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Structural requirements of the human sodium-dependent bile acid transporter (hASBT): Role of 3- and 7-OH moieties on binding and translocation of bile acids

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Abstract

Bile acids (BAs) are the end products of cholesterol metabolism. One of the critical steps in their biosynthesis involves the isomerization of the 3 β -hydroxyl (-OH) group on the cholestane ring to the common 3 α -configuration on BAs. BAs are actively recaptured from the small intestine by the human Apical Sodium-dependent Bile Acid Transporter (hASBT) with high affinity and capacity. Previous studies have suggested that no particular hydroxyl group on BAs is critical for binding or transport by hASBT, even though 3 β -hydroxylated BAs were not examined. The aim of this study was to elucidate the role of the 3 α -OH group on BAs binding and translocation by hASBT. Ten 3 β -hydroxylated BAs (Iso-bile acids, iBAs) were synthesized, characterized, and subjected to hASBT inhibition and uptake studies. hASBT inhibition and uptake kinetics of iBAs were compared to that of native 3 α -OH BAs. Glycine conjugates of native and isomeric BAs were subjected to molecular dynamics simulations in order to identify topological descriptors related to binding and translocation by hASBT. Iso-BAs bound to hASBT with lower affinity and exhibited reduced translocation than their respective 3 α -epimers. Kinetic data suggests that, in contrast to native BAs where hASBT binding is the rate-limiting step, iBAs transport was rate-limited by translocation and not binding. Remarkably, 7-dehydroxylated iBAs were not hASBT substrates, highlighting the critical role of 7-OH group on BA translocation by hASBT, especially for iBAs. Conformational analysis of gly-iBAs and native BAs identified topological features for optimal binding as: concave steroidal nucleus, 3-OH “on-” or below-steroidal plane, 7-OH below-plane, and 12-OH moiety towards-plane. Our results emphasize the relevance of the 3 α -OH group on BAs for proper hASBT binding and transport and revealed the critical role of 7-OH group on BA

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Supporting Information Available: ¹H, ¹³C NMR data, MRM scanning parameters for target compounds, conformational analysis, RMSD values, 2D-plots, and representative conformers are available free of charge *via* the Internet at <http://pubs.acs.org>.

translocation, particularly in the absence of a 3 α -OH group. Results have implications for BA prodrug design.

Keywords

Bile acid transporter; iso-bile acids; conformational analysis; translocation; inhibition; permeability

INTRODUCTION

Bile acids (BAs) are the end-products of cholesterol metabolism.^{1, 2} These amphipatic molecules recirculate between the liver and small intestine with only a minimal daily fecal loss.³ This remarkably efficient process is mediated in the small intestine by the human Apical Sodium-dependent Bile acid Transporter (hASBT; SLC10A2), a 348 amino acid protein located in the apical membrane of the ileocytes.^{4, 5} hASBT displays high affinity and high capacity for native BAs binding and transport.^{3, 5, 6}

BAs are biosynthesized from cholesterol by a series of enzymatic modifications including the isomerization of the 3 β -hydroxyl group present on the C₃ of the cholestane nucleus.⁷ Thus, all native BAs bear a 3 α -hydroxyl substituent in the cholane skeleton. Iso-bile acids (iBAs) are 3 β -hydroxy epimers of native BAs commonly found in cecal contents, urine, and blood but not in bile.^{8, 9} Shefer et al, have demonstrated that iso-chenodeoxycholic acid (iCDCA), its 7 β -hydroxyl epimer iso-ursodeoxycholic acid (iUDCA), and their taurine conjugates, are efficiently absorbed in rats after an intraluminal infusion into either the duodenum or the cecum.¹⁰ In the same line, iUDCA has been orally administrated to patients in the treatment of primary biliary cirrosis (PBC) as an alternative to ursodeoxycholate (UDCA).^{11, 12} However, surprisingly, it is not known if iUDCA and other iBAs are actively transported by hASBT.

In 1966, Lack et al. studied the permeation of BA derivatives across everted guinea pig intestinal segments. By employing derivatives with different hydroxylation patterns, it was concluded that no particular hydroxyl group was necessary for transport.¹³ However, 3 β -hydroxy bile acids were not studied. Subsequently, a QSAR model for ASBT binding was developed by Baringhaus et al. using a series of bile acid-like and non-steroidal hASBT inhibitors.¹⁴ However, their data set did not include native or 3 β -hydroxylated BAs, and yet it was concluded that a 3 α -hydroxyl group was not necessary for binding/transport of BAs. Hence, the role of the 3-hydroxyl stereochemistry on hASBT binding/transport of BAs merits further investigation, particularly since iBAs display diminished ability to activate the Farnesoid X Receptor (FXR) nuclear receptor, which impacts numerous metabolic pathways.^{15–19} Additionally, enantiomeric BAs have shown differential interactions with FXR, and other nuclear receptors, compared to their natural BA counterparts.²⁰

The objective of this research was to systematically evaluate the influence of 3 β -hydroxyl group configuration of BAs on hASBT binding affinity and translocation. We synthesized five iBAs corresponding to the 3 β -hydroxy epimers of the five important native BAs. Their glycine conjugates (i.e. gly-iBAs) were also prepared, since high passive permeability of

unconjugated BAs precludes their active uptake component to be detected.²¹ iBAs and gly-iBAs were subjected to hASBT inhibition and uptake studies. In order to explore the conformational space sampled by glycine conjugate of both iBAs and their native epimers, molecular dynamics simulations were performed and topological descriptors identified that relate to hASBT binding and translocation. 3 β -hydroxy BAs showed lower affinity and impaired translocation by hASBT compared to native BAs. Interestingly, 3 β -hydroxy isomerization unveiled the relevance of the 7-hydroxyl group on hASBT-mediated BA translocation. Results offer mechanistic insights about BA transport and conformational requirements for BA binding and translocation by hASBT.

EXPERIMENTAL SECTION

Materials

[³H]-Taurocholic acid (10 μ Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Taurocholic acid (TCA), cholic acid (CA), deoxycholic acid (DCA), lithocholic acid (LCA), and glycine benzyl ester hydrochloride were from Sigma Aldrich (St. Louis, MO). Chenodeoxycholate (CDCA) and ursodeoxycholate (UDCA) were obtained from TCI America (Portland, OR). Geneticin, fetal bovine serum (FBS), trypsin, and DMEM were purchased from Invitrogen (Rockville, MD). All other reagents and chemicals were of the highest purity commercially available.

Chemistry

Routine mass spectrometry (MS) was performed in a LCQ ESI-MS (Thermo Scientific, Waltham, MD). NMR spectra were recorded in a Varian Inova 500 MHz (Varian Inc., Palo Alto, CA) in CDCl₃, MeOD, d₆-DMSO, or mixtures. Chemical shifts are reported in ppm relative to TMS. Abbreviations are as follows: s=singlet; d=doublet; and m=multiplet. Column chromatography was performed using Merck silica gel 60 (0.040–0.063 mm). TLC was conducted on precoated plates with silica gel 60 F-254 (Whatman, Sandford, ME). Plates were developed by spraying with 5% phosphomolybdic acid in EtOH and heating at 120 °C until blue spots appeared. All reactions were performed under nitrogen atmosphere. Purity of intermediates was confirmed by TLC and MS. Purity of final products was assessed by TLC, ¹H NMR and ¹³C NMR spectra, MS, and LC-MS/MS analysis and confirmed the absence of 3 α -hydroxylated-BA impurity in the iBA targets (**1–5**) and their respective glycine conjugates (**6–10**). ¹H NMR and ¹³C NMR of final products, along with ¹H NMR of native bile acids for comparison, are available as Supporting Information (Fig. S1–S25).

