

Structural Requirements of the ASBT by 3D-QSAR Analysis Using Aminopyridine Conjugates of Chenodeoxycholic Acid

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The human apical sodium-dependent bile acid transporter (ASBT) is a validated drug target and can be employed to increase oral bioavailability of various drug conjugates. The aim of the present study was to investigate the chemical space around the 24-position of bile acids that influences both inhibition and uptake by the transporter. A series of 27 aminopyridine and aminophenol conjugates of glutamyl-chenodeoxycholate were synthesized and their ASBT inhibition and transport kinetics (parametrized as K_i , K_t , and J_{max}) measured using stably transfected ASBT-MDCK cells. All conjugates were potent ASBT inhibitors. Monoanionic conjugates exhibited higher inhibition potency than neutral conjugates. However, neutral conjugates and chloro-substituted monoanionic conjugates were not substrates, or at least not apparent substrates. Kinetic analysis of substrates indicated that similar values for K_i and K_t implicate substrate binding to ASBT as the rate-limiting step. Using 3D-QSAR, four inhibition models and one transport efficiency model were developed. Steric fields dominated in CoMFA models, whereas hydrophobic fields dominated CoMSIA models. The inhibition models showed that a hydrophobic or bulky substitute on the 2 or 6 position of a 3-aminopyridine ring enhanced activity, while a hydrophobic group on the 5 position was detrimental. Overall, steric and hydrophobic features around the 24 position of the sterol nucleus strongly influenced bile acid conjugate interaction with ASBT. The relative location of the pyridine nitrogen and substituent groups also modulated binding.

INTRODUCTION

Bile acids are primarily absorbed in the terminal ileum via the apical sodium-dependent bile acid transporter (ASBT; SLC10A2) (1, 2), which displays high transport capacity and efficiency (3). This transporter may also be employed as a prodrug target for enhancing oral drug bioavailability of poorly permeating compounds, where drug is conjugated to bile acid and bile acid allows for the conjugate to be taken up by ASBT (3). However, the lack of knowledge of ASBT substrate requirements has impeded progress in rational prodrug design to target ASBT. Structure–activity studies have largely employed ASBT inhibition rather than substrate uptake, perhaps due to inhibition studies being more facile. Various transport studies have been performed over 30 years using animal tissues and organs, but the interpretability of the bile acid transport by ASBT has been confounded by other intestinal and hepatic transporters (4).

Our laboratory previously developed a stably transfected ASBT-MDCK monolayer to evaluate ASBT-mediated transport of solutes (5). Using a series of native bile acids to evaluate ASBT binding properties, we found that chenodeoxycholate (CDCA) was the most potent inhibitor among native bile acids and conjugation through C-24 of CDCA with taurine or glycine enhanced binding affinity (6). A series of C-24 CDCA conjugates were then synthesized using glutamic acid or lysine as a linker, and the results showed that only conjugates with one negative charge and a single bulky ester substituent proximal or distal to the C-24 region were ASBT substrates (7). Furthermore, a C-24 bile acid prodrug of valacyclovir enhanced

oral bioavailability by 2-fold, compared to acyclovir in rats (8). Overall, ASBT permits bile acid conjugates with substitutions at the C-24 side chain region, but the chemistry space extending beyond the C-24 region needs to be further explored, especially for ASBT substrates. In fact, several quantitative structure–activity relationships studies have been reported for general ASBT inhibition. Thus, we sought to apply 3D-QSAR approaches to elucidate the substrate requirements in the space extending beyond the C-24 region using comparative molecular field analysis (CoMFA) (9) and comparative molecular similarity indices analysis (CoMSIA) (10). These algorithms are particularly useful when the structure of a target protein is unknown and may be applied for lead compound optimization (11).

The objective of this study was to synthesize and characterize substrate uptake and inhibition properties of C-24 bile acid probe moiety conjugates, and subsequently develop comprehensive 3D-QSAR models to elucidate the substrate requirements on positions beyond C-24 region. Pyridine and phenol are common chemical scaffolds in drug structures (12). Therefore, C-24 aminopyridine and aminophenol conjugates of glutamyl-CDCA were synthesized and their inhibition and uptake kinetics were evaluated. Pyridine and phenol probes were functionalized using different ring substitutions in order to study chemical substituent effect on ASBT inhibition and uptake. Using a combination of inhibition and uptake studies, we differentiate ASBT inhibitors from substrates and developed 3D-QSAR models for both classes of bile analogues. These studies provide new insight into the substrate requirements for ASBT uptake and shed light on the design of bile acid conjugates that can increase intestinal drug permeability and bioavailability.

EXPERIMENTAL PROCEDURES

Materials. [³H]-Taurocholic acid was purchased from Perkin-Elmer (Waltham, MA). Taurocholic acid was purchased from Sigma (St. Louis, MO). CDCA was obtained from TCI America

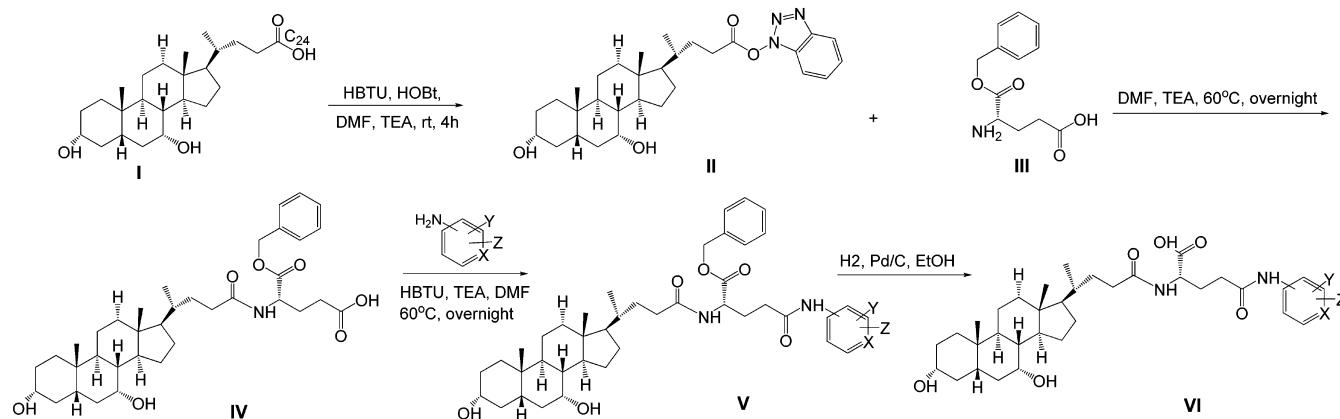
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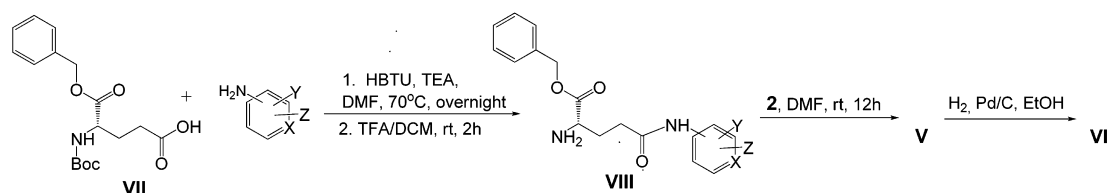
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Scheme 1. General Synthetic Approaches to Obtain CDCA-Glutamyl-Pyridine or CDCA-Glytamyl-Phenol Conjugates^a

Approach 1.



Approach 2.



^a X = N, H; Y = H, CH₃, OCH₃, NH₂, OH, Cl, F, Br; Z = H, CH₃.

(Portland, OR). Protected glutamic acid analogues were from Novabiochem (Gibbstown, NJ). Aminopyridine analogues were purchased from Sigma Aldrich (St. Louis, MO) and Matrix Scientific (Columbia, SC). Fetal bovine serum (FBS), trypsin, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen Corporation (Carlsbad, CA). All other reagents and chemicals were of the highest purity commercially available.

Synthetic Procedures. A series of aminopyridines were conjugated to CDCA using glutamic acid as a linker. Two synthetic approaches were employed (Scheme 1). Among the native bile acids, CDCA was selected on the basis of its favorable transport across ASBT-MDCK monolayers (6).

