistinguishable uptake in the presence or absence of sodium (Student's t-test, $p>0.05$). However, it may be possible that active transport was occurring, but masked by the compounds' high passive permeability (Table 3). The K_t of the CA was 54.9 μM , and K_t of UDCA was 1.55 μM . Interestingly, the normalized $J_{\max}$ of UDCA was 14-fold lower than that for taurocholate, suggesting little contribution of NTCP to UDCA uptake.

Like with K_i , K_t potency were inversely related to the number of the hydroxyl groups. CA and its conjugate showed higher K_t (e.g. about 18 μM) than other bile acids. LCA conjugates exhibited the lowest K_t values (e.g. about 5 μM), while LCA was not an apparent substrate, perhaps due to its high passive permeability.

Interestingly, normalized $J_{\max}$ of CA and its conjugates was generally higher than CDCA, UDCA and LCA and their conjugates, indicating three hydroxyl groups promote transporter capacity. In terms of the transport efficiency (i.e. normalized $J_{\max}/K_t$), taurine conjugates were slightly higher than glycine conjugates, and followed the order: LCA>CDCA>UDCA>CA. Regarding CDCA-L-Val-OH and CDCA-L-Glu- γ -Benzyl Ester and steric bulk reduced both transport capacity and transport efficiency, but not K_t . Overall, uptake studies indicate that the K_t of bile acid and bile acid conjugates were dominated by hydroxyl pattern, while transport efficiency was determined by both hydroxyl pattern and C-24 steric interaction.

3.3 Common feature pharmacophore of NTCP substrates

The 14 native bile acids and bile acid analogs were used as the training set to develop a common feature pharmacophore. Among them, chenodeoxycholic acid and lithocholic acid were used to generate excluded volumes. The resulting model was composed of three hydrophobes, one hydrogen bond donor, one negative ionizable feature and three excluded volumes (Figure 4). The model was used to search the SCUT database which retrieved 297 drugs with Fit values from 0.00322 to 3.87. Among them, 11 drugs were previously identified as NTCP inhibitors, including ezetimibe, irbesartan, and losartan (Dong et al., 2013). Ezetimibe, irbesartan, and losartan were predicted as NTCP substrates with Fit values of 2.28, 0.377, and 2.22 (Figure S1 and Table S3), respectively. In particular, four of the five

previously known NTCP substrates (i.e. pitavastatin, atorvastatin, fluvastatin, and micafungin) were retrieved, reflecting favorably on the pharmacophore. However, the known substrate rosuvastatin was not retrieved.

3.4 Irbesartan and losartan are substrates of NTCP

Ezetimibe, losartan and irbesartan were assessed as NTCP substrates. Fenofibrate and bendroflumethiazide were not retrieved by the pharmacophore but were also tested since we had previously observed them to be inhibitors (Dong et al., 2013). At each concentration of losartan and irbesartan, uptake was greater in the presence than absence of sodium, indicating irbesartan and losartan were substrates of human NTCP (Figure 5). In addition, taurocholate inhibited irbesartan and losartan uptake in a concentration dependent fashion, further supporting the contribution of NTCP to irbesartan and losartan uptake (Figure 6). The K_t of irbesartan and losartan were 32.7 μ M and 38.5 μ M respectively (Table 4). However, J_{max} was only about 20% that of taurocholate J_{max} suggesting they are likely weaker substrates. Meanwhile, ezetimibe, fenofibrate, and bendroflumethiazide were found not to be NTCP substrates. Hence, the pharmacophore accurately predicted irbesartan, losartan, fenofibrate and bendroflumethiazide, but was incorrect for ezetimibe.