General Synthetic Procedures

Iso-BAs (**1–5**) and their respective glycine conjugates (**6–10**) were obtained by the route depicted in Scheme 1 as exemplified by the preparation of iso-chenodeoxycholic acid glycine amide (gly-iCDCA, **7**). Briefly, methyl ester derivatives (BAMe) were prepared as previously reported by Dayal et al.,²² followed by chromatographic purification using mixtures of chloroform/acetone as mobile phase. BAMe were peracetylated by reacting with an excess acetic anhydride in pyridine, followed by chromatographic purification with mixtures of ethyl acetate/hexanes as eluant. Next, the 3 α -acetyl protecting group was

selectively removed from fully protected precursors by reacting with catalytic acetyl chloride in methanol as previously described,²³ purified and treated under Mitsunobu conditions to obtain the 3 β -formate intermediate.²⁴ Pure 3 β -formate intermediates were fully deprotected in one step by refluxing in methanolic 5% KOH,²⁵ precipitated at pH 2–3, and recrystallized from either water or mixtures water/methanol to obtain products **1–5** as white solids (overall yield 25–46%). Gly-iBAs (**6–10**) were prepared from iBAs by using standard peptide coupling chemistry followed by catalytic hydrogenolysis of intermediate benzyl esters in overall yields ranging from 85–92%.

3 β -hydroxy-5 β -cholanolic acid (iLCA, **1) MS (m/z)**—375.3 (M-H). ¹H NMR (CDCl₃/MeOD): δ 0.65 (s, 3H), 0.93 (d, 3H, J = 6.5 Hz), 0.96 (s, 3H), 4.11 (s, 1H, 3 α); ¹³C NMR (MeOD): δ 12.72, 18.98, 22.39, 24.36, 25.44, 27.63, 28.02, 28.68, 29.39, 31.15, 32.21, 32.49, 34.52, 36.30, 36.86, 37.23, 37.95, 41.27, 41.72, 44.09, 57.63, 58.10, 67.91, 178.31.

3 β , 7 α -dihydroxy-5 β -cholanolic acid (iCDCA, **2) MS (m/z)**—391.3 (M-H). ¹H NMR (CDCl₃/MeOD): δ 0.67 (s, 3H), 0.93 (d, 3H, J = 2.1 Hz), 3.85 (s, 1H, 7 β), 4.05 (s, 1H, 3 α); ¹³C NMR (CDCl₃/MeOD): δ 11.44, 17.97, 20.73, 22.94, 23.42, 27.24, 27.97, 29.63, 30.88, 30.91, 31.94, 34.04, 35.28, 35.37, 35.85, 36.05, 39.16, 39.53, 42.49, 50.23, 55.72, 66.49, 68.20, 177.00.

3 β , 7 β -dihydroxy-5 β -cholanolic acid (iUDCA, **3) MS (m/z)**—391.4 (M-H). ¹H NMR (CDCl₃/MeOD): δ 0.65 (s, 3H), 0.90 (d, 3H, J = 6.4 Hz), 0.95 (s, 3H), 3.50 (m, 1H, 7 α), 4.02 (s, 1H, 3 α); ¹³C NMR (CDCl₃/MeOD): δ 11.91, 18.18, 21.39, 23.60, 26.61, 27.26, 28.42, 29.45, 30.96, 34.05, 34.38, 35.21, 36.53, 37.03, 38.57, 40.11, 43.15, 43.57, 54.93, 55.85, 66.15, 71.05, 177.05.

3 β , 12 α -dihydroxy-5 β -cholanolic acid (iDCA, **4) MS (m/z)**—391.3 (M-H). ¹H NMR (MeOD): δ 0.63 (s, 3H), 0.89 (s, 3H), 0.93 (d, 3H, J = 6.1 Hz), 3.93 (s, 1H, 12 β), 4.03 (s, 1H, 3 α). ¹³C NMR (MeOD): δ 13.37, 17.74, 24.31, 25.00, 27.37, 28.01, 28.47, 28.78, 30.23, 31.10, 32.16, 32.44, 34.10, 34.48, 35.89, 36.85, 37.37, 37.99, 40.58, 47.73, 48.26, 67.98, 74.22, 178.28.

3 β , 7 α , 12 α -trihydroxy-5 β -cholanolic acid (iCA, **5) MS (m/z)**—407.3 (M-H). ¹H NMR (MeOD/d₆-DMSO): δ 0.73 (s, 3H), 0.96 (s, 3H), 1.04 (d, 3H, J = 6.5 Hz), 3.80 (s, 1H, 7 β), 3.95–3.98 (partially overlapped s, 2H, 3 α +12 β). ¹³C NMR (MeOD/d₆-DMSO): δ 11.47, 16.10, 21.98, 22.29, 24.92, 26.46, 26.69, 27.80, 28.94, 30.19, 33.50, 34.65, 34.56, 35.25, 35.48, 40.85, 45.57, 45.92, 65.34, 67.02, 71.73, 175.53.

3 β -hydroxy-5 β -cholanolic acid glycine amide (gly-iLCA, **6) MS (m/z)**—432.40 (M-H). ¹H NMR (d₆-DMSO/MeOD): δ 0.79 (s, 3H), 1.06 (d, 6H, J = 8.8 Hz), 3.89 (s, 2H), 4.05 (s, 1H, 3 α). ¹³C NMR (d₆-DMSO/MeOD): δ 12.00, 18.40, 20.96, 23.94, 24.09, 26.23, 26.70, 27.68, 27.99, 29.95, 31.72, 32.31, 33.47, 34.90, 35.20, 35.54, 36.29, 40.61, 42.56, 55.98, 56.40, 65.01, 171.55, 173.43.

3 β , 7 α -dihydroxy-5 β -cholanolic acid glycine amide (gly-iCDCA, **7) MS (m/z)**—448.32 (M-H). ¹H NMR (MeOD): δ 0.70 (s, 3H), 0.97 (m, 6H), 3.80 (s, 1H, 7 β), 3.88 (s,

2H), 3.97 (s, 1H, 3 α). ^{13}C NMR (MeOD): δ 12.35, 19.06, 22.23, 23.97, 24.79, 28.66, 29.37, 31.10, 33.23, 33.51, 33.94, 35.64, 36.82, 36.99, 37.59, 40.85, 41.22, 41.94, 43.87, 51.72, 57.50, 67.86, 69.43, 173.27, 177.26.

