Synthesis and *In Vitro* Evaluation of Gabapentin Prodrugs That Target the Human Apical Sodium-Dependent Bile Acid Transporter (hASBT)

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ABSTRACT: Gabapentin is a zwitterionic drug that exhibits low and variable oral absorption at therapeutic doses. The human apical sodium-dependent bile acid transporter (hASBT; *SLC10A2*) is a potential prodrug target to increase oral drug absorption. The objective was to evaluate several bile acid conjugates of gabapentin as potential prodrugs that target hASBT. Five analogues were synthesized and varied in ionic nature and the presence or absence of glutamic acid linker between the bile acid and drug. Analogues were evaluated for their inhibition and uptake properties using stably transfected hASBT–MDCK cells. The two monoanionic conjugates were potent hASBT substrates, with high affinity (K_m of 16.3 and 5.99 μM) and high capacity (V_{max} of 0.656 and 0.842 pmol/cm^2/s). The dianionic conjugate inhibited hASBT with moderate potency but was not a substrate. The two monoanionic conjugates were catalytically degraded in Caco-2 homogenate and rat liver microsomes. Each yielded gabapentin from prodrug. These two conjugates are novel prodrugs of gabapentin and illustrate prodrugs that can be designed to target hASBT. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:1184–1195, 2011

Keywords: transporters; prodrugs; gabapentin; apical sodium-dependent bile acid transporter; bile acid; active transport; bioavailability; permeability; stability

INTRODUCTION

Gabapentin is a structural analogue of gamma-amino butyric acid (GABA). It is indicated to treat neuropathic pain and used as adjunctive therapy for partial seizures in adults with epilepsy.[1] Gabapentin absorption and pharmacokinetics are dose dependent and highly variable between patients. In humans, gabapentin bioavailability decreases from about 60% at a 300 mg dose to about 35% or less at doses to treat neuropathic pain (i.e., 1000 mg). Although the mechanism of uptake of gabapentin is still not clear, the reasons for low and highly variable gabapentin absorption are speculated to be due to low capacity and saturable amino acid transporters involved in its uptake in the upper part of the small intestine.[2,3] The high doses required for neuropathic pain treatment lead to saturation of transporter uptake.[4–6] Rapid gabapentin excretion and short half life (about 5–7 h) warrants frequent dosing, causing noncompliance in epileptic patients.[7]

To overcome these pharmacokinetic limitations, gabapentin enacarbil has been designed as a gabapentin prodrug. It is absorbed by high-capacity nutrient transporters throughout the small and large intestines, and is rapidly and extensively converted by nonspecific esterases to gabapentin, delivering dose-proportional exposure up to 2100 mg twice daily.[8] The prodrug also shows dose-proportional gabapentin exposure at doses up to 6000 mg.[9] Gabapentin enacarbil at 1200 mg significantly improved restless legs syndrome symptoms compared with placebo.[10]

Since the major limitation in gabapentin absorption results from its zwitterionic nature[11] and its facilitated uptake by low-capacity transporters, an alternative approach to enhance gabapentin absorption is through targeting high-capacity membrane transport

Abbreviations used: hASBT, human apical sodium-dependent bile acid transporter; CDCA, chenodeoxycholic acid; glu-CDCA, glutamic acid CDCA amide; OSU, N-hydroxysuccinimide; OBT, benzotriazole; HBTU, benzotriazol-1-yloxytris-1,1,3,3 tetramethyl uranium hexafluorophosphate; TEA, triethylamine; RT, room temperature; MDCK, Madin–Darby canine kidney; CaCo-2, colon adenocarcinoma cell line.

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systems in the gut. The human apical sodium-dependent bile acid transporter (hASBT; *SLC10A2*) is one such potential target that can be exploited for prodrug delivery. hASBT is a member of solute carrier genetic superfamily and is involved in the absorption of bile acids.[12] It has high intestinal expression and is a key player in enterohepatic circulation of bile acids.[13,14] hASBT transports 2 to 4 g of bile acids 6 to 10 times daily, giving an overall turnover rate of 20 to 30 g of bile salts per day, depicting the tremendous efficiency and capacity of this transporter. ASBT plays a major role in cholesterol homeostasis and maintaining the bile acid pool. A cholesterol-rich diet reduces ASBT expression in mice and in Caco-2 cells. The modulation of ASBT by cholesterol is an adaptive response that decreases ASBT expression. hASBT has been previously identified as a potential prodrug target to enhance oral drug absorption of low permeable drugs,[15,16] although lack of a high-resolution hASBT crystal structure has impeded understanding of hASBT structural requirements.[17]

One prodrug strategy is to couple drug to a natural substrate to create a molecule that mimics the three-dimensional structure of natural ligand of a transporter.[18] Bile acids are natural substrates for hASBT. Their unique structural properties and the large bile acid pool in the body affords them as suitable potential carriers for drug delivery. Various approaches have been employed to conjugate drugs to bile acids to create novel molecules that retain recognition by the bile acid transporters.[15,16] One such example is acyclovir, which has low oral bioavailability because of low permeability. Acyclovir was coupled to bile acid using valine as a linker to target hASBT. Relative to uptake of acyclovir alone, the permeability of the prodrug was 16-fold higher in cells *in vitro*. *In vivo*, the oral bioavailability was enhanced twofold as compared to the drug alone. This strategy clearly indicated that ASBT can be used to enhance the oral bioavailability of low-permeability drugs.[19]

Various structural prerequisites that are essential for recognition by hASBT have been determined previously.[17,20,21] It was demonstrated that monoanionic, neutral, and cationic bile acid conjugates were potent inhibitors of hASBT, indicating that a single negative charge was not essential for binding to hASBT, although was preferable for translocation. Dianionic compounds were neither inhibitors nor substrates of hASBT. Based on the structural requirements, five gabapentin analogues were synthesized, where drug was coupled to chenodeoxycholic acid (CDCA). Among the five analogues, two were neutral, two were monoanionic, and one was dianionic. Since gabapentin shows incomplete and highly variable uptake by amino acid transporter(s), high hASBT efficiency and capacity was hypothesized to improve gabapentin oral bioavailability.