Rosuvastatin was previously reported to be a substrate of human NTCP, which contributed 35% to rosuvastatin's hepatic uptake (Ho et al., 2006). Here in the present study, rosuvastatin's K_t was measured to be 72.8 μ M (Table 4 and Figure S2), which is comparable with the previous report of 65 μ M (Ho et al., 2006). Irbesartan and losartan exhibited K_t values that were twice as potent as rosuvastatin K_t . Rosuvastatin did not map to the common feature pharmacophore (Table S3), while irbesartan and losartan did map. Rosuvastatin missed one hydrophobe, and one negative ionizable feature. In contrast, irbesartan and losartan mapped these two features better (Figure S3). The J_{max} of rosuvastatin was about 2-fold higher than the J_{max} of either irbesartan or losartan, while the transport efficiency was 0.00648 μ M⁻¹, which is only slight greater than the about 0.005 μ M⁻¹ values for irbesartan and losartan. Overall, the study results showed that NTCP may also contribute to irbesartan and losartan uptake into hepatocytes.

4. Discussion

We assessed the NTCP uptake of 12 native bile acids, two bile acid conjugates, and five drugs in order to elucidate the substrate requirements of human NTCP. The majority of our current understanding of human NTCP substrate requirements is based on rat and rabbit NTCP. However, species effects motivated us to systematically probe the substrate requirements of human NTCP.

4.1 Inhibition of NTCP

Various native unconjugated, as well as taurine and glycine conjugated bile acids, were initially measured for their inhibition potency. Almost all 18 native bile acids inhibited human NTCP, except DHCA which is consistent with a previous report (Hagenbuch and Meier, 1994). Inhibition results showed a very similar pattern from mouse and rabbit, where the binding affinity to NTCP was inversely related to the number of the hydroxyl groups.

For example, CA and its conjugates had higher K_i than CDCA, UDCA and HDCA. LCA and its conjugates generally showed the lowest K_i values (Kramer et al., 1999; Saeki et al., 2002). However oxidization of hydroxyl group on C-7 (i.e. DHCA) did not alter the binding affinity to NTCP in rabbit (Kramer et al. 1999), but hampered binding to human NTCP here, suggesting species specific structure-activity relationship of NTCP. In addition, the orientation of hydroxyl groups at C-7 (α vs β) did not impact binding affinity, K_i .

4.2 Uptake of NTCP

Of the 18 bile acids subjected to inhibition studies, 12 native bile acids and both bile acid conjugates were subsequently assessed for NTCP uptake. The Michaelis-Menten constant K_t exhibited the same trend as K_i . LCA and its conjugates showed the highest binding affinity to NTCP. The K_t of GUDCA (13.0 μ M) and TUDCA (9.53 μ M) were comparable to previous values of 15 μ M and 10 μ M, respectively (Maeda et al. 2006; Mita et al. 2006). In terms of transport efficiency as measured by J_{max}/K_t , LCA may be a more efficient drug carrier than other bile acids for prodrug design. Hence, for low dose prodrug, LCA may be a preferred drug carrier. However, LCA showed a relative low transport capacity, as measured by J_{max} , suggesting that high dose prodrug may result in less drug uptake into hepatocytes than using other bile acids as the drug carrier. In addition, LCA's higher passive permeability may also be a concern for a prodrug to achieve liver specific delivery.

Previously, UDCA was significantly transported by NTCP at low concentration while there is no significant uptake by NTCP at high UDCA concentration (König et al. 2012). Findings here suggested that this pattern is due to UDCA's moderate transport efficiency, which is a major factor at low substrate concentrations when the transporter is not saturated. However, at UDCA high concentration, its low transport capacity and high passive permeability afford UDCA to be a relatively poor substrate.

Interestingly, unconjugated bile acids are taken up by rat Ntcp in the order of CDCA > UDCA > CA (Mita et al., 2005). From studies here, this sequence is reversed for human NTCP, as CA had the highest uptake and CDCA showed no NTCP uptake. Moreover, conjugation of a bulky group with side chain (i.e. Glu- γ -Benzyl Ester, Val-OH) appeared to cause steric hindrance at the C-24 region, dramatically reducing NTCP transport capacity and transport efficiency. Meanwhile, conjugation of taurine or glycine enhanced transport capacity and transport efficiency. This observation reiterates important species differences and likely different pharmacophores.