N-Hydroxybenzotriazole (HOBt) Esters of CDCA (II). CDCA **I** (5 g) was activated with HOBt (0.5 equiv) and HBTU (1.2 mmol) in 20 mL of anhydrous dimethylformamide (DMF) along with 1.2 equiv of triethylamine (TEA) at RT for 4 h to form CDCA-OBt ester **II**. The reaction was terminated by adding 50 mL of water. Subsequently, the reaction mixture was extracted with ethyl acetate (EtOAc) (3×). The combined organic layer was washed with brine (1×), dried with anhydrous sodium sulfate, filtered, and evaporated under vacuum to yield a white fluffy solid CDCA-OBt ester **II** (90% yield). Mass spectrometry (MS) showed appropriate peaks: [M+H]⁺ 510.81, [M+Na]⁺ 532.81. **II** was used in approaches A and B.

Synthesis of CDCA-α-Benzyl-Glutamyl Probe (V). Approach 1 was applied as shown in Scheme 1. **II** (5.0 g) was reacted with 0.8 equiv of L-α-benzyl glutamic acid **III** in 30 mL of anhydrous DMF along with 1 equiv of TEA at 60 °C overnight. Reaction was terminated by adding 50 mL of water. The reaction mixture was extracted with EtOAc (3×). The combined organic layer was washed with 1 N HCl (3×) and brine (1×). Then, the organic extract was dried with anhydrous sodium sulfate, filtered, and evaporated under vacuum to yield off-white solid glutamyl-CDCA conjugates **IV** (75% yield). MS showed appropriate peaks: [M+H]⁺ 612.81, [M+Na]⁺ 634.81.

IV (1.0 g) was activated with 1.2 equiv of HBTU in 15 mL of anhydrous DMF along with 1.2 equiv of TEA for 10 min

followed by addition of appropriate aminopyridine or aminophenol probes at 60 °C overnight. Reaction was terminated by adding 30 mL of water. The reaction mixture was extracted with EtOAc (3×). The combined organic layer was washed with 1 N HCl (3×), 1 N NaOH (3×), and brine (1×). Then, the organic extract was dried with anhydrous sodium sulfate and filtered. The EtOAc extract was evaporated under vacuum to yield thick, yellow oil. The crude product was further purified by silica gel column chromatography using a mobile phase of EtOAc and hexane (95:5). Fractions were collected and solvent evaporated to yield the neutral conjugate **V**. Purity of the conjugate was characterized by TLC and MS techniques.

Approach 2 was applied as shown in Scheme 1. First, 2.0 g of N-boc-α-benzyl-glutamic acid **VII** was coupled to aminopyridine or aminophenol probes (1.1 equiv) using HBTU (1.2 equiv) as coupling reagents in 10 mL of anhydrous DMF along with 2.2 equiv of TEA at 70 °C overnight. Reaction was terminated by adding 30 mL of water. The reaction mixture was extracted with EtOAc (3×). The combined organic layer was washed with 1 N HCl (3×), 1 N NaOH (3×), and brine (1×). Then, the organic extract was dried with anhydrous sodium sulfate, filtered, and evaporated under vacuum to yield orange oil. The crude product was further purified by silica gel column chromatography using a mobile phase of EtOAc and hexane (90:10). Fractions were collected and solvent evaporated to give a solid, white intermediate. Purity of the intermediate conjugates was characterized by TLC and MS techniques. The intermediate was then selectively N-deprotected using 25% trifluoroacetic acid (TFA) in anhydrous dichloromethane (DCM) at RT for 1–2 h. Solvent was evaporated under vacuum to yield off-white solid **VIII**.

VIII (1.0 g) was reacted with 1.2 equiv of CDCA-OBt ester **II**, along with 1 equiv of TEA in 15 mL of anhydrous DMF at RT overnight. Reaction was terminated by adding 50 mL of water. The reaction mixture was purified as described above (see synthetic approach **I**) to yield the neutral conjugate **V**.

Synthesis of CDCA-Glutamyl Probe (VI). The neutral conjugates **V** were subjected to catalytic hydrogenation for the removal of the benzyl ester using 10% Pd/charcoal in ethanol at 50 psi overnight. Suspension was filtered through Celite and solvent evaporated under vacuum to yield the final conjugates **VI**. For chloro- and bromo-substituted neutral conjugates **V**, the benzyl ester was removed in 10 mL 33% hydrobromic acid in acetic acid at RT for 2 h. Reaction was terminated by adding 30 mL of EtOAc. The reaction mixture was extracted with water (3 \times). The organic layer was washed with brine (1 \times), dried with anhydrous sodium sulfate, filtered, and evaporated under vacuum to yield the final conjugates **VI**.

Characterization of Conjugates. Identity and purity of bile acid conjugates was characterized by TLC, MS, and ^1H NMR (proton, COSY). MS was performed on a LCQ ESI-MS (Thermo Fisher Scientific Inc., Waltham, MA). ^1H NMR spectra were recorded on a Varian Inova 500 MHz (Varian Inc., Palo Alto, CA). The mass spectra in all cases gave a molecular ion in agreement with the calculated mass of the conjugates. No CDCA impurity was detected in any final conjugate (**13**). LC-MS/MS and ^1H NMR results are compiled in Supporting Information Table S1. The chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) ($\delta = 0.0$ ppm).

Cell Culture. Stably transfected ASBT-MDCK cells were grown as previously described (**14**). Briefly, cells were grown at 37 $^\circ\text{C}$, 90% relative humidity, 5% CO_2 atmosphere and fed every two days. Media comprised DMEM supplemented with 10% FBS, 50 units/mL penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin. Cells were passaged after reaching 90% confluency.

Inhibition Study. To characterize compound binding affinities to ASBT, cis-inhibition studies of taurocholate uptake were conducted as previously described (**15**). Briefly, cells were seeded in 12 well cluster plates (Corning; NY) at a density of 1.5 million cells/well. Inhibition studies were performed on the fifth day in Hank's Balance Salts Solution (HBSS). Cells were exposed to donor solution containing 2.5 μM taurocholate (spiked with 0.5 $\mu\text{Ci}/\text{mL}$ [^3H]-taurocholate) in the presence of a conjugate at eight concentrations (0–100 μM) for 10 min. Cells were lysed using NaOH. Cell lysate was counted for taurocholate radioactivity using a liquid scintillation counter (Beckmann Instruments, Inc., Fullerton, CA). For neutral conjugates, 2.5% DMSO was included in transport buffer, which has been shown to not affect ASBT kinetics (**16**).

Uptake Study. The uptake of conjugates by ASBT, representing cellular absorption mediated by the transporter, was evaluated. Cells were seeded as above for inhibition studies. Uptake studies employed HBSS buffer or modified HBSS buffer (i.e., sodium-free). Since ASBT is a sodium-dependent transporter, studies using sodium-free buffer enabled passive permeability measurement of conjugate. Cells were exposed to donor solution contain eight different conjugate concentrations (1–500 μM). Subsequently, cells were lysed as previous described (**16**) by adding 0.25 mL acetonitrile to each well, following evaporation at RT. A solution (1 mL) containing 50% water and 50% acetonitrile was added to each well for 10 min. Sample was stored in -80 $^\circ\text{C}$ until analysis by LC-MS/MS. Taurocholate uptake was measured on each study occasion as a control.

Analytical Methods. The conjugate was 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 used was a Phenomenex Luna C8 (50 \times 2.0 mm, 3 μm ; Phenomenex; Torrance, CA, USA) heated to 45 $^\circ\text{C}$. The mobile phase was A, 0.1% formic acid in water; and B, 0.1% formic acid in acetonitrile, with a flow rate of 0.4 mL/min. The injection volume was 10 μL . Detection was achieved under positive ion electrospray tandem mass spec-

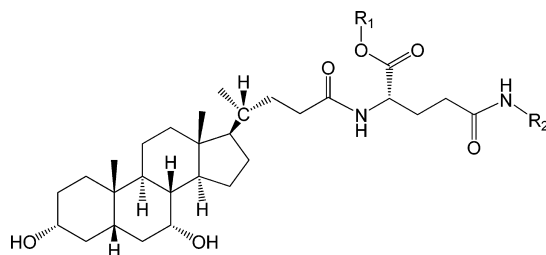


Figure 1. General structure of CDCA-glutamyl-probe (pyridines, phenol) conjugates. Database alignment employed the sub-structure CDCA-glutamate.

trometry using $[\text{M}+\text{H}]^+$ peak for all conjugates. The mobile phase and retention time for each conjugate are detailed in Supporting Information Table S1. The limit of quantification for all conjugates was between 1 and 2.5 nM.