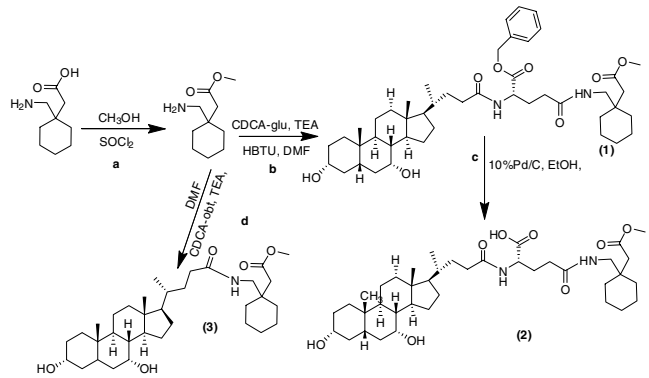
EXPERIMENTAL SECTION

Materials

[^{3}H]-Taurocholic acid (10μCi/mM) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Missouri). Taurocholate was obtained from Sigma Aldrich (St. Louis, Missouri). CDCA was obtained from TCI America (Portland, Oregon). Protected glutamic acid analogs were purchased from Novabiochem (Gibbstown, New Jersey). Gabapentin was obtained from spectrum chemicals (New Brunswick, New Jersey). Geneticin, fetal bovine serum (FBS), trypsin, and DMEM were purchased from Invitrogen (Rockville, Maryland). All other reagents and chemicals were of the highest purity commercially available.

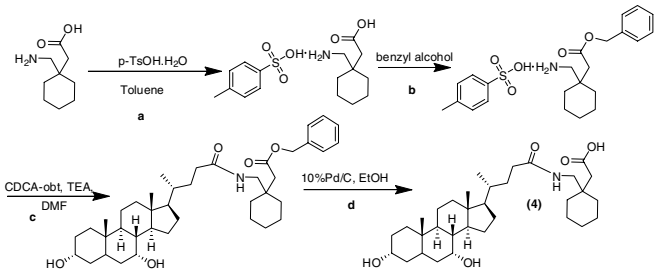
Synthesis of Gabapentin Analogues of Chenodeoxycholic Acid

Synthetic schemes for the activation of carboxylic acid have been described previously.[17,21] Briefly, the acid of bile acid was activated either by forming a benzotriazole ester (OBT) or N-hydroxysuccinimide ester (OSU) and separated by extraction. The activated bile acid was then coupled to α-benzyl ester of glutamic acid through peptide-coupling chemistry methods.[17] Either the activated CDCA or its amide with glutamic acid was then coupled to gabapentin to obtain the monoanionic, neutral and dianionic conjugate. Gabapentin carboxylate was protected either as its methyl ester or as its benzyl ester, as shown in synthetic Schemes 1 and 2. The identity and purity of all final targets were confirmed by thin layer chromatography (TLC), mass spectrometry (MS), and one-dimensional (1D) C-13 NMR.



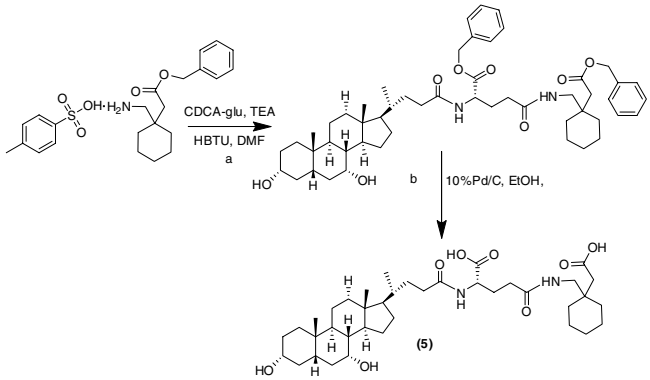
(a) 0ºC 1 h, RT 1 h, 70ºC overnight (b) 60ºC 24–48 h (c) H_2, 10% Pd/C, 40–50 psi 3–4 h (d) 60ºC 24–48 h.

Scheme 1. Synthesis of neutral and monoanionic conjugates of gabapentin methyl ester of glu-chenodeoxycholic acid (CDCA) and CDCA. CDCA-α-benzyl-glu-gabapentin methyl ester (**1**) was deprotected to yield the monoanionic CDCA-glu-gabapentin methyl ester **(2)**. CDCA-gabapentin-methyl ester **(3)** is neutral and lacks a glutamic acid linker.



(a) RT 12 h (b) 100° C 48 h (c) 60° C 48 h (d) H_2, 10% Pd/C, 40–50 psi 6–12 h.

Scheme 2. Synthesis of CDCA-gabapentin **(4)**.



(a) 60° C 48 h (b) H_2, 10% Pd/C, 40–50 psi 6–12 h.

Scheme 3. Synthesis of dianionic gabapentin conjugate, CDCA-glu-gabapentin **(5).**

Synthesis of Gabapentin Methyl Ester

To a magnetically stirred solution of gabapentin (5.84 mM) in methanol on dry ice, 1.2 eq of thionyl chloride (0.5 mL) was added dropwise. The reaction was allowed to react for 1 h at 0°C and then 1 h at room temperature (RT). The reaction mixture was then refluxed at 70°C overnight. After the reaction was complete, methanol was evaporated under reduced pressure and crude product was triturated in ether. The resulting white crude material was extracted into 50 mL volumes of EtOAc (3×) and washed with 15 mL of 1N $NaHCO_3$ (3×), 15 mL of water (3×), and 15 mL of brine (1×). The organic layer was dried over Na_2SO_4 and filtered. Removal of EtOAc under reduced pressure yielded a white fluffy solid. MS showed appropriate peaks: [M + 1] 186.3, [M + Na] 208.3.

Synthesis of Bile Acid Conjugates of Gabapentin

The protected gabapentin methyl ester was coupled to either OBT of CDCA or its amide with glutamic acid, as presented in Scheme 1. After the reaction was complete, the neutral compounds were extracted with 60 mL volumes of EtOAc (3×), and washed with 15 mL of water (3×) and then 15 mL of brine (1×). The organic layer was dried over Na_2SO_4 and filtered. Removal of EtOAc under reduced pressure yielded crude oily product. The conjugates were further purified by flash column chromatography starting with ethylacetate and hexane (80:20), increasing to 100% ethylacetate. The products eluted out in ethylacetate. TLC plates were stained using 10% w/v phosphomolybdic acid in absolute ethanol, which showed single spots. The neutral compounds (**1** and **3**) were used as such. The monoanionic compound **2** was obtained via catalytic hydrogenation to remove the α-benzyl ester (10% Pd/charcoal in EtOH at 40–50 psi for 3–4 h). MS showed appropriate peaks as given below. Several attempts to hydrolyze methyl ester from **2** and **3** to obtain targets **4** and **5** were unsuccessful because of simultaneous hydrolysis of amide bonds. Hence, Scheme 2 and 3 were employed to obtain compounds **4** and **5**.