4.3 Pharmacophore for NTCP substrates

Greupink et al. and our group have previously developed common features pharmacophores to elucidate the inhibitor requirements of human NTCP using either bile acids (Greupink et al., 2012) or FDA approved drugs (Dong et al., 2013). Greupink's pharmacophore was developed from four bile acids and estrone sulfate and possessed three hydrophobes and two hydrogen bond acceptors. Meanwhile, our model was derived from FDA approved drugs and featured two hydrophobes and one hydrogen bond acceptor. However, regarding identifying substrates, a limitation of the inhibitor pharmacophores is that the substrate also needs to be translocated by the transporter, beyond only binding to transporter. Therefore it

is a multi-step, likely multi-site recognition process which may require different molecular features.

Due to the very limited number of drugs currently identified as NTCP substrates, the pharmacophore was developed using bile acids and bile acid analogs. The model was able to retrieve the majority of the known five NTCP substrates which are FDA approved drugs. Five previously known inhibitors were evaluated as NTCP substrates. Of the five, three were predicted by the pharmacophore to be substrates (i.e. irbesartan, losartan, ezetimibe) and two were predicted to be non-substrates (e.g. fenofibrate and bendroflumethiazide). Of the five, only the prediction of ezetimibe was incorrect. Hence, although the pharmacophore was derived from bile acids and bile acid analogs, it was able to predict NTCP substrates from FDA approved drugs.

The overlap of two hydrophobes between our current substrate pharmacophore and our previous common feature inhibitor pharmacophore (Dong et al., 2013) suggested that these two hydrophobes perhaps were important for NTCP binding while other features (e.g. hydrogen bond donor, negative ionizable feature) were important for substrate translocation (Figure S4).

5. Conclusions

In conclusion, 18 native bile acids and 2 bile acid conjugates were assessed on NTCP for their inhibition and uptake. We have shown that the steroid hydroxyl pattern dramatically affects inhibition, as steric interactions determined NTCP transport capacity and efficiency. A pharmacophore was developed to elucidate NTCP substrate requirements and was used to reliably identify NTCP substrates. Both irbesartan and losartan were transported by NTCP which suggested a potential role of NTCP in their drug disposition. Such a pharmacophore could be useful to distinguish between compounds that are inhibitors and useful as HBV treatments and likely should avoid being substrates in order to have clinical efficacy or causing drug-drug interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CA	cholate
DCA	deoxycholate
CDCA	chenodeoxycholate

UDCA	ursodeoxycholate
HDCA	hyodeoxycholate
LCA	lithocholate
DHCA	dehydrocholate
GCA	glycocholate
GDCA	glycodeoxycholate
GCDCA	glycochenodeoxycholate
GUDCA	glycoursodeoxycholate
GLCA	glycolithocholate
Glu	Glutamic acid
HEK	human embryonic kidney
NTCP	sodium taurocholate cotransporting polypeptide
SCUT	Clinician's Pocket Drug Reference
TCA	taurocholate
TDCA	taurodeoxycholate
TCDCa	taurochenodeoxycholate
TUDCA	tauroursodeoxycholate
THDCA	taurohyodeoxycholate
TLCA	tauroolithocholate
Val	valine

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Figure 1.
Structure of native bile acids and bile acid conjugates. Compounds differed in substitution at C-3 (R₁), C-7 (R₃), C-12 (R₂) and C-24 (X).

Figure 2.

Inhibition of taurocholate uptake by glyoursodeoxycholate (GUDCA). Taurocholate uptake into NTCP-HEK293 cells was reduced by GUDCA in a concentration-dependent manner. Inhibition studies were conducted using seven concentrations of GUDCA (0-200 µM). Closed circles indicate observed data points, while the solid line indicates model fit. Data were presented as mean (+SEM) of three measurements at each concentration.

Figure 3.

Concentration-dependent uptake of glyoursodeoxycholate (GUDCA) into NTCP-HEK293 cells. Closed circle indicated total drug uptake by NTCP in the presence of sodium, and hence reflect total uptake. Open circle indicated passive uptake of the drug in the absence of sodium, and hence intent to reflect only passive uptake. The solid line indicated model fit of the total uptake. Data were presented as mean (+SEM) of three measurements at each concentration.