Kinetic Analysis. Inhibition data were analyzed in terms of inhibition constant K_i and used a modified Michaelis–Menten competitive inhibition model, as previously described, taking into account aqueous boundary layer (ABL) resistance (**17**).

Uptake data were analyzed in terms of K_t , J_{max} , $\text{norm}J_{\text{max}}$, and P_p , where J_{max} is the transport capacity; $\text{norm}J_{\text{max}}$ is normalized conjugate J_{max} against taurocholate J_{max} from the same occasion; K_t is the Michaelis–Menten coefficient for ASBT-mediated transport; and P_p is the conjugate passive permeability coefficient. For **18**, estimated K_t was less than 0.1 μM , such that K_t was assigned a value of 0.1 μM , due to the limited ability to accurately estimate potent K_t values (**17**). Data were analyzed using WinNonlin Professional (Pharsight Corporation; Mountain View, CA).

3D-QSAR Analysis. 3D-QSAR models were developed for K_i and $\text{norm}J_{\text{max}}/K_t$. The inhibition constant K_i reflects compound binding affinity to ASBT, with smaller values denoting greater binding. $\text{norm}J_{\text{max}}/K_t$ reflects the efficiency of conjugate translocation by ASBT.

The implementation of the CoMFA and CoMSIA techniques was performed as described previously (**18**, **19**). Briefly, conjugates were sketched with Sybyl 7.2 (Tripos Inc., St. Louis, MO). Each structure was minimized using the Powell method and Tripos force field (**20**) with maximum iterations of 2000 steps and termination gradient of 0.001 kcal/mol. Partial atomic charges were computed by the Gasteiger–Hückel method (**21**). Conjugates were aligned via “database alignment” based on a template structure in Figure 1. The reference compound during alignment was **12** for K_i models and **15** for the $\text{norm}J_{\text{max}}/K_t$ model. A regularly placed grid of 2.0 \AA was created around aligned molecules. An sp^3 carbon atom with charge +1.00 was used as a probe atom. The steric and electrostatic fields were truncated at +30.00 kcal/mol. In contour maps of models, areas with above 80% favorability among the fields being viewed were regarded as beneficial for the biological activity, while those with field values below 20% were regarded as detrimental. The attenuation factor was 0.3. Models were obtained using the partial-least-squares (PLS) analysis (**22**). Leave-one-out cross validation (LOO) was performed to determine the optimum number of components, and nonvalidation PLS was applied to obtain the final model. Models were evaluated by the cross-validated standard coefficient q^2 and correlation coefficient r^2 . A model with high q^2 (above 0.5), high r^2 , low standard error of prediction (SEP), and low standard error of estimate (SEE) was considered to possess good predictability. Models were also evaluated using a test set of four randomly selected conjugates.

RESULTS AND DISCUSSION

Synthesis. A series of aminopyridine and aminophenol conjugates of glutamyl-CDCA were synthesized. The general

Table 1. Structures and the Biological Activities of the Bile Acid Conjugates^a




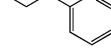



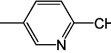





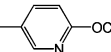
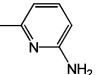




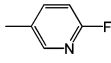

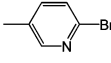
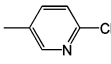



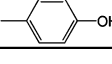
Comp.	R ₁	R ₂	K _i (μM)	K _i (μM)	normJ _{max} ^b	normJ _{max} /K _i (μM ⁻¹)	P _p × 10 ⁷ (cm/s)	ClogP
1	-H		5.28 (±0.84)	9.88 (±2.33)	0.616 (±0.033)	0.0620 (±0.0151)	5.36 (±0.26)	2.87
2 ^c	-H		1.51 (±0.24)	1.06 (±0.71)	0.298 (±0.010)	0.281 (±0.189)	2.01 (±0.21)	2.87
3	-H		8.73 (±1.49)	21.6 (±4.8)	0.435 (±0.029)	0.0201 (±0.0047)	2.28 (±0.22)	2.87
4	-H		7.13 (±1.18)	17.4 (±3.0)	0.330 (±0.074)	0.0190 (±0.0054)	1.70 (±0.15)	1.90
5	-H		4.66 (±0.53)	11.2 (±3.6)	0.0791 (±0.0097)	0.00705 (±0.00243)	1.19 (±0.09)	2.72
6	-H		11.1 (±1.1)	77.3 (±26.7)	0.300 (±0.043)	0.00388 (±0.00146)	2.57 (±0.28)	2.72
7	-H		23.6 (±1.4)	21.2 (±4.4)	0.413 (±0.036)	0.0195 (±0.0044)	2.16 (±0.21)	3.37
8	-H		7.87 (±1.00)	24.6 (±4.4)	0.293 (±0.013)	0.0119 (±0.0022)	2.01 (±0.13)	3.37
9	-H		2.81 (±0.21)	9.80 (±3.83)	0.196 (±0.012)	0.0200 (±0.0079)	4.65 (±0.29)	3.37
10	-H		6.61 (±0.98)	7.44 (±2.20)	0.716 (±0.042)	0.0962 (±0.0290)	4.45 (±0.54)	3.87
11	-CH ₂ C ₆ H ₅		122 (±19)	ND ^d	ND	ND	ND	5.21
12	-H		1.91 (±0.41)	92.2 (±14.7)	0.511 (±0.034)	0.00554 (±0.00096)	1.55 (±0.21)	3.22
13	-CH ₂ C ₆ H ₅		28.1 (±4.43)	ND	ND	ND	ND	4.56
14	-H		8.94 (±1.11)	1.20 (±0.38)	0.242 (0.013)	0.202 (±0.065)	3.53 (±0.16)	3.71
15	-H		13.2 (±2.2)	4.44 (±0.42)	1.44 (±0.017)	0.324 (±0.031)	3.78 (±0.19)	2.54
16	-H		3.07 (±0.36)	2.80 (±1.19)	0.404 (0.025)	0.144 (±0.062)	2.57 (±0.33)	3.39
17	-CH ₂ C ₆ H ₅		17.8 (±2.0)	NM ^e	NM	NM	62.3 (±4.1)	4.73
18 ^c	-H		5.80 (±0.68)	0.1	2.67 (±0.24)	26.7	8.77 (±4.83)	2.53

Table 1. Continued

Comp.	R ₁	R ₂	K _i (μM)	K _t (μM)	normJ _{max} ^b	normJ _{max} /K _t (μM ⁻¹)	P _p × 10 ⁷ (cm/s)	ClogP
19	-H		6.84 (±0.76)	4.30 (±1.21)	0.361 (±0.052)	0.0840 (±0.0265)	11.6 (±0.5)	3.13
20	-H		5.74 (±1.02)	11.7 (±2.4)	0.307 (±0.023)	0.0262 (±0.0057)	4.40 (±0.18)	3.13
21	-H		21.1 (±3.13)	33.0 (±28.1)	0.670 (±0.050)	0.0203 (±0.017)	48.9 (±2.5)	3.85
22 ^c	-H		4.96 (±0.77)	83.6 (±43.2)	3.47 (±0.63)	0.0415 (±0.0227)	100 (±4)	3.85
23	-H		7.92 (±1.4)	NM	NM	NM	33.8 (±0.9)	3.7
24	-H		7.87 (±1.72)	NM	NM	NM	39.7 (±0.7)	2.85
25 ^c	-H		3.54 (±0.50)	5.57 (±1.09)	1.46 (±0.06)	0.262 (±0.052)	6.01 (±0.34)	3.1
26	-H		2.43 (±0.39)	3.29 (±1.15)	0.464 (0.151)	0.141 (±0.067)	7.95 (±0.80)	2.87
27	-H		7.18 (±0.98)	15.3 (±2.4)	0.437 (0.027)	0.0286 (±0.0036)	7.65 (±0.73)	2.87

^a Data were summarized as mean (±SEM) of three measurements. ^b To correct for variation in ASBT expression levels across studies, J_{max} of each bile acid was normalized against taurocholate J_{max} from the same occasion. ^c **2**, **18**, **22**, and **25** were used as the test set. The other 23 compounds were used as the training set. ^d Not determinable owing to high passive permeability. ^e Not measurable due to lack of transportation by ASBT.

structure is depicted in Figure 1, and all conjugate structures are drawn in Table 1. The synthetic strategy was based on coupling the carboxyl group at the C-24 position of CDCA to the amino group of the common drug scaffolds pyridine or phenol, using glutamic acid as a linker (Scheme 1). A negative charge around the C-24 region was designed to promote ASBT translocation (7, 23). The corresponding neutral analogues with α-benzyl protection on the glutamic acid linker were also synthesized. Unsubstituted, monosubstituted, and disubstituted pyridine and phenol were employed as functionalized probe moieties that yielded conjugates with varying chemistry in the region beyond C-24.