Compound **1**. MS: [M + 1] 779.3. ^{13}C NMR (DMSO-d6): δ 11.65, 18.37, 20.26, 21.06, 21.06, 22.72, 23.16, 25.49, 26.95, 27.78, 30.56, 31.49, 31.59, 32.03, 32.29, 32.54, 32.54, 34.75, 34.85, 34.99, 35.32, 36.8, 39.10, 39.60, 39.78, 39.94, 40.11, 41.43, 41.92, 44.32, 50.02, 50.96, 51.77, 55.63, 65.77, 70.33, 127.70, 127.70, 127.99, 128.38,128.38, 136.03, 171.49, 171.80, 171.99, 172.95. Compound **2**. MS: [M + 1] 689.6 ^{13}C NMR (DMSO-d6): δ 11.65, 18.37, 20.24, 21.03, 21.03, 22.70, 23.15, 25.47, 27.26, 27.77, 30.55, 31.48, 31.82, 32.09, 32.26, 32.52, 32.52, 34.73, 34.82, 34.96, 35.30, 36.7, 39.60, 39.78, 39.94, 40.11, 41.41, 44.27, 41.90, 50.01, 50.93, 51.52, 55.64, 66.14, 70.31, 171.66, 171.80, 172.68, 173.57. Compound **3**. MS: [M + 1] 560.3 ^{13}C NMR (DMSO-d6): δ 11.65, 18.32, 20.29, 21.09, 21.09, 22.75, 23.20, 25.54, 27.15, 27.83, 30.60, 31.79, 32.32, 32.39, 32.54, 32.54, 34.78, 34.87, 34.97, 35.35, 36.88, 39.60, 39.78, 39.94, 41.46, 40.11 41.96, 50.07, 51.04, 55.72, 66.21, 70.38, 171.84, 172.88.

For all compounds, peaks 39.60, 39.78, and 39.94 were obscured by DMSO but were obtained by heteronuclear multiple bond correlation spectroscopy (HMBC) of CDCA-glu-gabapentin and were also evident in C-13 NMR of compound **1**.

Synthesis of Benzyl-Protected Conjugates of Gabapentin

In 20 mL of toluene, 1.71 g (10 mM) of gabapentin and 1.1 eq (2 g) *p*-toluenesulfonic acid monohydrate (PTSA) were magnetically stirred for 6 to 12 h. Toluene was evaporated under reduced pressure, which azeotropically removed all water from the reaction vessel. To the *p*TsOH salt of gabapentin was added 20 mL of benzyl alcohol, which was then heated at 100°C for 48 h. The solution was allowed to cool. Fifty milliliters of ether was added, which led to a white solid precipitate. The white solid was filtered and washed several time with ether. MS showed

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appropriate peak at [M + 1] 262.5. The protected benzyl ester of gabapentin was coupled to either OBT of CDCA or its amide with glutamic acid, as presented in Schemes 2 and 3 which led to neutral conjugates. These were then purified by flash column chromatography as described above using a gradient of ethylacetate and hexane. The monoanionic and dianionic conjugates were then obtained by catalytic hydrogenation to remove the α-benzyl ester (10% Pd/charcoal in EtOH at 40 psi for 1 h). The mixture was filtered over celite, and ethanol was evaporated to yield white fluffy solids of conjugates **4** and **5**.

Compound **4**. MS: [M + 1] 546.3 ^{13}C NMR (DMSO-d6): δ 11.60, 18.30, 20.22, 21.05, 21.05, 22.70, 23.13, 25.54, 27.19, 27.83, 30.54, 31.73, 32.26, 32.33, 32.60, 32.60, 34.72, 34.81, 34.91, 35.29, 36.53, 39.60, 39.78, 39.94, 40.48, 41.40, 41.90, 50.01, 55.63, 66.14, 70.33, 172.93, 173.16. Compound **5**. MS: [M − 1] 673.5. ^{13}C NMR (DMSO-d6): δ 11.64, 18.38, 20.23, 21.03, 21.03, 22.68, 23.14, 25.33, 27.11, 27.79, 30.54, 31.48, 31.80, 32.09, 32.26, 32.56, 32.56, 34.73, 34.81, 34.97, 35.30, 36.2, 39.60, 39.78, 39.94, 40.11, 41.41, 44.27, 41.90, 50.01, 50.93, 55.64, 66.14, 70.31, 168.2, 172.2, 172.9, 173.9.

Binding Affinity Studies

Stably transfected hASBT–MDCK cells were cultured as previously described.[22] Briefly, the cells were grown at 37°C, 90% relative humidity, 5% CO_2 atmosphere, and fed every 2 days. Culture media consisted on DMEM supplemented with 10% FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin. Geneticin was added at 1 mg/mL to maintain selection pressure. The cells were passaged after 4 days or after reaching 90% confluency.

The binding affinities of gabapentin bile acid conjugates were evaluated through *cis*-inhibition of ^{3}H-taurocholic acid uptake into hASBT–MDCK monolayers in 12-well plates (3.8 cm^2, Corning Costar, Corning, New York), as previously described.[21] Being insoluble in buffer, even with 2.5% DMSO, neutral compounds were not evaluated. Briefly, cells were seeded at a density of 1.5 million/well. The cells were induced on day 4 with 10 mM sodium butyrate for about 12 to 17 h prior to experiment. The cells were washed three times with Hank's balanced salt solution [HBSS; Sigma Aldrich (St. Louis, MO, USA)], followed by an addition of donor solution containing taurocholic acid (2.5 μM with 0.5 μCi/mL ^{3}H-taurocholate) and conjugate (0–100 μM for monoanions and 0–1000 μM for dianion). Inhibition studies were conducted at 37°C for 10 min on an orbital shaker (50 rpm). At the end of 10 min,[23] donor solution was removed and the cells were washed three times with chilled sodium-free buffer (where NaCl was replaced by 137 mM tetraethylammonium chloride). Two hundred and fifty microliters of 1N NaOH was added to lyse the cells (2 h), neutralized with 250 μL of 1N HCl, and counted for radioactivity using an LS6500 liquid scintillation counter (Beckmann Instruments, Inc., Fullerton, California).

Inhibition data were regressed using a modified Michaelis–Menten equation to determine the inhibition constant K_i, as previously described,[20] including parallel studies on each occasion to estimate K_m, V_{max}, and P_p for taurocholic acid.