3 β ,7 β -dihydroxy-5 β -cholanolic acid glycine amide (gly-iUDCA, 8) MS (m/z)—

448.38 (M-H). ^1H NMR (MeOD): δ 0.72 (s, 3H), 0.99 (d, 6H, J = 7.8 Hz), 3.44 (m, 1H, 7 α), 3.88 (s, 2H), 3.99 (s, 1H, 3 α). ^{13}C NMR (MeOD): δ 12.90, 19.27, 22.83, 24.67, 28.11, 28.59, 29.78, 30.93, 33.27, 33.95, 35.45, 35.79, 36.87, 38.44, 38.67, 41.69, 41.94, 44.48, 44.91, 56.69, 57.60, 67.36, 72.01, 173.07, 176.91.

3 β , 12 α -dihydroxy-5 β -cholanolic acid glycine amide (gly-iDCA, 9) MS (m/z)—

448.34 (M-H). ^1H NMR (CDCl₃/MeOD): δ 0.64 (s, 3H), 0.90 (s, 3H), 0.94 (d, 3H, J = 5.6 Hz), 3.91 (partially overlapped s, 3H, 12 β + CH₂-), 4.02 (s, 1H, 3 α). ^{13}C NMR (CDCl₃/MeOD): δ 12.58, 16.99, 23.47, 23.62, 25.92, 26.57, 27.21, 27.44, 28.75, 31.44, 32.76, 33.13, 34.56, 35.30, 35.76, 36.44, 40.96, 46.39, 46.79, 48.18, 66.74, 73.00, 171.81, 175.13.

3 β ,7 α , 12 α -trihydroxy-5 β -cholanolic acid glycine amide (gly-iCA, 10) MS (m/z)—

464.30 (M-H). ^1H NMR (MeOD): δ 0.73 (s, 3H), 0.96 (s, 3H), 1.05 (d, 3H, J = 6.5 Hz), 3.80 (s, 1H, 7 β), 3.89 (s, 2H), 3.96 (partially overlapped s, 2H, 3 α +12 β). ^{13}C NMR (MeOD): δ 13.26, 18.00, 23.86, 24.40, 27.26, 28.57, 28.84, 30.04, 31.10, 33.24, 33.97, 35.70, 36.47, 36.96, 37.62, 33.66, 41.10, 41.93, 43.15, 47.64, 48.15, 67.65, 69.09, 73.93, 173.01, 176.85.

Cell Culture

Stably-transfected hASBT-MDCK cells were cultured as previously described.²⁶ Briefly, cells were grown at 37 °C, 90% relative humidity, 5% CO₂ atmosphere and fed every 2 days. Culture media consisted of DMEM supplemented with 10% FBS, 50 units/mL penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin. Geneticin was added at 1 mg/mL to maintain selection pressure. Cells were passaged after 4 days or after reaching 90% confluency.

Inhibition Assay

To characterize hASBT binding affinities of products **1–10**, *cis*-inhibition studies of taurocholate (TCA) uptake were conducted as described. Stably-transfected hASBT-MDCK cells were grown on 12-well plates (3.8 cm², Corning, Corning, NY) and grown under conditions described above. Briefly, cells were seeded at a density of 1.0 million/well and induced with 10 mM sodium butyrate 12–15 h at 37 °C prior to study on day 4. Cells were washed thrice with Hank's balanced salt solution (HBSS) prior to uptake assay. Studies were conducted at 37 °C, 50 rpm for 10 min in an orbital shaker. Incubation time was optimized to achieve both steady-state and sufficient analytical sensitivity. Uptake buffer consisted of HBSS, which contains 137 mM NaCl (pH 6.8). Cells were exposed to donor solutions containing TCA (2.5 μM + 0.5 $\mu\text{Ci}/\text{mL}$ [^3H]-TCA) and iBA or gly-iBA (1–200 μM , $n=3$) for 10 min. Cells were washed thrice with chilled sodium-free uptake buffer, lysed with 0.25 mL of 1N NaOH for at least two hours and then neutralized with 0.25 mL of 1N HCl. Lysate was then counted for associated radioactivity (i.e. [^3H]-TCA) using an LS6500 liquid scintillation counter (Beckmann Instruments, Inc., Fullerton, CA). Data were analyzed in terms of inhibition constant K_i (μM) as described below.

Uptake Studies

Uptake studies were performed to obtain kinetic parameters that relate to gly-iBA binding and subsequent translocation into the cell monolayer. Stably-transfected hASBT-MDCK cells were grown, seeded, and induced as described above. Cells were washed thrice with Hank's balanced salt solution (HBSS) or modified HBSS prior to uptake assay. Cells were exposed to gly-iBA (1–200 μ M, $n=3$) and studies conducted at 37 °C, 50 rpm for 10 min in an orbital shaker. Substrate concentration and assay time were optimized to achieve steady-state and adequate analytical sensitivity. Uptake buffer consisted of either HBSS, which contains 137 mM NaCl, or a sodium-free, modified HBSS where NaCl was replaced by 137 mM tetraethylammonium chloride (pH 6.8). Since ASBT is sodium dependent, studies using sodium-free buffer allowed for the measurement of passive uptake of gly-iBAs **6–10**. At the end of the assay, active uptake was stopped by washing the cells thrice with chilled sodium-free buffer. Cells were then lysed with 0.5 mL of acetonitrile allowing for complete evaporation and reconstituted with 0.5 mL of a 1:1 mixture of acetonitrile and 10 mM ammonium formate (pH 7.2) containing 50 ng/mL of TCA as internal standard. Cell lysate was transferred into silanized microcentrifuge tubes and centrifuged at 10000 rpm for 2 min. 100 μ L of supernatant was transfer into an HPLC vial adapted with a 100 μ L insert. **6–10** were quantified by HPLC-MS and HPLC-MS/MS as described below. Gly-iBAs were stable in both uptake buffer and cell lysates as judged by absence of unconjugated native or iso-BAs (data not shown). Uptake parameters Michaelis-Menten transport constant (K_t , μ M), maximum substrate flux ($J_{\max}$, pmol/cm²/s), and passive substrate permeability (P_p , cm/s) were estimated as described under kinetic analysis.

Gly-iBA Quantitation by HPLC-MS/MS

The HPLC system used included a CTC PAL autosampler (LEAP Technologies, Carrboro, NC) and an Agilent 1100 system, comprised of a solvent degasser and binary pump (Agilent Technologies, Palo Alto, CA) and a TL-105 HPLC column heater (Timberline Instruments, Boulder, CO). Chromatography was performed on a Phenomenex Luna C8(2) reversed-phase column (3 μ m, 2.1 $\times$ 50 mm), with a C8 guard cartridge, both obtained from Phenomenex, Inc. (Torrance, CA). The mobile phase consisted of (A) 100% HPLC-grade water with 10 mM ammonium acetate and (B) 100% acetonitrile containing 10 mM ammonium acetate. Ten μ L of each standard or sample dissolved in 50/50:buffer/acetonitrile were injected onto the column. The gradient for gly-iCDCA (**7**), gly-iUDCA (**8**), gly-iDCA (**9**), and gly-iCA (**10**) began at 10% B, increased to 20% B over 0.1 min, then from 20% to 40% B over 2.9 min, increased to 90% B over 0.1 min, and then held at 90% B for 0.4 min, before returning to 10% B over 0.5 min, for a total runtime of 4.0 min. The gradient for gly-iLCA (**6**) began by holding at 10% B for 0.1 min, increased linearly to 90% B over 3.4 minutes, and then held at 90% B for 0.2 minutes before equilibrating back to 10% B over 1.3 minutes, for a total runtime of 5.0 minutes. Mass spectrometry was performed using a hybrid triple quadrupole-linear ion trap mass spectrometer, 4000 QTRAPTM LC/MS/MS system (AB Sciex, Foster City, CA). The negative ion mode of 4000 QTRAPTM mass spectrometer was calibrated using PPG3000 (AB Sciex). The instrument was run in the negative ion multiple reaction monitoring (MRM) mode. The scan parameters for MRM of

gly-iBAs can be found as Supporting Information (Table S1). AB Sciex/MDS SCIEX Analyst software (version 1.4.2) was used for data acquisition and processing.