ASBT Inhibition. Table 1 lists K_i values of the 27 conjugates, which included 24 pyridine-based conjugates and 3 phenol-based conjugates. Neutral conjugates with α-benzyl protection on C-24 were poorly water-soluble. Therefore, among the 24 neutral conjugates that were synthesized, only **11**, **13**, and **17** allowed K_i to be measured.

All 24 monoanionic conjugates were potent inhibitors with K_i ranging from 1.51 to 23.6 μM with CDCA-glutamyl-3-aminopyridine **2** being the most potent inhibitor. They are in the range of native bile acids. The three neutral conjugates were less potent than their corresponding monoanionic conjugates by approximately 10–20-fold.

ASBT Uptake. While all 27 conjugates exhibited high inhibition potency, only **22** showed to be substrates. Table 1 lists kinetic parameters K_t, normJ_{max}, normJ_{max}/K_t, and P_p. K_t represents transporter affinity. To accommodate variation in ASBT expression levels across studies, J_{max} of each bile acid was normalized against taurocholate J_{max} from the same occasion yielding normJ_{max}. The normJ_{max}/K_t value represents the ASBT

mediated transport efficiency. Neutral compound **17** exhibited a high passive permeability, such that it was not able to be assessed as a substrate or not. The other two neutral compounds, **11** and **13**, were not further evaluated. Results from **17** were consistent with previous findings that a negative charge around the C-24 region is required for translocation (7).

K_t values ranged from 0.1 μM for **18** to 92.2 μM for **12**. normJ_{max} ranged from 0.0791 for **5** to 2.67 for **18**. normJ_{max}/K_t ranged from 0.00388 μM⁻¹ for **6** to 26.7 μM⁻¹ for **18**. P_p ranged from 1.19 × 10⁻⁷ cm/s for **5** to 100 × 10⁻⁷ cm/s for **22**. Figure 2A shows the uptake profile of **18**, which was the most potent substrate in terms of both K_t and normJ_{max}; Figure 2B shows the nonsubstrate **23**.

Ring substituent position had an important effect on transport efficiency. For example, for 3-aminopyridine conjugates, substitution at position 2 led to more potent substrate compared to other positions, e.g., **5** (11.2 μM) vs **7** (21.2 μM), and **18** (0.1 μM) vs **19** (4.30 μM) when substitution is moved from 2 to 5 position. Substitution at position 6 significantly decreased activity, when **8** (24.6 μM) is compared with **5** and **7**; **20** (11.7 μM) is compared with **18** and **19**; **12** (92.2 μM) is compared with **5**; **22** (83.6 μM) is compared with **21** (33.0 μM). The type of substituent group also influenced K_t. For example, all fluoro-substituted conjugates were potent substrates, bromo-substituted conjugates were weak substrates, while chloro-substitution abolished uptake.

Comparison of Inhibition Kinetics vs Transporter Kinetics. Transporter-mediated translocation minimally involves substrate binding to the transporter, transporter conformation changes involving substrate translocation, dissociation of substrate from transporter, and transporter conformation restoration.

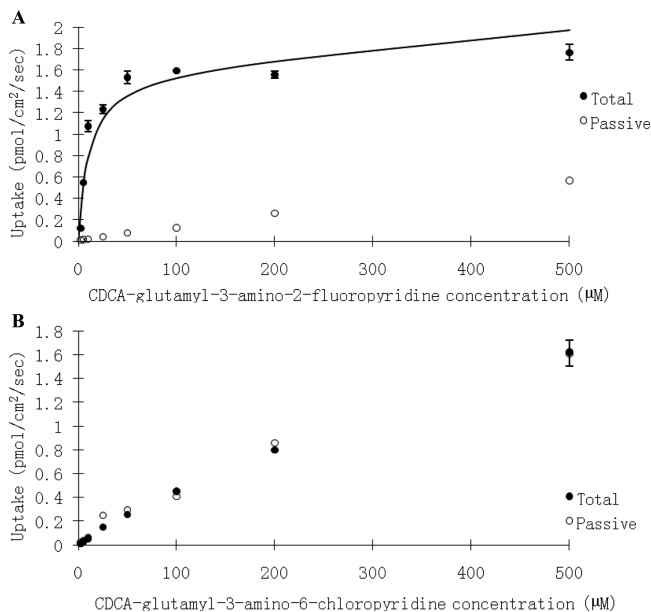


Figure 2. Concentration-dependent uptake of (A) CDCA-glutamyl-3-amino-2-fluoropyridine **18** and (B) CDCA-glutamyl-3-amino-6-chloropyridine **23** into ASBT-MDCK monolayers. The conjugate was measured at varying concentrations (2.5–500 μM) in the presence and absence of sodium to delineate ASBT-mediated and passive uptake components. Data indicate total uptake in the presence of sodium (\bullet) and passive uptake in the absence of sodium (\circ). Uptake of **18** was several-fold higher in the presence of sodium than in the absence of sodium, reflecting ASBT-mediated uptake. Uptake of **23** was the same in the presence and absence of sodium; passive uptake was high, such that the compound was either not a substrate or not measurable as a substrate due to high passive permeability.

The initial binding step is assessed by K_i . Results show that ASBT inhibition requirements were different from the ASBT-mediated transport requirements. A potent inhibitor may be a weak substrate, such as **6**, **12**, and **22**. Figure 3A illustrates the relationship of transporter affinity K_t and inhibition potency K_i for all 22 substrates. Figure 3B excludes **6**, **12**, and **22**, where K_t was notably weaker than K_i . For these 19 conjugates in Figure 3B, there was a modest association between K_t and K_i with linear slope = 0.995, $r^2 = 0.403$, and $p = 0.003$. Qualitatively, most conjugates yielded similar potency of K_t and K_i , suggesting that conjugate binding was broadly the slow kinetic step for conjugate translocation. These results agree with previous native bile acid findings (6). It should be noted that **14** and **18** yielded K_t values that were over 7-fold more potent than K_i although they both were potent inhibitors and substrates (e.g., K_i and $K_t < 10 \mu\text{M}$). For **6**, **12**, and **22**, where K_t was far less potent than K_i , postbinding events were rate-limiting.

Figure 4 shows that transport capacity was not associated with inhibitory potency. Capacities of most conjugates spanned a narrow range and were less than taurocholate. However, $\text{norm}J_{\text{max}}$ of **15**, **18**, **22**, and **25** were higher than that of taurocholate. Supporting Information Figures S1 and S2 show that transport capacity and transport efficiency were not related to K_t or K_i , respectively.

ASBT Inhibition 3D-QSAR Models. Figure 5 shows the superimposition of all 27 compounds for inhibition model development. Twenty-three compounds were included in the training set. The remaining, randomly selected four compounds (i.e., **2**, **18**, **22**, and **25**) were used as the test set. Four 3D-QSAR models, including one CoMFA and three CoMSIA models, were developed using $\text{p}K_i$ data (Table 2). CoMSIA-1 included electrostatic and steric fields, whereas CoMSIA-2 included an additional hydrophobic field. CoMSIA-3 included the three molecular descriptors from models 1 and 2, as well as hydrogen bond donor and acceptor fields.