Prodrug Uptake into hASBT–MDCK Cells

Uptake studies were performed in 12-well cell culture polystyrene plates (Corning Costar). The 12-well plates were seeded with hASBT–MDCK at a density of 1.5×10^6 cells/well and induced on day 4 with 10 mM sodium butyrate for 12 to 15 h prior to uptake experiments. Uptake studies were performed in a similar manner to above inhibition studies over 10 min.[23] The concentration of conjugates was varied from 1 to 250 μM for monoanions and 0 to 1000 μM for the dianion. To terminate prodrug uptake, the cells were washed three times with chilled sodium-free buffer. Additional identical studies were performed without sodium, to measure passive uptake of the conjugate on each occasion. Also, on each occasion, taurocholate V_{max} and Pp were measured to normalize for hASBT expression. Prodrug V_{max} was divided by taurocholate V_{max} to yield normalized V_{max} of prodrug ($normV_{max}$).

Quantification of Conjugates by LC/MS/MS

Cell lysis procedure and extraction assay have been previously optimized in our laboratory using taurocholate uptake into hASBT–MDCK monolayers.[24] Briefly, the cells were lysed by adding 300 μL of acetonitrile, which was then evaporated (2–3 h). The conjugate was extracted using 1:1 acetonitrile and HPLC grade water, spiked with internal standard. The anilinyl conjugate of glu-CDCA was used as internal standard, as it bears structural similarity to the prodrugs. The conjugate extract was collected from the wells in silanized vials and stored in −80°C until analyzed.

The prodrug concentration was determined in the cell lysate by liquid chromatography tandem MS (LC/MS/MS). Compound uptake was quantified on a Finnigan Surveyor HPLC system and a Finnigan TSQ quantum Discovery Max mass spectrometer (Thermo Fisher Scientific; Waltham, MA, USA). The column was a Phenomenex Luna C8, 50 × 2 mm, 5μ, heated to 40°C. The flow rate was 0.4 mL/min. The gradient of 5 to 95% acetonitrile was employed over 0.2 to 4 min, and the overall run time for all conjugates was 8 min. The initial mobile phase concentration was 50:50 acetonitrile:water up to 0.2 min, followed by increase in organic to 95%. The mobile phase included

0.1% formic acid as a modifier for all prodrugs. Injection volumes were 10 μL. Detection was achieved under positive ion electrospray tandem MS using $[M + H]^+$ peak, as a positive mode provided the greatest sensitivity for the synthesized prodrugs. The multiple reaction monitoring (MRM) transitions for internal standard were 597.3 > 83.7, 320.77; for compound **2**, MRM transitions were 689.6 > 154.26, 191.64; for compound **4**, MRM transitions were 546.23 > 94.69, 153.78; and for compound **5**, MRM transitions were 675.3 > 155.86, 266.31. The method was linear for prodrugs over the concentration range of 1 to 5000 μM for conjugate **2** ($r^2 = 0.9996$), of 10 to 2500 μM for conjugate **4** ($r^2 = 0.998$), of 25 to 2500 μM for conjugate **5** ($r^2 = 0.999$).

In Vitro Stability

Prodrugs were subjected to chemical stability assessment, as well as metabolic stability assessment. Chemical stability testing exposed prodrugs to 0.1 N HCl acid for 1 h and pH 6.8 HBSS buffer for 1 h. Compounds (5 μM) were incubated in acid or buffers at 37°C for 1 h in an orbital shaker. Sample was collected after 1 h, diluted with 50% acetonitrile, and analyzed by LC/ MS/MS, as described earlier.

Metabolic stability studies for the two monoanions were performed using Caco-2 homogenate and rat liver microsomes. Prodrug (1 μM) was incubated with either Caco-2 homogenate or rat liver microsome (2 mg/mL) at 37°C. Samples were quenched by adding 200 μL of chilled acetonitrile spiked with internal standard. The samples were then centrifuged at 10,000 rpm (i.e. 8944 g) at 4°C for 5 min and stored at −80°C prior to analysis. The samples were analyzed for disappearance of prodrug and appearance of drug by LC/MS/MS, as described below.

Regarding preparation of Caco-2 homogenates, Caco-2 cells were grown in 225 cm^2 flasks for 3 weeks at 37°C and 5% CO_2. The cells were grown in Dulbecco's modified Eagle medium (DMEM), 10% FBS, and 1% nonessential amino acids. The cells were washed with 10 mL of Dulbecco's phosphate buffered saline (DPBS) three times. The cells were scraped off the flasks on an ice tray and reconstituted in 10 mL of DPBS. The cell suspension was transferred to a sterile centrifuge tube and stored at −80°C overnight. The frozen cells were thawed in an ice bath and then sonicated by using sonic dismembrator (model F60; Fisher Scientific, Pittsburgh, Pennsylvania) at 5 to 6 W (RMS). Crude Caco-2 homogenates were centrifuged at 10,000 rpm for 10 min at 4°C. The 1.5-mL aliquots of supernatant were collected in centrifuge tubes and stored at −80° C until used. The total protein was quantified using the Bradford protein assay (Bio-Rad Laboratories, Hercules, California) and was about 0.4 mg/mL.

LC/MS/MS Methods for Stability Studies

Amount of prodrug remaining was determined in a similar manner as used earlier to quantify prodrug in uptake studies (i.e., under same conditions). However, the MRM transitions were 546.4 > 154.04, 510.44 for **4**; 689.3 > 154.03, 165.21 for **2**; and 597.3 > 83.92, 159.02 for internal standard.

To measure drug generation from prodrug, gabapentin concentration was quantified via LC/MS/MS. The column was aquasil C8, 5μ, heated to 40°C. The mobile phase contained a mixture of 80:20 water:acetonitrile with 0.1% formic acid, and the flow rate was 0.4 mL/min. The internal standard was (S)-(+)-aminocyclohexanepropionic acid at a concentration of 500 nM. The MRM transitions were 171.94 > 55.12, 126.13 for the internal standard; and 172.01 > 67.06, 154.12 for gabapentin. Total run times were 8 min. The method was linear over the concentration range of 1 to 2500 μM (r^2 of 0.998).