Kinetic Analysis

Data from TCA uptake inhibition were fitted to eqn 1 in WinNonlin 5.2 (Pharsight, Mountain View, CA) where J is substrate flux (pmol/cm²/s), I is inhibitor concentration (μM), and S is substrate concentration (μM). Eqn 1 is a modified version of the classical competitive inhibition model that accounts for the presence of an apical boundary layer (P_{ABL}).²⁷ Only K_i was estimated in eqn 1, while other parameters relative to the substrate (J_{max} , P_p , and K_t) were fixed and obtained from parallel TCA uptake studies performed on the same occasion. P_{ABL} was set to 1.5×10^{-4} cm/s.²⁷ Eqn 1 was chosen over other models (e.g. non-competitive inhibition) based on Akaike's Information Criterion (data not shown).

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

Data from gly-iBA acid uptake studies were simultaneously fitted to eqn 2 and eqn 3 by nonlinear regression in WinNonlin 5.2 (Pharsight, Mountain View, CA). In this way, kinetic parameters of hASBT-mediated active uptake (K_t and J_{max}) were obtained, as well as passive permeability (P_p) of gly-iBA into monolayer.

$$J = \frac{P_{ABL} \cdot P_p \cdot S}{P_{ABL} + P_p} \quad (2)$$

$$J = \frac{P_{ABL} \cdot \left(\frac{J_{max}}{K_t + S} + P_p \right)}{P_{ABL} + \frac{J_{max}}{K_t + S} + P_p} \cdot S \quad (3)$$

Statistical Analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Affinity data for each native BA and its respective iso-BA counterpart were compared using Student's t-test. Difference was considered statistically significant if $p < 0.05$.

In-silico Conformational Analysis

Geometrically optimized 3D structures for compounds **6–10** and their corresponding native BA counterparts were generated with Gaussian03 software (Gaussian, Inc., Wallingford CT), at the B3LYP 6-31G (d,p) level of theory.²⁸ Topology and parameters for molecular dynamics simulations were obtained from the SwissParam server.²⁹ Periodic cells were constructed with Visual Molecular Dynamics (VMD) program version 1.9.³⁰ All molecules were aligned using the steroidal nucleus as reference to work within the same spatial

coordinates. Compounds in their ionized form were solvated leaving at least 15x15x15 Å between the center of mass of each ligand and cell limits, and neutralized by random addition of Na⁺ and Cl⁻ ions up to a concentration of 137 mM of NaCl. The average dimension of the periodic cells were ~50x35x35 Å each containing an average of ~86,300 total atoms. Systems were subjected to energy minimization followed by a 15 ns molecular dynamics simulation (MD) where the first 5 ns allowed equilibration such that the remaining 10 ns were considered for production. MD simulations were performed with NAMD,³¹ using the CHARMM27 force-field with TIP3P water model,³² periodic boundary conditions, and the Particle Mesh Ewald (PME) for electrostatic forces calculation.³³ The Langevin approximation was used to maintain the temperature (300 °K) and pressure (1 atm).³⁴ SHAKE algorithm was used to constrain hydrogen bonds distances allowing the use of a 2 fs integration time.³⁵ Trajectory analyses were performed with VMD v1.9 to calculate all atom root-mean square deviation (RMSD) and several topological descriptors (e.g. distances, angles, dihedrals). In order to obtain representative structures of each compound, conformational clustering was performed using WORDOM v0.21.³⁶ Clusters were obtained using a positional RMSD cut-off of 3.0 Å based on the steroidal scaffold atoms. The central member of each cluster was selected as the representative structure.

RESULTS

hASBT Inhibition Data

Binding affinities of unconjugated (**1–5**) and glycine conjugated (**6–10**) iBAs were assessed by *cis*-inhibition of taurocholate active uptake in stably transfected hASBT-MDCK monolayers. The inhibitory potencies (i.e. K_i) of compounds, along with native BA data for comparison, are summarized in Table 1 and 2. Inhibitory affinities of unconjugated iBAs ranged from 5.36 to 53.8 μM (iLCA and iCA, respectively). In all cases, except for iUDCA, the inhibitory potency of iBA was weaker than the corresponding native BA. The impact of 3-hydroxyl epimerization (α to β) on BA derivatives was the highest for iCDCA with a K_i 6-fold less inhibitory potency than CDCA. Absence of native BAs as impurities in target iBAs as judged by LC/MS/MS, along with stability and relative potency of the iBAs, excludes such impurities to bias kinetic data.³⁷ iBAs were non-toxic to cells during the 10-min incubation period (data not shown).

Interestingly, iUDCA showed similar K_i to UDCA (*p* > 0.05) being both the weakest inhibitors of the dihydroxylated BA derivatives. The rank order for potency of iBAs was iLCA > iCDCA ≈ iDCA > iUDCA > iCA, somewhat similar to the order of native bile acids, where LCA ≈ CDCA > DCA > CA > UDCA. The inhibitory potency of iBAs was inversely proportional to the number of hydroxyls substitutions on the cholane scaffold. 7-hydroxyl stereochemistry effect on iBAs was similar to its effect on native BA binding affinity, where 7α-hydroxyl is preferred over the 7β-epimer.²¹ However, this effect was attenuated for iBAs, such that iCDCA was only 2-fold more potent than iUDCA, rather than 20-fold for their native counterparts. Figure 1A illustrates the inhibition profile of iCDCA.