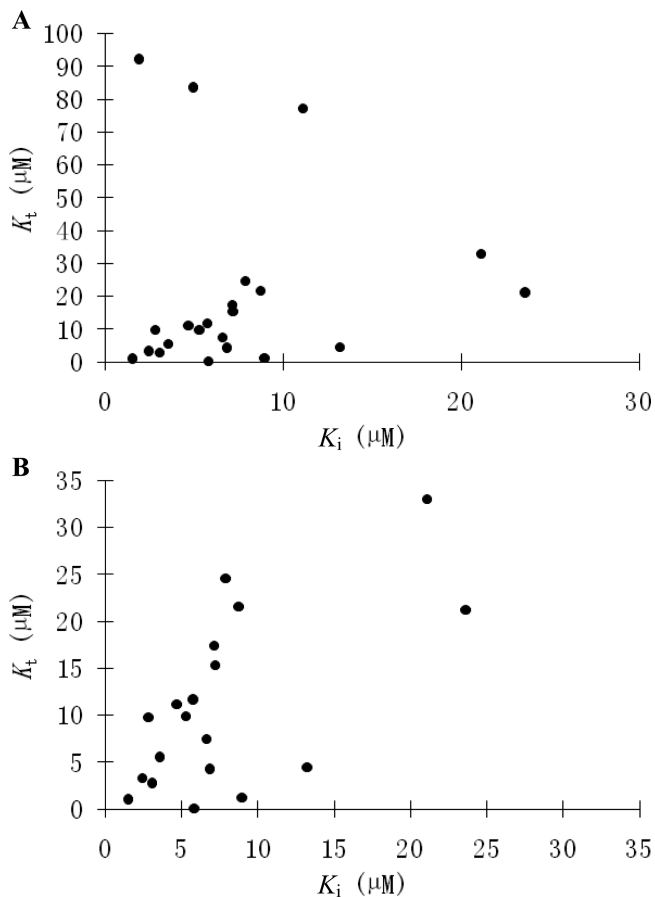


Figure 3. Relationship of K_t and K_i . (A) For all 22 bile acid conjugates that were substrates, K_t vs K_i are plotted. **6**, **12**, and **22** exhibited several-fold higher K_t values than K_i values. (B) With **6**, **12**, and **22** removed, most conjugates yielded similar K_t and K_i . Linear regression showed slope = 0.995, $r^2 = 0.403$, and $p = 0.003$. **14** and **18** yielded $K_t \ll K_i$, although all were $< 10 \mu\text{M}$.

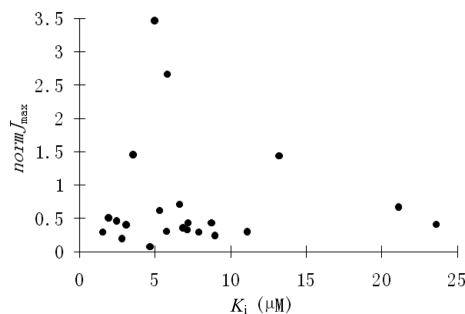


Figure 4. Relationship between $\text{norm}J_{\text{max}}$ and K_i . Transporting capacity was not associated with inhibitory potency. Linear regression showed a slope of $-0.008 \mu\text{M}^{-1}$ and $r^2 = 0.003$. **15**, **18**, **22**, and **25** yielded J_{max} higher than taurocholate. All other conjugates exhibited J_{max} less than taurocholate J_{max} , and ranged from 0.0791 to 0.716.

CoMFA and CoMSIA models were comparable in terms of their predictability of $\text{p}K_i$, with CoMFA-1 ($q^2 = 0.675$, $r^2 = 0.965$) and CoMSIA-3 ($q^2 = 0.612$, $r^2 = 0.958$) being the best inhibition models (Table 2). Figure 6 shows the relationship of observed versus predicted $\text{p}K_i$ for training set and test set of CoMFA-1 and CoMSIA-3. The corresponding data are listed in Supporting Information Table S2. Both models accurately predicted activities of three out of the four test compounds. **2** was underpredicted by both models, where the observed K_i was $1.51 \mu\text{M}$ and predicted K_i were $8.13 \mu\text{M}$ and $11.7 \mu\text{M}$ by CoMFA-1 and CoMSIA-3, respectively.

Table 2 also shows the relative contributions of specific fields to the inhibition activities. For CoMFA-1, the steric feature

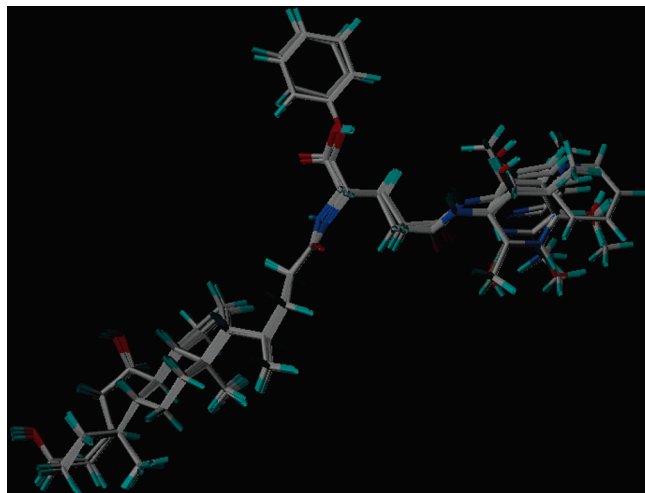


Figure 5. Superimposition of training set and test set compounds for inhibition model development.

Table 2. Result from CoMFA and CoMSIA Analysis

	CoMFA-1 ^a	CoMSIA-1 ^a	CoMSIA-2 ^a	CoMSIA-3 ^a	CoMFA-2 ^b
q^2	0.675	0.388	0.572	0.612	0.538
SEP ^c	0.255	0.349	0.292	0.278	0.550
r^2	0.965	0.879	0.959	0.958	0.999
SEE ^c	0.004	0.155	0.091	0.091	0.025
NOC ^c	5	5	5	5	9
Fraction					
steric	0.731	0.377	0.120	0.084	0.612
electronic	0.269	0.623	0.378	0.202	0.388
hydrophobic			0.502	0.408	
donor				0.131	
acceptor				0.173	

^a Models for pK_i . ^b Model for $\log(\text{norm}J_{\text{max}}/K_i)$. ^c SEP: standard error of prediction; SEE: standard error of estimate; NOC: number of components.

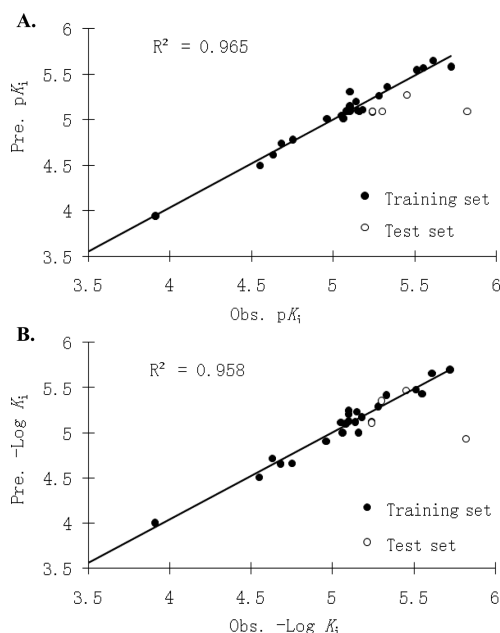


Figure 6. Observed versus predicted pK_i plot of the CoMFA-1 (A) and CoMSIA-3 (B) using 23 training set conjugates (●) and 4 test set conjugates (○). The solid line is the regression for the training set. The regression line has a slope = 0.965 and $r^2 = 0.965$ for CoMFA-1, and 0.959 and 0.958 for CoMSIA-3, respectively.

dominated (73.1% contribution) the electrostatic feature. For all CoMSIA models, the steric feature was less important than the electrostatic feature. For example, the contributions of steric

and electrostatic features in CoMSIA-1 were 37.7% and 62.3%, respectively. The different effects of steric and electrostatic fields between CoMFA and CoMSIA are attributed to their energy function forms. The contribution of the steric feature in CoMSIA is further reduced when the hydrophobic field was added, with 12.0% and 8.4% for CoMSIA-2 and CoMSIA-3, respectively. For these two models, the hydrophobic was the most determining feature that contributed 50.2% and 40.8%, respectively. Considering the improved q^2 in CoMSIA-2 and CoMSIA-3 vs CoMSIA-1, the hydrophobic feature was the major contributor to improve pK_i predictability. Hydrogen bond donor and acceptor in CoMSIA-3 contributed 13.1% and 17.3%, respectively, and account for the slight q^2 increase from 0.572 in CoMSIA-2 to 0.612 in CoMSIA-3.