RESULTS AND DISCUSSION

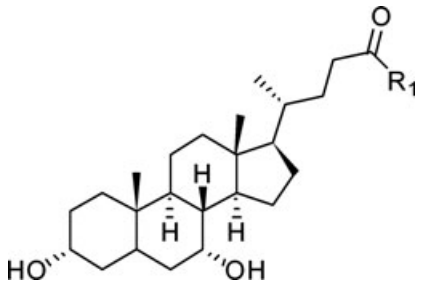
Synthesis of Gabapentin Prodrugs

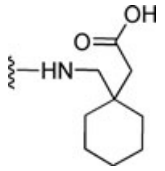
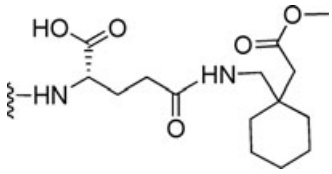
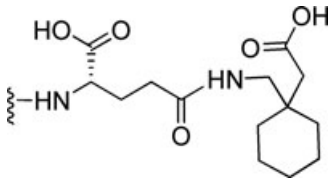
Five bile acid conjugates of gabapentin were synthesized and included two neutral, two monoanionic, and one dianionic compound. Compounds **1** to **5** are denoted CDCA-α-benzyl-glu-gabapentin methyl ester, CDCA-glu-gabapentin methyl ester, CDCA-gabapentin-methyl ester, CDCA-gabapentin, and CDCA-glu-gabapentin, respectively.

Charge has previously been shown to impact conjugate inhibition and substrate activity for CDCA conjugates.[17] In Scheme 1, compound **2** was designed by employing CDCA as the targeting moiety, and glutamic acid as a linker to afford a single negative charge near the bile acid's C-24 position. Gabapentin carboxylate was protected by methyl ester to have an overall charge of −1 on compound **2**. Compound **1** was the intermediate prior to deprotection of the benzyl ester to yield compound **2**, such that compound **1** is neutral. Compound **3** differs from compound **2**: in that compound **3** lacks the glutamic acid linker, and hence is neutral. The two neutral conjugates **1** and **3** were insufficiently water soluble to allow for hASBT testing.

Various attempts were made to obtain compounds **4** and **5** from compounds **2** and **3**, respectively. However, selective hydrolysis of the methyl ester was not achieved due to simultaneous cleavage of the amide bond. Schemes 2 and 3 illustrate the alternative, successful approach that yielded compounds **4** and **5**. Compounds **4** and **5** are monoanionic and dianionic, respectively, and differ in the absence or presence of a glutamic acid linker.

Table 1. Structures and Kinetic Parameters for Gabapentin Prodrugs



Compound Number	R_1	Charge	K_i (μM)	K_m (μM)	V_{max} (pmol/cm^2/s)	Normalized V_{max}	$P_P \times 10^6$ (cm/s)
4		−1	2.85 ± 0.44	16.3 ± 4.7	0.842 ± 0.083	2.15 ± 0.23	11.5 ± 0.3
2		−1	6.46 ± 0.92	5.99 ± 1.45	0.656 ± 0.030	1.68 ± 0.09	1.11 ± 0.01
5		−2	34.8 ± 11.9	NS	NS	NS	0.109 ± 0.008

Compounds are arranged in order of inhibition potency.
NS denotes compound was not a substrate.

Inhibition of Taurocholate Uptake into hASBT–MDCK Cells

The three negatively charged analogues, CDCA-glu-gabapentin methyl ester (compound **2**), CDCA-gabapentin (compound **4**), and CDCA-glu-gabapentin (compound **5**) were evaluated for the inhibition of hASBT using taurocholate as a substrate. Each inhibited taurocholate uptake into hASBT–MDCK monolayers in a concentration-dependent manner. Their kinetic parameters are listed in Table 1 in order of inhibitory potency. The two monoanions exhibited inhibitory potency similar to native bile acids (about 5 μM). The dianion showed moderate inhibitory potency, with K_i = 34.8 μM. Figure 1 plots the inhibition profiles. In panel A, neither gabapentin nor gabapentin methyl ester inhibited taurocholate, while CDCA-glu-gabapentin methyl ester decreased taurocholate uptake. CDCA-gabapentin (panel B) and CDCA-glu-gabapentin (panel C) each also inhibited taurocholate uptake. Inhibition results of the two monoanionic compounds are consistent with prior observations of monoanions to inhibit hASBT.[17] The dianionic gabapentin conjugate inhibited hASBT with moderate potency.

Conjugate Uptake into hASBT–MDCK Cells

The gabapentin conjugates were further evaluated for uptake into hASBT–MDCK cells. Figure 2 represents the concentration-dependent uptake of potential prodrugs into hASBT–MDCK monolayers. In panels A and B, CDCA-glu-gabapentin methyl ester uptake and CDCA-gabapentin uptake were greater in the presence of sodium than in the absence of sodium, indicating active uptake by the transporter. In panel C, the dianion CDCA-glu-gabapentin was equally low in the presence and absence of sodium and was not a substrate.

Kinetic parameters using the modified Michaelis—Menten equation are listed in Table 1. For CDCA-glu-gabapentin methyl ester, K_m = 5.99 μM and