Figure 4.

Common feature pharmacophore of NTCP substrates. The pharmacophore employed 14 native bile acids and bile acid analogs. Pharmacophore features three hydrophobic features (cyan), one hydrogen bond donor (purple), one negative ionizable feature (dark blue) and three excluded volume (grey). Lithocholic acid (shown as stick format) was mapped to the pharmacophore.

Figure 5.

Concentration-dependent uptake of irbesartan (A) and losartan (B) into NTCP-HEK293 cells. Closed circle indicated total drug uptake by NTCP in the presence of sodium. Open circle indicated passive uptake of the drug in the absence of sodium. The solid line indicated model fit of the total uptake. Data were presented as mean (+SEM) of three measurements at each concentration. In panel A, there was a small difference between total and passive uptake due in part to the high passive permeability of irbesartan. In panel B, there was a larger difference between total and passive uptake due in part to the low passive permeability of losartan.

Figure 6.

Inhibition of irbesartan uptake or losartan uptake by taurocholate. Uptake of irbesartan (open bar) or losartan (closed bar) uptake into NTCP-HEK293 cells was reduced by taurocholate in a concentration-dependent manner. Data were presented as mean (+SEM) of three measurements at each concentration.

* Percent of drug uptake was statistically reduced by taurocholate (Student's t-test, $p < 0.05$).

Table 1

Functional groups at C-3, C-6, C-7, C-12, and C-24 for native bile acids and two chenodeoxycholate conjugates. Figure 1 illustrates the structure of native bile acid and bile acid analogs.

Bile acids	R ₁ (C-3)	R ₂ (C-6)	R ₃ (C-7)	R ₄ (C-12)	X (C-24)
CA	-OH	-H	-OH	-OH	-OH
DCA	-OH	-H	-H	-OH	-OH
CDCA	-OH	-H	-OH	-H	-OH
UDCA	-OH	-H	-OH (β)	-H	-OH
HDCA	-OH	-OH	-H	-H	-OH
LCA	-OH	-H	-H	-H	-OH
DHCA	=O	-H	=O	=O	-OH
GCA	-OH	-H	-OH	-OH	-NHCH ₂ COOH
GDCA	-OH	-H	-H	-OH	-NHCH ₂ COOH
GCDCA	-OH	-H	-OH	-H	-NHCH ₂ COOH
GUDCA	-OH	-H	-OH (β)	-H	-NHCH ₂ COOH
GLCA	-OH	-H	-H	-H	-NHCH ₂ COOH
TCA	-OH	-H	-OH	-OH	-NH(CH ₂) ₂ SO ₃ H
TDCA	-OH	-H	-H	-OH	-NH(CH ₂) ₂ SO ₃ H
TCDCa	-OH	-H	-OH	-H	-NH(CH ₂) ₂ SO ₃ H
TUDCA	-OH	-H	-OH (β)	-H	-NH(CH ₂) ₂ SO ₃ H
THDCA	-OH	-OH	-H	-H	-NH(CH ₂) ₂ SO ₃ H
TLCA	-OH	-H	-H	-H	-NH(CH ₂) ₂ SO ₃ H
CDCA-L-Val-OH	-OH	-H	-OH	-H
CDCA-L-Glu-γ-Benzyl Ester	-OH	-H	-OH	-H

All hydroxyl (-OH) groups are in α position, except when noted as β position. Abbreviations for bile acids are : CA, cholate; DCA, deoxycholate; CDCA, chenodeoxycholate; UDCA, ursodeoxycholate; HDCA, hyodeoxycholate; LCA, lithocholate; DHCA, dehydrocholate; GCA, glycocholate; GDCA, glycodeoxycholate; GCDCA, glycochenodeoxycholate; GUDCA, glyoursodeoxycholate; GLCA, glycolithocholate; TCA, taurocholate; TDCA, taurodeoxycholate; TCDCa, taurochenodeoxycholate; TUDCA, taoursodeoxycholate; THDCA, taurohyodeoxycholate, TLCA, tauroolithocholate

Table 2

Impact of native bile acid hydroxylation pattern and conjugation on human NTCP inhibition. Figure 1 illustrates the native bile acid structure.