3D-QSAR Contour Maps for Inhibition. Figures 7 and 8 illustrate features of the CoMFA-1 and CoMSIA-3 models, respectively. Contour maps were drawn relative to CDCA-glutamyl-3-amino-2,6-dimethylpyridine **12**.

CoMFA Contour Maps. The steric contours from CoMFA-1 (Figure 7A) reveal that bulky groups at the 2 or 6 position (green region) of 3 aminopyridine afford beneficial steric interactions for inhibition, whereas bulky substitutes on position 5 (yellow region) are detrimental. The contours explain the higher activity of 2,6-dimethyl-substituted **12** ($K_i = 1.94 \mu\text{M}$) over 2-methyl substituted **8** ($7.87 \mu\text{M}$) and 6-methyl substituted **5** ($4.66 \mu\text{M}$). The two green regions also accounted for the higher activity of **5** and **8** than **6** ($11.1 \mu\text{M}$) and **7** ($23.64 \mu\text{M}$), where the methyl group is moved from 2 or 6 to 4 or 5. The same pattern is evident for bromo substitution. When bromine is moved from position 6 in **22** to 5 in **21**, the activity decreased from 4.96 to $21.1 \mu\text{M}$. The electrostatic contour from CoMFA-1 (Figure 7B) indicates that an electropositive group near the $-\text{COO}$ (blue region) will be favorable. Considering that a hydroxyl hydrogen on a carboxylic group bears a positive charge, this blue area reflects that a carboxylic acid near C-24 improved inhibition more than the corresponding conjugates with α -benzyl protection. Inhibition potency decreased from 6.61 to $122 \mu\text{M}$ comparing **10** to **11**, from 1.94 to $28.1 \mu\text{M}$ comparing **12** to **13**, and from 3.07 to $17.78 \mu\text{M}$ comparing **16** to **17**. These findings agree with previous observations that a negative charge near C24 region is preferred (7). In Figure 7B, the red contour region behind the 2 position and a blue contour region near the 6 position of the 3-aminopyridine ring indicate that an electro-negative or electropositive group enhanced inhibition.

CoMSIA Contour Maps. Steric contour maps from CoMSIA-3 (Figure 8A) reveal that the green regions are patterned similarly to those from CoMFA-1 (Figure 7A). However, CoMSIA-3 provides two additional yellow contours above the carboxylate group indicating that a steric interaction near $-\text{COO}$ is unfavorable. The yellow areas are consistent with the blue electrostatic contour of CoMFA-1 (Figure 7B), where α -benzyl protection of the carboxylate decreased the inhibition potency (i.e., **10** vs **11**, **12** vs **13**, and **16** vs **17**). Electrostatic contours (Figure 8B) are consistent with the patterns observed in CoMFA-1 (Figure 7B), e.g., red and blue regions around 2 and 6 position of the 3-aminopyridine ring. Hydrophobic contour plots (Figure 8C) indicate that hydrophobic substitution on the 2 or 6 position enhances inhibition (orange regions), whereas such substitution on the 5 position reduces potency (white contour). This result is consistent with steric contours of CoMFA-1 (Figure 7A). Hydrogen bond donor contours (Figure 8D) suggest that a $-\text{OH}$ in the carboxyl group can be regarded as a hydrogen bond donor. The cyan area near the $-\text{COO}$ group agrees with the steric (Figure 8A) and hydrophobic contours (Figure 8C), indicating that the α -benzyl group on $-\text{COO}$ remarkably reduced the inhibition activities. The purple area near the pyridine ring indicates that a hydrogen-bond donor

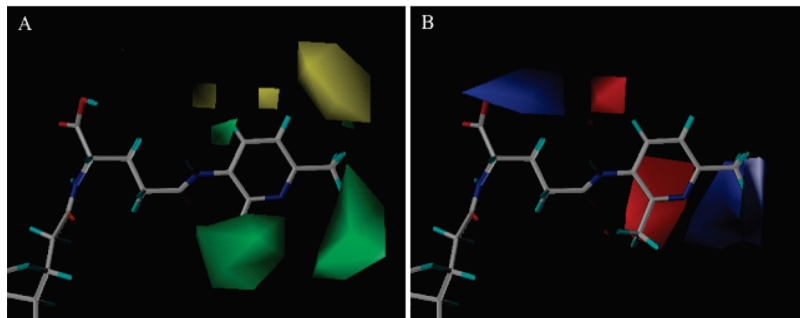


Figure 7. Steric (A) and electrostatic (B) contour maps around compound CDCA-glutamyl-3-amino-2,6-dimethylpyridine **12** for CoMFA-1 inhibition model. In panel A, green contours show where bulky substitute favored inhibition, while yellow contours were disfavored. In panel B, blue region represents favorable electropositive interaction for inhibition, while red shows disfavored electropositive character.