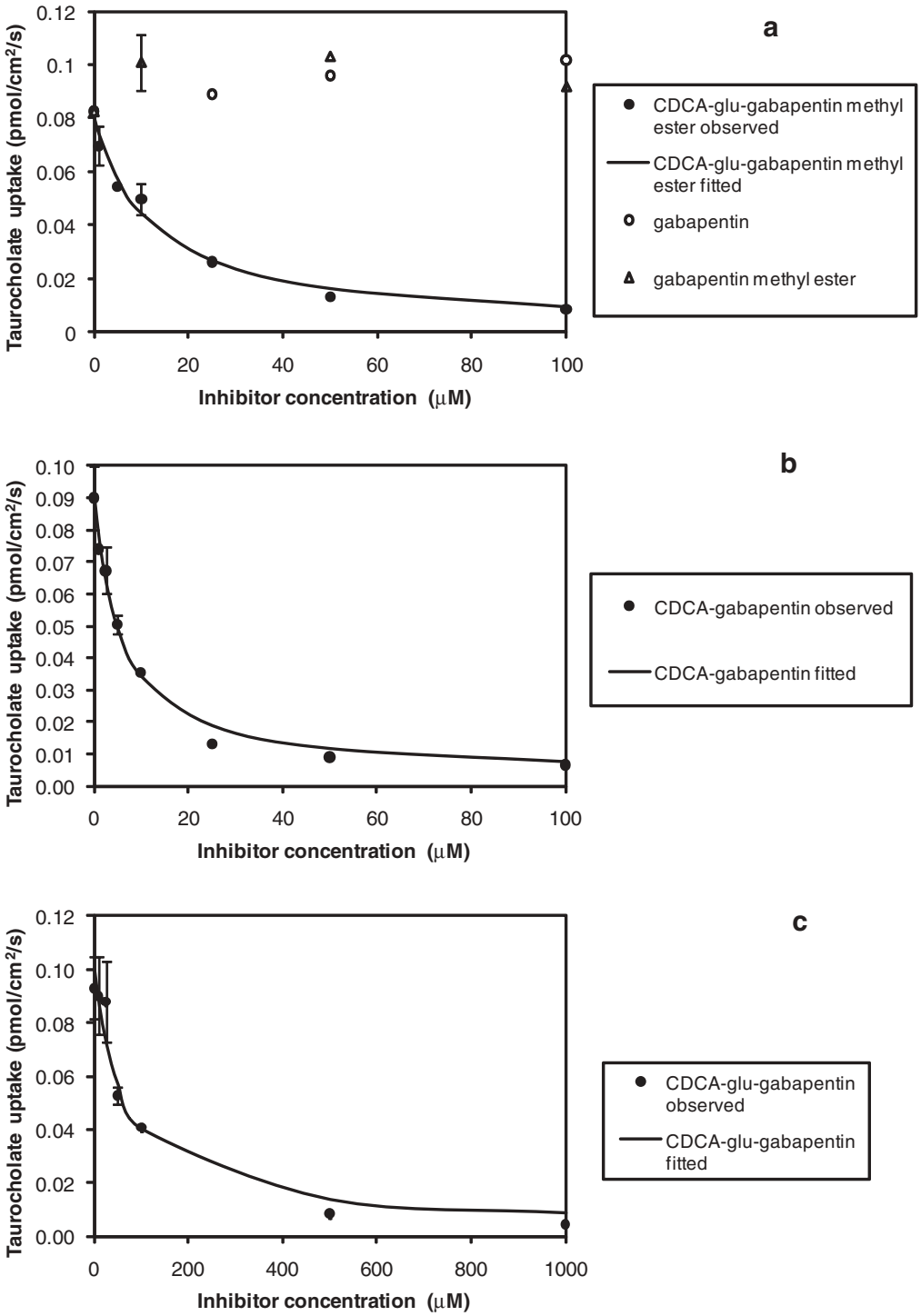


Figure 1. Inhibition profiles of three potential gabapentin prodrugs. (a) CDCA-glu-gabapentin methyl ester, (b) CDCA-gabapentin, and (c) CDCA-glu-gabapentin. Each inhibited taurocholate uptake into hASBT-MDCK monolayers. In panel A, neither gabapentin nor gabapentin methyl ester inhibited taurocholate.

V_{max} = 0.656 pmol/cm^2/s indicate high affinity and capacity of hASBT for this substrate, suggesting the compound to be a potential hASBT prodrug. In Table 1, the normalized V_{max} value is V_{max} for the conjugate normalized for functional ASBT expression, using taurocholate as the reference; conjugate V_{max} is divided by taurocholate V_{max} from the same study occasion to give conjugate normalized V_{max}. The normalized V_{max} of CDCA-glu-gabapentin methyl ester and CDCA-gabapentin were each about 2 (i.e., twofold higher than taurocholate V_{max}). As a reference, taurocholate's K_m is about 5 μM, such that the K_m of

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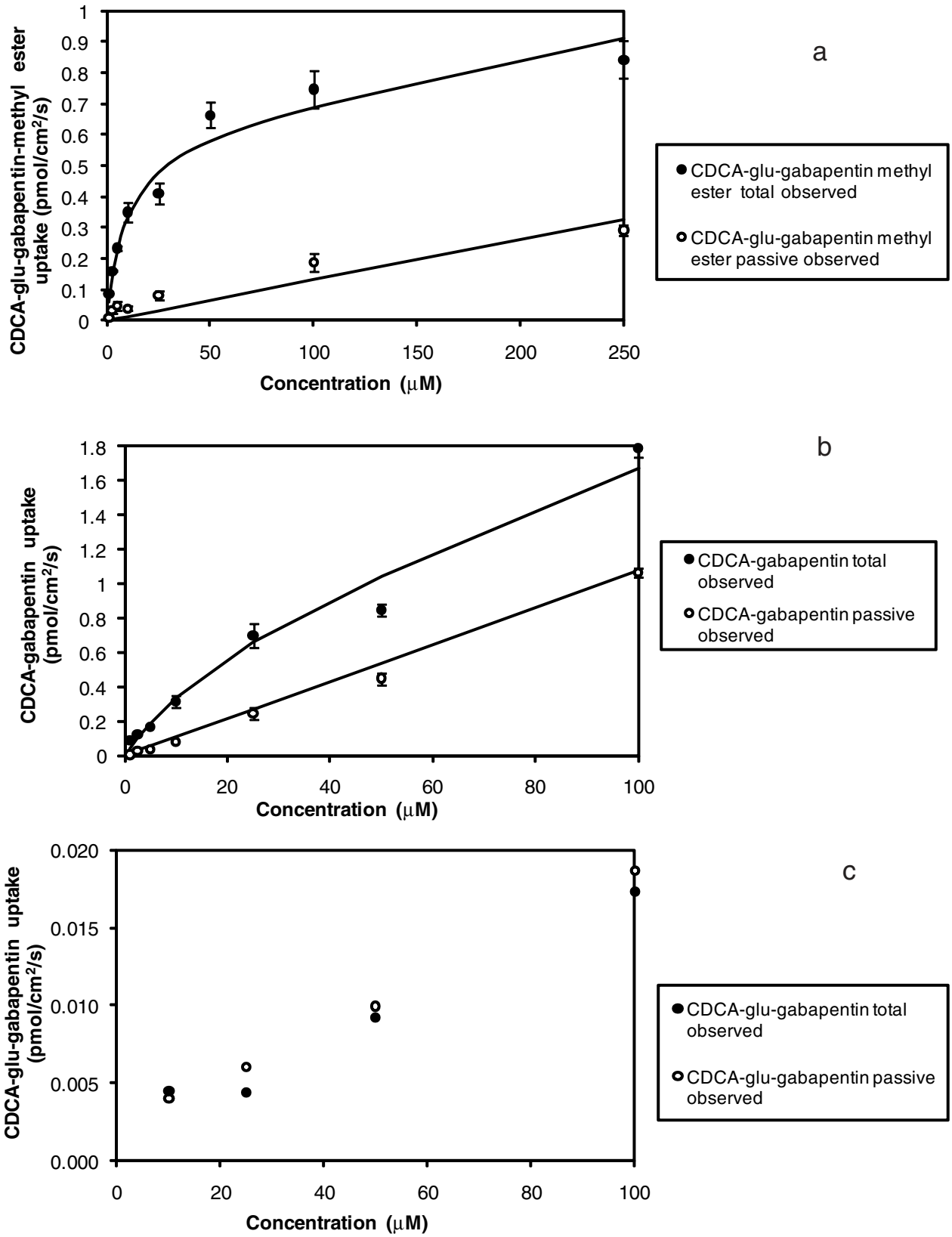