Parent bile acid	Hydroxylation pattern	K_i (μ M)		
		Unconjugated	Taurine	Glycine
Cholate	Trihydroxy (3 α , 7 α , 12 α)	41.6+5.3	30.2+3.0	29.6+1.7
Hyodeoxycholate	Dihydroxy(3 α , 6 α)	10.4+0.5	7.07+0.45	NM ^a
Chenodeoxycholate	Dihydroxy(3 α , 7 α)	3.09+0.12	8.13+0.70	13.2+1.5
Ursodeoxycholate	Dihydroxy(3 α , 7 β)	10.9+0.72	13.5+1.2	15.0+1.4
Deoxycholate	Dihydroxy(3 α , 12 α)	8.54+0.38	13.3+1.4	13.6+1.2
Lithocholate	Monohydroxy(3 α)	4.09+0.70	4.28+0.25	7.29+1.15

^aNM indicates not measured.

Table 3

Uptake parameters of native bile acids or bile acid analogs for human NTCP.

Parent bile acid	C-24 conjugate	K_t (μM)	Normalized J_{max}	Normalized J_{max}/K_t (μM^{-1})	P_p (cm/s) $\times 10^6$
Cholate	Unconjugated	54.9 +13.8	0.523 +0.081	0.00953 +0.00281	4.56+0.06
	Taurine	19.8 +2.5	0.708 +0.057	0.0358 +0.0053	1.34+0.14
	Glycine	15.8 +2.7	0.508 +0.041	0.0321 +0.0060	0.417+0.080
Chenodeoxycholate	Unconjugated	NS ^a	NS ^a	NS ^a	27.6+1.5
	Taurine	8.12 +1.20	0.621 +0.058	0.0765 +0.0134	4.35+0.70
	Glycine	10.9 +1.8	0.726 +0.091	0.0666 +0.0138	2.17+0.32
	L-Valine	9.62 +1.47	0.330 +0.031	0.0343 +0.0062	1.47+0.09
	L-Glu- γ -Benzyl Ester	11.7 +4.3	0.233 +0.036	0.0199 +0.0079	7.70+0.23
Ursodeoxycholate	Unconjugated	1.55 +1.00	0.0511 +0.0093	0.0330 +0.0221	26.3+1.1
	Taurine	9.53 +2.21	0.409 +0.038	0.0429 +0.0107	0.625+0.083
	Glycine	13.0 +2.0	0.460 +0.143	0.0353 +0.0122	0.934+0.044
Lithocholate	Unconjugated	NS ^a	NS ^a	NS ^a	33.9+3.3
	Taurine	6.50 +1.87	0.495 +0.053	0.0761 +0.0234	3.37+0.63
	Glycine	4.87 +3.49	0.384 +0.070	0.0789 +0.0584	6.67+0.95

^aNS indicates not substrate of NTCP due to indistinguishable uptake in the presence or absence of sodium due to high passive permeability or possibly not a substrate.

Table 4

Uptake parameters of irbesartan, losartan and rosuvastatin for human NTCP.

Drug	K_t (μM)	J_{max} (pmol/sec/cm^2)	Normalized J_{max}	Normalized J_{max}/K_t (μM^{-1})	P_p (cm/s) $\times 10^6$
Irbesartan	32.7+5.9	0.430+0.026	0.172 +0.022	0.00526 +0.00116	6.27 +0.14
Losartan	38.5+4.9	0.483+0.022	0.193 +0.024	0.00501 +0.00089	1.48 +0.12
Rosuvastatin	72.8+10.0	0.849+0.063	0.472 +0.036	0.00648 +0.0010	0.330 +0.006