In the glycine conjugated series, K_i values varied from 4.53 to 23.3 μM (gly-iCDCA and gly-iCA, respectively). 3-hydroxyl epimerization showed the highest impact on gly-iLCA (**6**) with a 12-fold weaker inhibitory potency compared to gly-LCA. Inhibition potency

ranking was gly-iLCA $\approx$ gly-iCDCA > gly-iUDCA > gly-iDCA > gly-iCA and showed some similarity to that of native BAs, which was gly-LCA > gly-CDCA > gly-DCA $\approx$ gly-UDCA > gly-CA.²¹ In general, glycine conjugation increased affinity of iBAs except for the 7-dehydroxylated derivatives gly-iLCA and gly-iDCA ($p > 0.05$). As with conjugated native BAs, the pattern of gly-iBA binding affinities suggested that 12 α -hydroxylation hinders gly-iBAs binding to hASBT.

hASBT Transport Data

Uptake studies were conducted to evaluate the ability of gly-iBAs to serve as substrates of hASBT. Transport studies were not conducted for unconjugated iBAs since previous data on unconjugated BAs have shown that their high passive permeability precludes obtaining hASBT transport kinetic estimates.²¹ Gly-iCDCA, gly-iUDCA, and gly-iCA were hASBT substrates with Michaelis-Menten K_t ranging from 59.5 to 163 μ M (Table 2). Active uptake parameters for gly-iLCA were not determinable, due to its high passive permeability. In fact, gly-iLCA P_p was 20-fold that of gly-LCA (Table 2). This situation differs from that of gly-iDCA whose cellular uptake was insensitive to the presence of sodium, and displayed a similar P_p to gly-DCA, suggesting gly-iDCA was not transported by hASBT (Table 2). In all cases, the iBA was the weaker substrate, compared to its corresponding native bile acid, in terms of K_t . Similar to the binding scenario, although much more pronounced, the highest impact of 3-hydroxyl epimerization on transport affinity was on gly-iCDCA which was 90-fold weaker than gly-CDCA. Figure 1B illustrates the uptake profile of gly-iCDCA. Of note, and unlike the situation for native BAs, 7-hydroxyl epimerization had only a minor effect on transport affinity of gly-iBA (gly-iCDCA vs gly-iUDCA).

Normalized $J_{\max}$ of gly-iBA was lower than normalized $J_{\max}$ of the corresponding native BA (Table 2). For example, gly-iCDCA and gly-iUDCA $J_{\max}$ values were 18- and 16-fold lower than $J_{\max}$ values for gly-CDCA and gly-UDCA, respectively. The rank order for substrate $J_{\max}$ of gly-iBAs was gly-iCA > gly-iCDCA $\approx$ gly-iUDCA $\gg$ gly-iDCA, highlighting the role of 7-hydroxyl group on iBA transport. This pattern differed from that of native BAs which was gly-CDCA $\approx$ gly-LCA $\approx$ gly-DCA > gly-UDCA $\approx$ gly-CA.²¹

Regarding passive permeability, no clear pattern was observed when comparing gly-iBAs and their respective native counterpart. The rank order for gly-iBA P_p was gly-iLCA > gly-iUDCA $\approx$ gly-iDCA $\approx$ gly-iCA > gly-iCDCA, unlike that of native bile acids which is gly-UDCA $\approx$ gly-LCA > gly-DCA $\approx$ gly-CDCA > gly-CA. gly-iCDCA and gly-iLCA displayed extreme P_p values (0.058 and 16.6×10^{-6} cm/s), approximately one order of magnitude lower and higher than the average values for derivatives, respectively. Although glycine conjugates of BAs are fully dissociated at the buffer pH regardless of steroidal hydroxylation pattern,³⁸ gly-iLCA was unique in that it exhibited solubility less than 200 μ M (about 50 μ M).

Conformational Analysis

In order to gain further insight into the conformational space sampled by conjugated native and isomeric BAs, 15 ns MD simulations were performed over compounds **6–10** and their corresponding 3 α -hydroxylated counterpart. In all cases, average all-atom RMSD values

stayed within 1.2–2.4 Å (Supporting Information Fig. S26). Trajectories analysis included the following topological descriptors (Fig. 2): i) curvature of the steroidal scaffold ($O_3-C_{10}-C_{17}$); ii) facial disposition (α or β) of hydroxyl groups O_3 ($O_3-C_5-C_{10}-C_{19}$), O_7 ($O_7-C_7-C_{14}-H_{14}$), and O_{12} ($O_{12}-C_{12}-C_9-H_9$); and iii) facial disposition/distance of carboxylate group relative to the steroidal nucleus ($H_{14}-C_{14}-C_{17}-C_{26}$)/(H₁₄-C₂₆), respectively.

Regarding the curvature of the steroidal scaffold (angle $O_3-C_{10}-C_{17}$), inspection of the probability distributions for each compound (not shown) led us to identify three different possible conformations: concave ($<135^\circ$), stretched ($135^\circ-160^\circ$), and flat ($>160^\circ$). In general native gly-BAs sampled a concave nucleus in more than 86% of the trajectory, while gly-iBAs showed a preference for a stretched conformation (more than 84% of the conformers). Two compounds markedly differed from this pattern: gly-UDCA and gly-iCA. While the former displayed a strong preference for a stretched and flat steroidal skeleton, accounting for more than 98% of the sampled conformers, the latter was the only iso-BAs derivative with 40% of the conformers sampling a flat steroidal nucleus (Supporting information, Table S2).

Analysis of the facial orientation of the common 3-hydroxyl group ($O_3-C_5-C_{10}-C_{19}$) on BA derivatives identified the following spatial positioning relative to the steroidal nucleus: below-plane ($>135^\circ$), “on-plane” ($90^\circ-135^\circ$), and above-plane ($<90^\circ$). In the gly-iBAs series, 3- and 3,7-dihydroxylated derivatives displayed a strong preference for an O_3 group “on-plane” ($>90\%$), while 12-hydroxylated gly-iDCA and gly-iCA were able to sample the above-plane conformation with a 26 and 44% occurrence, respectively. On the other hand, no clear pattern was identified for native gly-BAs, although data showed that: i) 3-mono hydroxylated gly-LCA prefers the below-plane orientation (79%); ii) addition of a –OH group on C_7 , reorients O_3 towards plane (94% for 7 α -hydroxylated gly-CDCA) or above the plane (83% for 7 β -hydroxylated gly-UDCA); and iii) 12-hydroxylated derivatives position O_3 sampling the “on-plane” and below-plane conformation similarly (54 and 44%, respectively for gly-CA).

Facial orientation of 7-hydroxyl group relative to steroidal nucleus ($O_7-C_7-C_{14}-H_{14}$) could be grouped into the following categories: below-plane ($0^\circ-30^\circ$), towards-plane ($30^\circ-60^\circ$), and within steroidal cavity ($<0^\circ$). Gly-CDCA, gly-CA and their 3-isomeric counterparts (gly-iCDCA and gly-iCA) showed a strong preference for an O_7 group below the plane ($>86\%$). Gly-iCDCA and gly-iCA oriented their 7-hydroxyl group towards plane on 16% of the trajectory. Of note, native gly-CDCA and gly-CA were able to orient their O_7 within the steroidal cavity (“hidden”) in 8 and 12% of the dynamics, respectively. On the other hand, 7 β -hydroxylated derivatives gly-UDCA and gly-iUDCA oriented their O_7 above the plane ($>90^\circ$) with more than 99% occurrence.