Figure 2. Concentration-dependent uptake of potential prodrugs into hASBT–MDCK monolayers. (a) CDCA-glu-gabapentin methyl ester uptake and (b) CDCA-gabapentin uptake were greater in the presence of sodium (•) than in the absence of sodium (○), indicating each to be hASBT substrates. (c) CDCA-glu-gabapentin was not a substrate, as uptake was equally low in the presence and absence of sodium.

CDCA-glu-gabapentin methyl ester was the same as taurocholate.

Like CDCA-glu-gabapentin methyl ester, CDCA-gabapentin was a potent substrate, with K_m = 16.3 μM and V_{max} = 0.842 pmol/cm^2/s. The most notable difference between these two monoanions was passive permeability, which was low for CDCA-glu-gabapentin methyl ester (Pp = 1.11×10^{-6} cm/s) and moderate for CDCA-gabapentin (Pp = 11.5×10^{-6} cm/s). CDCA itself has a moderate-to-high passive permeability of 20.7×10^{-6},[20] but exhibits lower passive permeability when conjugated to glutamic acid.[17] The lower passive permeability for CDCA-glu-gabapentin methyl ester versus CDCA-gabapentin is not surprising due to conjugation in the former, while the lowest passive permeability of the dianion (Pp = 0.109×10^{-6} cm/s) was expected, given it has a negative charge of two.

In Table 1, the K_i and K_m values for CDCA-glu-gabapentin methyl ester were essentially identical, suggesting that substrate binding was the rate-limiting step in its overall translocation by hASBT. For CDCA-gabapentin, K_i was about sixfold more potent than K_m, implicating postbinding events as the rate-limiting step(s) in CDCA-gabapentin uptake. Postbinding events include steps that are required for substrate translocation but occur after substrate binds to the transporter at the extracellular surface; postbinding events include orientation of the transporter-substrate complex from extracellular orientation to intracellular orientation, release of substrate from the transporter-substrate complex into the cell, and re-orientation of the transporter. With a viewpoint that K_i and K_m reflect kinetics of substrate binding and overall translocation (i.e., binding and postbinding events), respectively, where smaller valued parameters denote faster kinetics, smaller K_i than K_m, implicate that binding is faster than postbinding events for CDCA-gabapentin uptake.

The uptake results indicate high apparent permeabilities for the two-monoanionic compounds, including higher prodrug permeability than gabapentin permeability. The apparent permeability of gabapentin across MDCK monolayers was reported to be 0.73×10^{-6} cm/s and 0.25×10^{-6} cm/s in apical-to-basolateral and basolateral-to-apical directions, respectively, which was described to be low and consistent with poor passive absorption.[6] The apparent permeabilities of CDCA-glu-gabapentin methyl ester and CDCA-gabapentin were several fold higher than these gabapentin values. From uptake studies (Fig. 2), the apparent permeabilities of CDCA-glu-gabapentin methyl ester at 1, 2.5, 5, 10, 25, 50, 100, and 250 μM were 87.5×10^{-6}, 63.8×10^{-6}, 43.3×10^{-6}, 33.9×10^{-6}, 16.1×10^{-6}, 12.7×10^{-6}, 6.95×10^{-6}, and 3.62×10^{-6} cm/s, respectively. Similarly, the apparent permeabilities of CDCA-gabapentin at 1, 2.5, 5, 10, 25, 50, and 100 μM were 188×10^{-6}, 48.2×10^{-6}, 38.4×10^{-6}, 31.6×10^{-6}, 29.5×10^{-6}, 18.2×10^{-6}, and 17.3×10^{-6} cm/s, respectively.

Overall, a single negative charge was preferred for substrate translocation, and promoted aqueous solubility. Although the dianionic compound bound to hASBT with moderate potency, it was not a substrate. These observations agree with previous findings where other dianions were found not to be hASBT substrates.[17]

In Vitro Stability

For the two substrates, stability was conducted in 0.1 N HCl, HBSS buffer with pH = 6.8, Caco-2 homogenate, and rat liver microsomes. Each CDCA-glu-gabapentin methyl ester and CDCA-gabapentin were stable for more than 1 h in 0.1 N HCl and HBSS buffer with pH = 6.8. Essentially no loss of either prodrug was observed. For CDCA-glu-gabapentin methyl ester, about 104.7 ± 9.1% and 114.6 ± 3.92% was remaining in 0.1 N HCl and HBSS, respectively. Similarly, for CDCA-gabapentin 102.9 ± 8.9% and 89.3 ± 4.9% was remaining in 0.1 N HCl and HBSS, respectively.

In Figure 3, the stability profiles of CDCA-glu-gabapentin methyl ester and CDCA-gabapentin in Caco-2 homogenate are plotted. In panel A, only 68.2% of prodrug remained after 15 min. An equivalent amount of gabapentin (32.6%) was produced after 15 min. Studies were also performed in rat liver microsomes (data not shown), where the loss of prodrug was measured. After 2 h, 56.5% of prodrug remained. The release of the drug in rat liver microsomes was not feasible due to matrix effects (i.e., interference of microsomal matrix with gabapentin quantification by LC/MS). CDCA-glu-gabapentin methyl ester exhibited slightly greater stability in rat liver microsomes. Compared to the observed chemical stability in acid and buffer, prodrug was unstable in Caco-2 homogenate and microsomes. Results from Caco-2 homogenate and rat liver microsomes show that CDCA-glu-gabapentin methyl ester was catalytically degraded, yielding the parent drug.