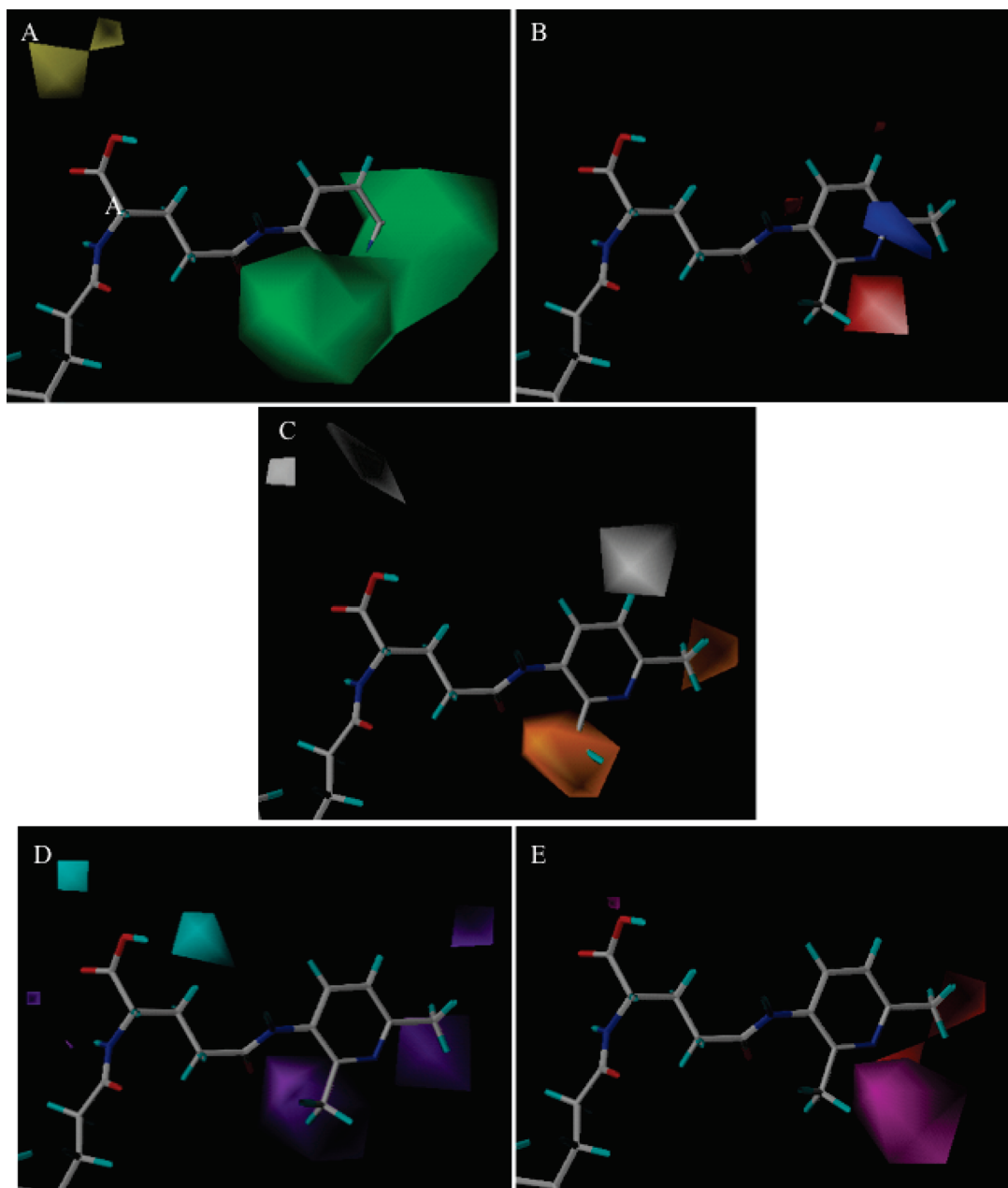


Figure 8. Steric (A), electrostatic (B), hydrophobic (C), hydrogen bond donor (D), and hydrogen bond acceptor (E) contour maps around CDCA-glutamyl-3-amino-2,6-dimethylpyridine **12** for CoMSIA-3 inhibition model. In panel A, green and yellow contours represent favorable and unfavorable bulky substituent interaction regions for inhibition. In panel B, blue and red contours represent regions that favor electropositive and electronegative groups, respectively. In panel C, orange and white contours are areas where hydrophobic groups enhance and diminish activity. In panel D, cyan and purple contours indicate regions where an inhibitor's hydrogen bond donor is favored and disfavored, respectively. In panel E, magenta and red contours represent favorable and unfavorable hydrogen-bond acceptor regions, respectively.

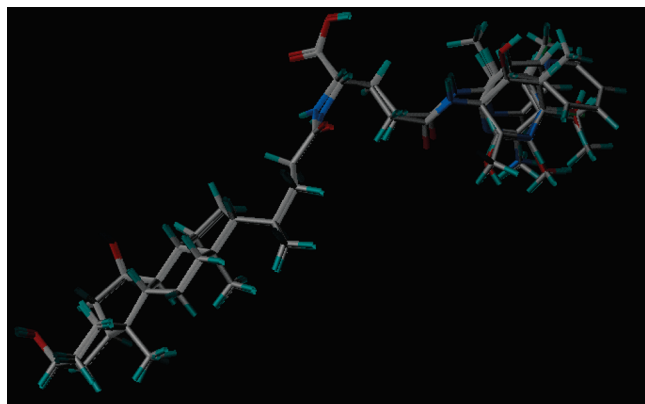


Figure 9. Superimposition of training set and test compounds for transport efficiency model development.

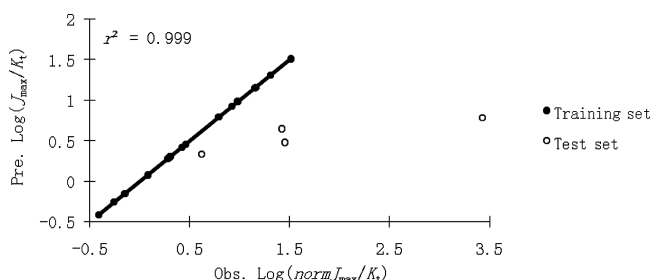


Figure 10. Observed vs predicted $\log(\text{norm}J_{\text{max}}/K_t)$ plot of the CoMFA-2 using 18 training set conjugates (●) and 4 test set conjugates (○). The solid line is the regression for the training set. The regression line has slope = 1.00 and $r^2 = 0.999$.

behind the 2 and 6 positions of the 3-aminopyridine ring is detrimental. Hydrogen bond acceptor contours (Figure 8E) determine favorable (magenta) areas near the pyridine nitrogen and a red (disfavored) area around the 6 position on 3-aminopyridine ring, reflecting the decreased activity from 2.43 to 7.18 μM when a hydroxyl group is moved from the meta position in **26** to the para in **27** on the phenol ring.

Comparison of CoMFA and CoMSIA Models for Inhibition. CoMFA-1 possessed slightly greater predictability than CoMSIA-3 in terms of q^2 (0.675 and 0.612, respectively). However, the advantage of CoMSIA is the employment of a Gaussian-type energy calculation and additional molecular descriptors, including hydrophobic fields, hydrogen bond acceptor, and hydrogen bond donor. For example, the influence of the α -benzyl group is not accommodated by the steric contour of CoMFA-1 (Figure 7A), but is accommodated by the steric and hydrophobic contours of CoMSIA-3 (Figure 8A,B). The hydrophobic contour of CoMSIA-3 had a similar pattern to the steric contour of CoMFA-1, indicating that the hydrophobic field of CoMSIA-3 may share common features with the steric field of CoMFA-1. The similarity is not surprising, because a large hydrophobic substitution may have significant steric effects.

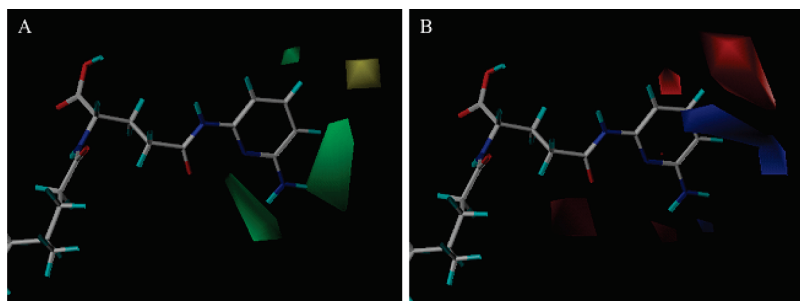


Figure 11. Steric (A) and electrostatic (B) contour maps around CDCA-glutamyl-2,6-diaminopyridine **15** for CoMFA-2 transport efficiency model. In panel A, green contours show where bulky substitute favored inhibition, while yellow contours were disfavored. In panel B, the blue region represents favorable electropositive interaction for inhibition, while red shows disfavored electropositive character.

ASBT Transport Efficiency Model. As with the inhibition models, **2**, **18**, **22**, and **25** were reserved as test compounds for transport efficiency modeling. Figure 9 shows the superimposition of all 22 compounds for transport efficiency model development. No QSAR model was derived from K_t data with significant q^2 .

A comprehensive CoMFA model (CoMFA-2) was derived from $\log(\text{norm}J_{\text{max}}/K_t)$ (Table 2). The q^2 and r^2 values were 0.538 and 0.999, respectively. Similar to CoMFA-1, steric fields dominated the electrostatic fields for transport efficiency with contributions of 61.2% and 38.8%, respectively. Figure 10 shows the relationship between observed and predicted uptake efficiencies of the training and test set (corresponding data in Supporting Information Table S2). The uptake efficiencies of test sets **22** and **25** were underestimated by 2- and 6-fold, respectively. **2** and **18** were significantly underestimated by approximately 9- and 400-fold, respectively. A potential contributing factor to the prediction error of **18** is that its uptake efficiency was over 100-fold greater than that of all other conjugates. The underestimated activity of compound **2** might be due to chemical or physical features beyond the predictive capacity of CoMFA and CoMSIA models.

CoMFA Model Contours for Transport Efficiency. Figure 11 shows steric and electrostatic contours of CoMFA-2 relative to CDCA-glutamyl-2,6-diaminopyridine **15**, the most efficient ASBT substrate within the training set ($\text{norm}J_{\text{max}}/K_t = 0.324 \mu\text{M}^{-1}$). As above, a large green contour near positions 5 and 6 on the pyridine ring in Figure 11A indicates that a bulky group is beneficial. Compared to other position 6 substituents, transport efficiency of **14** with a methoxy group at position 6 on the 3-aminopyridine ring ($\text{norm}J_{\text{max}}/K_t = 0.202 \mu\text{M}^{-1}$) was high compared to **8** (methyl substituted, $0.0119 \mu\text{M}^{-1}$), **20** (fluoro, $0.0262 \mu\text{M}^{-1}$), and **22** (bromo, $0.0415 \mu\text{M}^{-1}$). The other green region near 1 and 6 positions on the pyridine ring of **15** indicates that steric interaction is favorable. A yellow contour between 4 and 5 positions indicates that a steric substituent will decrease efficiency. For example, fluoro substitution exhibited higher efficiencies than corresponding bromo substitution (**19** vs **21**). Figure 11B shows the electrostatic contour. The large blue region near the 4 and 5 positions on the 2,6-aminopyridine ring reflects the lower efficiency of the para hydroxyl in **27** compared to the ortho hydroxyl in **25** or the meta hydroxyl in **26**. The small blue region under the 6 position of **15** agrees with its high efficiency, compared to compounds lacking a 6-amino substitute such as **1**. The red region near the 4 position of **15** indicates that an electronegative substitute at the 4 position on 2-aminopyridine could enhance the transport efficiency.