Similar conformational analysis over the 12-hydroxyl group ($O_{12}-C_{12}-C_9-H_9$) showed some preference for the towards-plane orientation in native derivatives gly-DCA and gly-CA ($>71\%$) compared to their 3-isomeric counterparts gly-iDCA and gly-iCA (48 and 64%, respectively). Noticeably, 29 and 23% of gly-DCA and gly-CA conformers positioned their O_{12} within the steroidal cavity, figures that rose to 52 and 36% for their respective 3-isomeric counterparts (Table S2). Figure 3 (**panels A, B and C**) are 2D-plots of topological

descriptors for gly-CA and gly-iCA. Similar plots for the other native-isoBA pairs in Supporting Information (Fig. S27).

Figure 4 displays the conformational space sampled by the carboxylate group of gly-iCDCA and gly-CDCA (faciality and distance) relative to the steroidal nucleus (H_{14}) in a polar set of coordinates. This representation is convenient since it relates both descriptors to a conformationally-restricted (α) hydrogen on the steroidal scaffold such that points (conformers) below the “horizon” (horizontal red line) orient their C_{26} group below the plane and *vice-versa*, while the H_{14} - C_{26} distance increases radially (0–15 Å). At the same time, points on the right-half side of the plot represent conformers with their C_{26} group towards C_7 while those on the left-half side orient the carboxylate towards C_{12} . Based on this, it is possible to divide the plot in four quadrants (I through IV), moving in a counter clock-wise fashion, starting at a dihedral angle H_{14} - C_{14} - C_{17} - C_{26} of 0° . In general, conjugated BA derivatives, oriented their carboxylate group preferentially towards C_7 (quadrants I+II 90%) rather than towards C_{12} (III+IV 10%), independently of their 3-hydroxyl conformation. In this regard gly-iCDCA (Fig. 4) was unique since more than 83% of its conformers placed their C_{26} group within quadrant I ($< 90^\circ$ relative to H_{14}). Polar plots for the remaining gly-iBA/gly-BA pairs in Fig. S28 (Supporting Information). Cluster analysis identified the center of topological clusters for each BA derivative. Typically, the most populated cluster contained up to 35% of the trajectory conformers. Gly-iCDCA and gly-iLCA were exceptions such that their most populated cluster represented 50 and 54% of the conformers, respectively. Figure 5 shows an overlay of conformers from cluster analysis of gly-iLCA and gly-iCDCA (**panels A and B**) and an overlay of the representative conformer (rc) of the most populated cluster for each derivative (**panel C**). Representative conformers of most populated clusters for gly-iBAs and their respective native counterpart are presented in Fig. S29 (Supporting Information).

DISCUSSION

Comparison of Inhibition and Uptake Data: Kinetic and Conformational Interpretation

Iso-BAs and gly-iBAs were weaker hASBT inhibitors and substrates than their corresponding native BA epimer. They generally showed both weaker inhibition, as measured by K_i , and impaired transport, as measured by K_t and J_{max} .

In a simplified view of transport across hASBT, BA first binds to hASBT to be subsequently translocated. In this simple view, binding and translocation are the two steps for overall transport. It's been previously concluded that native BA binding to hASBT was the slowest step in their transport across hASBT, since K_t was approximately equal to K_i .²¹ However, gly-iBAs did not follow this kinetic pattern. Rather, K_t was generally several-fold larger than K_i , suggesting that translocation was the rate-limiting step.³⁹ For example, K_t was about 10-fold higher than K_i for gly-iCDCA, gly-iUDCA, and gly-iCA. Consistent with this interpretation, all gly-iBA here studied displayed lower TCA-normalized J_{max} compared to native BAs. For example, gly-iCDCA, gly-iUDCA, and gly-iCA showed 18-, 16-, and 3-fold lower J_{max} , respectively. These data suggest that, unlike the situation with native gly-BAs, overall gly-iBAs transport across hASBT is translocation-limited. Although taurine conjugates of iBAs were not studied here, it is speculated that taurine conjugation on iBAs

will have a similar effect as glycine conjugation on iBAs, since taurine and glycine conjugation each had similar effects of BA binding and transport with hASBT.²¹

Results from conformational analysis showed that 3-hydroxy epimerization (α to β) had a strong impact over topological descriptors: global curvature of steroidal nucleus, positioning of 3-OH group relative to steroidal scaffold, and faciality of 7- and 12-OH groups. Firstly, iso-BAs derivatives displayed a more stretched steroidal nucleus compared to their native counterparts. This finding is in agreement with previous data from Poša and Kuhajda as well as Roda et al., who evaluated the influence of polar axial (ax) or equatorial (eq) groups (hydroxyl and oxo) on the steroidal nucleus of native and isomeric BA derivatives on their critical micelle concentration (cmc) values.^{40, 41} They found that replacing a 3 α -OH (eq) group by a 3 β -OH (ax) or an oxo-group, increases cmc values of BA derivatives. The stretched steroidal nucleus on iso-derivatives would decrease the fjord effect between monomers in the primary micelles (Small's model of β -side packing of monomers in primary micelles)⁴² and the axial 3 β -OH would be able to form hydrogen bonds with surrounding water molecules from both faces of the steroidal nucleus (α and β), thus reducing the driving force for micelle formation.⁴³ Secondly, 3-hydroxyl orientation of gly-iBAs displayed a tighter conformational space compared to native BA, positioning O₃ preferentially "on-plane" (7-hydroxylated derivatives) together with a minor "above-plane" orientation (12-hydroxylated members) relative to the steroidal nucleus. On the other hand, native gly-BAs showed greater conformational freedom for O₃, albeit without a clear pattern. In fact, while gly-LCA, gly-CDCA, and gly-UDCA respectively sampled the below-plane, "on-plane", and above-plane orientation preferentially, the 12-hydroxylated derivatives (gly-DCA and gly-CA) showed a bimodal distribution of conformers similarly populating both the "on-plane" and the above-plane faciality. Thirdly, 3-OH isomerization had opposite effects on 7 α - and 12 α -OH facial preference, being able to either decrease or increase the "within steroidal cavity" positioning, respectively. No reorientation was observed for the 7 β -OH moiety. Collectively, these data identified the preferred BA conformation for hASBT inhibition as: concave steroidal nucleus, 3-OH "on-" or below-plane, 7-OH below-plane, and 12-OH moiety towards-plane. Of note, the least potent native (UDCA) and isomeric (iCA) BA derivatives sampled stretched/flat steroidal nucleus similarly and had the largest population of conformers with 3-OH above-plane (Supporting Information, Table S2), suggesting the role of 3-hydroxyl group on BA binding to hASBT.

Regarding P_p of BA derivatives, most derivatives displayed values that were insensitive to 3-OH isomerization, with two notable exceptions: gly-iCDCA and gly-iLCA. Polar plots of C₂₆ relative to steroidal nucleus showed that gly-iCDCA (i.e. the least permeable derivative) had a strong preference for a carboxylate group below the plane and within 5–10 Å from the steroidal nucleus (Fig. 4), while gly-iLCA (i.e. the most permeable) showed no particular pattern. Cluster analysis of trajectories revealed that these two iso-BAs were the only derivatives with a representative conformer (rc) accounting for 50% or more of the entire dynamics (Fig. 5C). Inspection of the gly-iLCA (rc) model suggests the existence of an intermolecular hydrogen bond between carboxylate group and amide proton in a 5-member system arrangement. Both hydrogen bond distance (2.8 Å) and dihedral angle (N-H...O-C₂₆, 3.6°) are in agreement with recently published data by Kuhn et al., who found that 5-membered ring systems formed by intramolecular hydrogen bonds showed planar

conformation (small dihedrals) and long distances (2.5–3.0 Å).⁴⁴ In contrast, no such system was found for any of the (rc) of gly-iCDCA. Solvation energy calculations results for the (rc) of gly-iLCA, gly-iCDCA, and their respective native counterparts, between a low-dielectric media ($\epsilon=2$) and water ($\epsilon=80$) showed that gly-iLCA had the least negative energy differential (−74.6 kcal/mol) while gly-iCDCA had the most negative energy difference (−81.8 kcal/mol). Taken together, these data suggest that the very low and high P_p of gly-iCDCA and gly-iLCA are due to low conformational freedom together with favorable and unfavorable solvation energies, respectively.

Perhaps surprisingly, results here are the first to evaluate the role of 3-hydroxyl stereochemistry on bile acid inhibition and uptake by hASBT. iBAs are 3 β -hydroxy epimers of native BAs and constitute an important fraction of the BAs excreted daily in the feces (around 27%).⁸ Additionally, iBAs are also present in urine and blood (15–20%) but not in bile.⁹ Kinetic studies have demonstrated that orally absorbed iBAs (iCDCA and iUDCA) are efficiently epimerized in the liver, and not during intestinal transit, to yield native BAs.^{10–12} All native BAs bear a 3 α -hydroxy group in the cholane skeleton, suggesting that this structural feature might be critical for bile acid function and/or interaction with the various transporters/receptors responsible for the homeostasis of bile salts, such as hASBT. For example, iLCA has been shown to serve as a TGR5 agonist, similar to LCA.⁴⁵ On the other hand, iBAs are weak agonists of FXR.¹⁵ In a recent study, a knock-out mouse model that synthesized only iBAs exhibited loss of negative feedback regulation of FXR by bile acid.⁴⁶ This *in-vivo* observation is consistent with 3 α -hydroxyl group of bile acid being required for FXR activation.¹⁵ Results here showed that gly-iBAs displayed P_p similar to their native counterpart (except for gly-iLCA and gly-iCDCA), suggesting that iBAs could have potential as permeability-enhancing moieties in a pro-drug design presumably with less FXR-mediated intestinal effects.

In their seminal work on native BA structure-activity relationship, Lack and colleagues suggested that no particular hydroxyl group on the cholane skeleton is required.¹³ Subsequently, Kramer and coworkers developed a model for ASBT binding requirements, based on a data set that included non-steroidal compounds and BA derivatives with linkage via C₃. Their model identified five pharmacophoric elements: one hydrogen bond acceptor, one hydrogen bond donor, and three hydrophobic interaction points. Interestingly, it was concluded that the 3 α -hydroxyl group was not necessary for binding/transport even though iBAs were not examined.¹⁴ More recently, González et al., explored the role of the exofacial half of transmembrane domain (TM) 7 of hASBT on BA binding and translocation.⁴⁷ The authors prepared two irreversible inhibitors of hASBT by chemical modifications of CDCA (electrophilic BAs), systematically mutated amino acids residues in hASBT from Phe-287 (membrane limit) to Q-297 (membrane center) by cysteines, and performed MTS-biotin labeling experiments in presence and absence of electrophilic and native BAs in order to test their ability to interact with amino acids residues in this TM. All BA derivatives carrying a free 3 α -hydroxyl group were able to interact with glutamine 297 while 3 β -chloro-CDCA (without 3 α -hydroxyl moiety) was unable to protect Q-297 from MTS-biotin labeling suggesting the role of 3 α -OH group on BA binding to hASBT.

Thus, results here presented complement previous findings emphasizing the role of 3 α -hydroxyl group on BA binding to hASBT.

Role of 7-hydroxyl Group in Bile Acid Translocation

Uptake results directly highlighted the role of 7-hydroxy moiety on BAs on hASBT-mediated translocation. For example, 7-dehydroxylated gly-iDCA was not translocated by hASBT while its 7 α -hydroxylated homologue gly-iCA was a substrate. Thus, addition of a 7 α -hydroxyl group restores “translocability” to gly-iDCA. Likewise, gly-iLCA may not be a substrate, while its 7 α or 7 β -hydroxylated homologues (gly-iCDCA and gly-iUDCA, respectively) are actively transported by hASBT. In these cases, addition of a 7-hydroxy moiety, regardless of stereochemistry, resulted in BA derivative being translocated by hASBT. To the best of our knowledge, results here presented are the first to directly evaluate the significance of 7-hydroxyl group on BA translocation via hASBT using a set of native and 3-epimeric BA derivatives. Interestingly, only by isomerizing the 3 α -OH moiety, which from findings here otherwise dominates the global transport scenario, it was found that a hydroxyl group on C₇ is critical for proper BA translocation by hASBT. It should be emphasized that this discussion of translocation focuses on post-bind events, as all studied compounds were able to inhibit the transporter. Our result is in agreement with recent reports from Hussainzada et al. who indicated that Asp-124 on the extracellular loop 1 of hASBT interacts with the 7 α -hydroxy moiety of native BAs and serves as an hydrogen bond acceptor.⁴⁸ They observed that neutralization of Asp-124 by mutation to an alanine reduced ASBT inhibitory activity for 7 α -hydroxylated BAs without affecting DCA, which lacks a 7 α -hydroxy group. Meanwhile, the D124N mutation exhibited higher taurocholate affinity than the D124A mutant, emphasizing the role of the 7-hydroxyl group on BA translocation by hASBT, particularly in the absence of a 3 α -hydroxyl on the steroidal nucleus.

CONCLUSION

The objective was to systematically evaluate the influence of 3 β -hydroxyl configuration in BAs on hASBT binding affinity and translocation. Ten iBAs were synthesized and evaluated for hASBT inhibition and uptake, comparing data against their corresponding native bile acid counterpart. The 3 β -hydroxy epimers bound to hASBT with lower affinity and exhibited reduced translocation, compared to 3 α -hydroxylated BAs. In contrast to native bile acids, iBA translocation, and not hASBT binding, was the rate-limiting step in iBA transport. Conformational analysis identified topological features for optimal hASBT binding as: concave steroidal nucleus, 3-OH “on-” or below-plane, 7-OH below-plane, and 12-OH moiety towards-plane. Additionally, results here revealed the critical role of 7-hydroxyl group on BA translocation, particularly in the absence of a 3 α -hydroxy group. Iso-bile could have potential as permeability-enhancing moieties in a prodrug design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BA s	Bile acids
hASBT	apical sodium-dependent bile acid transporter
iBA s	iso-bile acids
gly-BA/gly-iBA	glycine conjugated bile acid/iso-bile acid
TCA	taurocholic acid
CA	cholic acid
DCA	deoxycholic acid
LCA	lithocholic acid
CDCA	chenodeoxycholic acid
UDCA	ursodeoxycholic acid
K_i	inhibition constant
K_t	Michaelis-Menten transport constant
J_{max}	maximum transport flux
P_p	passive permeability
P_{ABL}	apical-boundary layer permeability

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FIGURE 1.A **FIGURE 1.B** **Figure 1.**

Kinetic characterization of iso-chenodeoxycholic acid. Panel A shows the concentration-dependent inhibition profile of iCDCA ($K_i = 11.8 \pm 1.6 \mu\text{M}$). Panel B depicts the uptake profile of gly-iCDCA into hASBT-MDCK monolayer. hASBT-mediated uptake is sodium-dependent. Total uptake (filled square) was measured in the presence of sodium. Passive uptake (open square) was measured in the absence of sodium. hASBT-mediated uptake (filled circle) was computed as the difference between studies and reflects saturable uptake ($K_t = 59.5 \pm 8.8 \mu\text{M}$).