LogP Effects on ASBT Inhibition and Transport. Partition coefficient (LogP) is a measurement of compound solubility and lipophilicity (24). Since CoMFA and CoMSIA models indicated that steric and hydrophobic features strongly influenced conjugate interaction with ASBT, LogP may explain inhibition or transport efficiency. Calculated LogP (CLogP) was computed

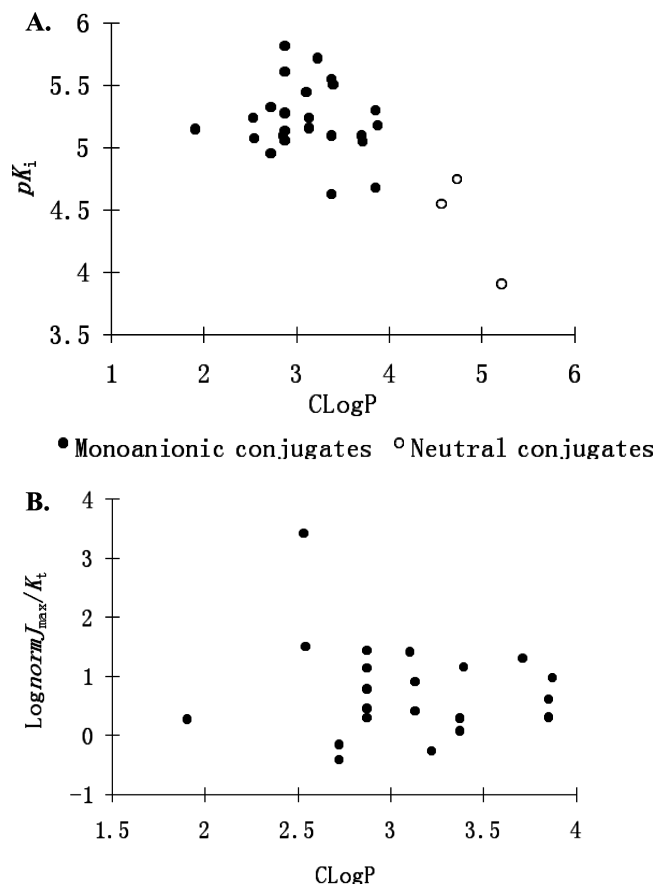


Figure 12. Influence of LogP on ASBT inhibition (A) and transport efficiency (B). (A) Observed pK_i vs CLogP shows a fairly negative correlation between pK_i and CLogP (slope = -0.331 , $r^2 = 0.367$, and $p = 0.0008$). However, there was no simple correlation ($r^2 = 0.021$) among the 24 monoanionic conjugates when the three neutral conjugates (i.e., the α -benzyl substituted conjugates **11**, **13**, and **17**) were eliminated. (B) Observed $\log(\text{norm}J_{\text{max}}/K_i)$ vs CLogP shows no relationship between $\log(\text{norm}J_{\text{max}}/K_i)$ and CLogP with $r^2 = 0.015$ and $p = 0.586$.

using SYBYL according to Hansch and colleagues (24, 25) (Table 1). Plots of observed pK_i vs CLogP (Figure 12A) shows a fairly negative correlation between pK_i and CLogP with $r^2 = 0.367$ and $p = 0.0008$ (i.e., lipophilicity reduced binding potency). Comparison of α -benzyl-substituted conjugates to their nonsubstituted counterparts (**11** vs **10**, **13** vs **12**, and **17** vs **16**) essentially accounted for this trend. However, elimination of these three neutral conjugates (open circles in Figure 12A) leads to no relationship between pK_i and CLogP ($r^2 = 0.021$) among the 24 monoanionic conjugates (filled circles in Figure 12A). This lack of simple correlation between inhibition and CLogP is supportive of COMFA and COMSIA findings that hydrophobicity played differing roles in conjugate binding (i.e., hydrophobic or bulky substitute on the 2 or 6 position of a 3-aminopyridine ring enhanced activity, while a hydrophobic group on the 5 position was detrimental).

There was no correlation between $\log(\text{norm}J_{\text{max}}/K_i)$ and CLogP, with $r^2 = 0.015$ (Figure 12B). Additionally, no correlation was observed between K_i vs CLogP and $\text{norm}J_{\text{max}}$ vs CLogP as well with $r^2 = 0.037$ and 0.005 , respectively. The lack of correlation between transport parameters and CLogP indicates that other physicochemical and structural variables impact transport beyond hydrophobicity, and further suggests a greater complexity in conjugate translocation compared to conjugate binding.

Comparison to Previous ASBT Models. Several computational models have been developed to study structural require-

ments of ASBT inhibition. Baringhaus and co-workers (26) explored the chemical features of ASBT inhibitors with 3D pharmacophore using structurally diverse molecules. 3D pharmacophore and Bayesian models were derived and applied by our lab to identify novel ASBT inhibitors among commercial drugs (15). However, the above studies do not systemically involve C-24 conjugated bile acid analogues, nor do they address the more complex process of ASBT-mediated uptake. Swaan and colleagues (27) applied CoMFA analysis on a series of bile acid-peptide analogues and found that inhibition was favored by an electron donor group between C-24 and C-27 and steric bulk at the end of the side chain. These results corroborate our present findings.

3D-QSAR models using a conformationally sampled pharmacophore (CSP) approach were developed for inhibition requirements using C-24 bile acid conjugates that employed piperidines as probe moieties (28). Results here using pyridines and phenols as probe moieties in part agree with these piperidine findings, where hydrophobicity favored inhibition activity. In contrast to piperidine findings, the results showed a position effect, where the relative location of the pyridine nitrogen and substituent groups modulated the conjugate ASBT inhibition and translocation.

C-24 bile acid conjugates have previously been shown to be ASBT substrates (7, 29–31). One of these studies was a SAR study involving a series of C-24 cholic acid conjugates and found that the C-24 side chain can be 14 Å in length or longer for translocation and that large hydrophobic moieties increase binding to ASBT (29). In another study, C-24 conjugation with glutamic acid and lysine also found that charge and hydrophobicity around the 24 position influenced translocation (7). Results of this study are in agreement with these prior findings that a negative change near C-24 promotes substrate translocation and that steric bulk at certain positions of the aminopyridine ring beyond C-24 also favors translocation.

CONCLUSION

With the view that bile acid conjugates can serve as prodrugs that target ASBT to increase oral drug permeability and bioavailability, a series of aminopyridine or aminophenol conjugates of glutamyl-bile acids were synthesized. Their ASBT inhibition and transport efficiency were evaluated to investigate their interaction beyond the C-24 region of CDCA with ASBT. Similar values for K_i and K_t indicate that substrate binding to ASBT was generally the rate-limiting step in overall substrate transport. 3D-QSAR models were developed for ASBT inhibition. The steric field dominated in the CoMFA model, whereas the hydrophobic field dominated other features in the CoMSIA models. Monoanionic conjugate exhibited higher inhibition potency than neutral conjugate. A hydrophobic or bulky substituent on the 2 or 6 position of a 3-aminopyridine ring enhanced inhibition, while a hydrophobic group on the 5 position was detrimental. Both inhibition models predicted test set inhibition. Recently, the value of inhibition-only QSAR models for drug transporters has been questioned (32), which prompted us to also develop ASBT models for transport efficiency. 3D-QSAR has not previously been applied to ASBT substrate studies. Similar to the inhibition models, the steric field was the predominant contributor; however, we demonstrate that steric contributions to ASBT transport cannot be explained simply by lipophilicity. Overall, these 3D-QSAR models provide new insight into the substrate requirements for ASBT uptake and shed light on the design of bile acid conjugates that can increase intestinal drug permeability and bioavailability.

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Supporting Information Available: Additional related tables and figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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