Figure 2.
Schematic conformational representation of gly-iBAs and their native counterparts, as exemplified by gly-iCA/gly-CA. Atoms included in the topological analysis are labeled following the bile acid numbering system.

FIGURE 3.A

FIGURE 3.B

FIGURE 3.C

Figure 3.

2D-plots of topological descriptors for gly-iCA (red) and gly-CA (blue) depicting the curvature of the steroidal scaffold (O₃-C₁₀-C₁₇, Panel A) and facial orientation of 7 α -(O₇-C₇-C₁₄-H₁₄, Panel B) and 12 α -hydroxyl (O₁₂-C₁₂-C₉-H₉, Panel C) groups against the facial positioning of 3-hydroxyl group (O₃-C₅-C₁₀-C₁₉).

Figure 4.

Polar plots of the facial orientation of carboxylate group/distance to the steroidal nucleus (H_{14}) for gly-iCDCA (red) and gly-CDCA (blue). Points below the horizontal red line are C_{26} groups below the steroidal plane and *vice-versa*. H_{14} - C_{26} distance increases radially (0–15 Å). Quadrants I and II represents C_{26} groups towards C_7 and quadrants III and IV include conformers with carboxylates towards C_{12} . While gly-CDCA sampled quadrants I and II indistinctively, gly-iCDCA showed a strong preference (>83%) for a C_{26} group within quadrant I (< 90° relative to H_{14}).

FIGURE 5.A **FIGURE 5.B** **FIGURE 5.C**

Figure 5. Cluster analysis of gly-iLCA and gly-iCDCA. Panels A and B show overlaid conformers for gly-iLCA and gly-iCDCA, respectively. Panel C displays an overlay of the representative conformer (rc) of the most populated cluster for gly-iLCA (green) and gly-iCDCA (purple).

Scheme 1.

Synthesis of 3β-hydroxy bile acids and their glycine conjugates as exemplified by the preparation of iso-chenodeoxycholic acid glycine amide. **a)** reflux, anh. MeOH, *p*-TsOH; **b)** acetic anhydride, pyr., rt; **c)** acetic chloride, anh. MeOH, rt; **d)** diisopropyl-azodicarboxylate (DIAD), triphenylphosphine, formic acid, anh. toluene, reflux; **e)** i. 5% KOH, MeOH, reflux; ii. HCl dil. pH 2–3; **f)** i. glycine *O*-benzyl ester hydrochloride, *O*-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU); diisopropylethylamine, dimethylformamide (DMF), rt; ii. H₂, 10% Pd/C, ethanol, rt.

Table 1Binding affinities of unconjugated bile and iso-bile acids to hASBT^{a,b}

Trivial name	R ₁	R ₂	R ₃	K _i (μM ±SEM)
LCA	-OH(α)	-H	-H	2.10 (0.22)
iLCA (1)	-OH(β)	-H	-H	5.36 (0.80)**
CDCA	-OH(α)	-OH	-H	1.94 (0.17)
iCDCA (2)	-OH(β)	-OH	-H	11.8 (1.6)**
UDCA	-OH(α)	OH(β)	-H	22.6 (3.0)
iUDCA (3)	-OH(β)	OH(β)	-H	24.7 (2.8) ^{ns}
DCA	-OH(α)	-H	-OH	8.31 (0.77)
iDCA (4)	-OH(β)	-H	-OH	11.4 (1.3)*
CA	-OH(α)	-OH	-OH	17.7 (1.4)
iCA (5)	-OH(β)	-OH	-OH	53.8 (14.0)*

^a Binding affinities of native bile acids are provided for comparison.²¹^b Statistical significance between iBA and corresponding BA affinity (** denotes $p < 0.01$; * denotes $0.01 < p < 0.05$; ns denotes not significant $p > 0.05$).

Table 2

Binding affinities and kinetic parameters of hASBT-mediated active uptake of glycine conjugates of bile and iso-bile acids ^{a,b}

Trivial name	K _i (μM ±SEM)	K _t (μM ±SEM)	Relative J _{max} ^c (% TCA J _{max} ±SEM)	P _p (cm/s ± SEM x 10 ⁶)
Gly-LCA	0.427 (0.017)	<0.1	405 (64)	0.865 (0.780)
Gly-iLCA (6)	5.07 (0.65)**	nd ^d	nd	16.6 (0.8)
Gly-CDCA	0.992 (0.089)	0.662 (0.337)	432 (58)	0.414 (0.201)
Gly-iCDCA (7)	4.53 (0.48)**	59.5 (8.8)	23.9 (2.3)	0.058 (0.07)
Gly-UDCA	3.41 (0.21)	11.5 (2.0)	226 (36)	1.01 (0.13)
Gly-iUDCA (8)	7.66 (1.19)*	75.3 (22.1)	14.0 (2.4)	0.690 (0.090)
Gly-DCA	3.22 (0.26)	1.10 (0.26)	349 (47)	0.520 (0.162)
Gly-iDCA (9)	12.4 (1.4)**	149 (75) ^{ns}	3.68 (1.14) ^e	0.730 (0.020)
Gly-CA	7.53 (0.44)	11.0 (1.9)	152 (24)	0.220 (0.157)
Gly-iCA (10)	23.3 (2.7)**	163 (37)	61.2 (9.9)	0.740 (0.120)

^a Binding affinities and uptake parameters of native bile acids are provided for comparison.²¹

^b Statistical significance between gly-iBA and corresponding gly-BA affinities (** denotes $p < 0.01$; * denotes $0.01 < p < 0.05$; ns denotes not significant $p > 0.05$)

^c Absolute J_{max} (pmol/cm²/s) for each gly-iBA were 0.017 ± 0.006 (gly-iLCA), 0.065 ± 0.004 (gly-iCDCA), 0.010 ± 0.003 (gly-iDCA), 0.038 ± 0.006 (gly-iUDCA), and 0.166 ± 0.024 (gly-iCA) respectively. TCA P_p and J_{max} were 1.56×10^{-6} cm/s and 0.271 ± 0.020 pmol/cm²/s, respectively.

^d Not determinable due to high passive permeability. Estimates for K_t and relative J_{max} were 6.82 (4.98) μM and 6.26 (2.27) percent, respectively.

^e Relative J_{max} was indistinguishable from zero ($p > 0.08$), such that gly-iDCA is not a substrate.