The corresponding stability profiles of CDCA-gabapentin are plotted in Figure 3 panel B. There was general agreement between prodrug loss and drug generation. After 15 min, 31.9% of prodrug degraded with a slightly less than equivalent amount of gabapentin (17.1%) produced. CDCA-gabapentin was more stable in rat liver microsomes compared to Caco-2 homogenate. In rat liver microsomes, 56.9% of prodrug remained after 2 h. Like for CDCA-glu-gabapentin methyl ester, CDCA-gabapentin was catalytically degraded and yielded drug from prodrug. Hydrolysis rates were much higher in Caco-2 cell

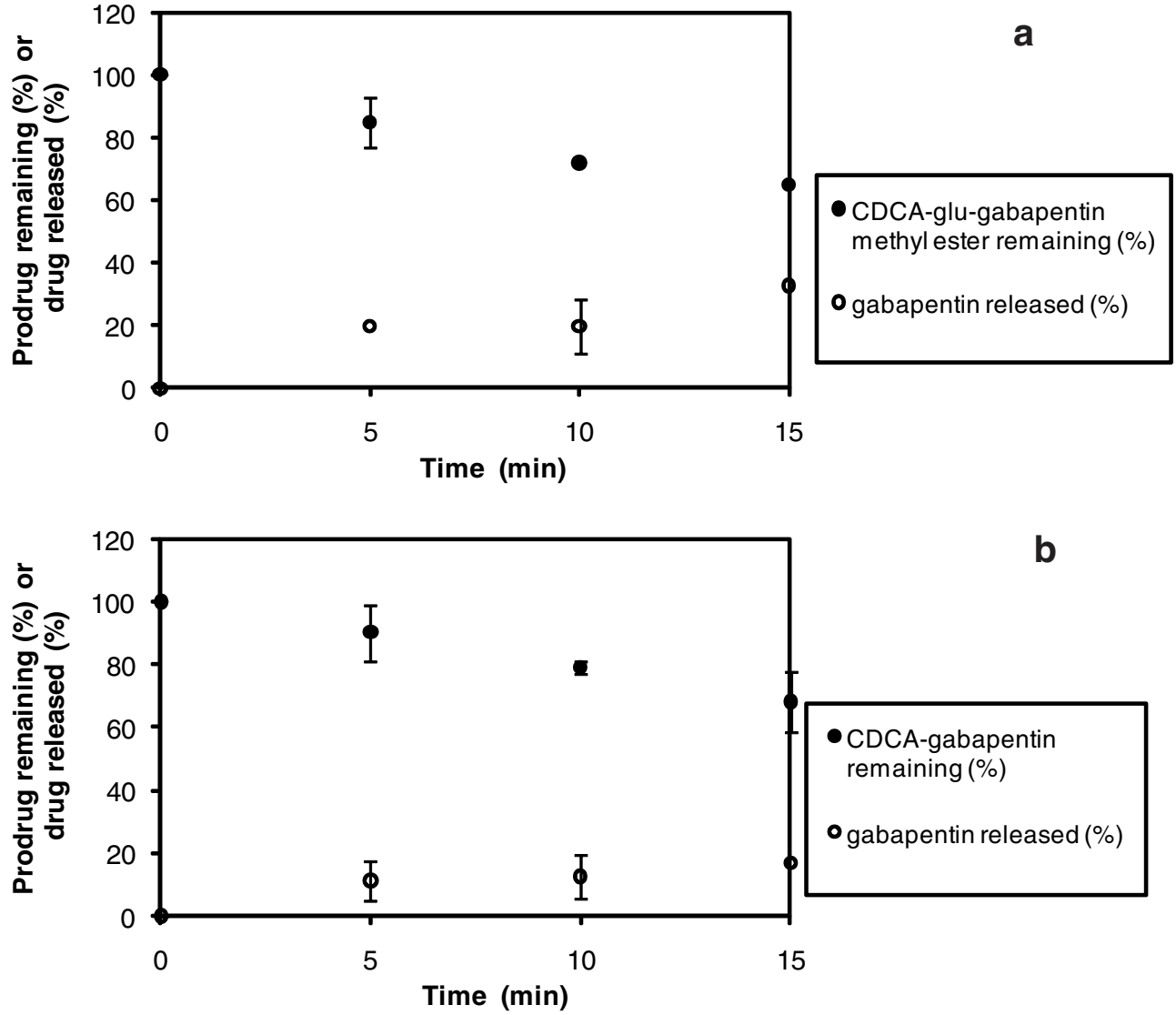

Figure 3. Catalytic activation of prodrugs in Caco-2 homogenate. (a) CDCA-glu-gabapentin methyl ester and (b) CDCA-gabapentin were evaluated. For each prodrug, prodrug concentration declined (●), while gabapentin was generated (○).

homogenates and liver microsomes as compared to chemical stability in buffers, indicating enzymatic bioconversion of the prodrugs.

Prodrug Design

Gabapentin is a model low-permeability drug because of its zwitterionic nature. Gabapentin is structurally similar to amino acids, which generally rely on amino acid transporters for absorption. *In vivo*, gabapentin is a substrate for saturable solute transporters in the intestine and brain. Its dose-dependent pharmacokinetics limits its oral bioavailability, especially at doses required to treat the neuropathic pain. In studies performed by Cundy et al.,[6] gabapentin transport was evaluated for various amino acid transporters, such as $B^{0,+}$, $ATB^{0,+}$, and LAT2 in transfected oocytes. Results showed that gabapentin was not a substrate for these transporters, although it was found to be a substrate for OCTN2. It has also been shown that the amino acid transporter LAT1 is not significantly expressed in human intestine. Thus, the overall mechanism for gabapentin's highly variable and incomplete absorption is still not clear.

A prodrug of gabapentin that increases gabapentin absorption is in development.[5,8] This prodrug was designed to be recognized by a low-affinity high-capacity nutrient transporter, such as monocarboxylate transporter type I (MCT-1) expressed along the intestine, and a high-affinity low-capacity, the sodium-dependent multivitamin transporter (SMVT), which is expressed largely in intestine and responsible for the absorption of essential cofactors. Analysis of the prodrug in MCT-1-specific component revealed a

saturable uptake with a K_m of approximately 220 μM and a V_{max} of 6 pmol per oocyte. The prodrug was also shown to be a substrate of SMVT with K_m of 3 μM and V_{max} equal to only 40% of natural substrate biotin. The prodrug showed low affinity for MCT-1 and low capacity for SMVT.[6]

An alternative strategy is employed here where the drug is coupled to a natural substrate for a transporter, such that the drug-substrate conjugate mimics the native substrate. Using CDCA as a targeting moiety, the prodrugs were strategically designed to target hASBT with the same high affinity and high capacity as native bile acids. The following features have previously been shown to be preferable for substrate uptake by ASBT: (1) the presence of negative charge proximal to C-24, (2) the presence of single negative charge, where two negative charges hampered activity, and (3) hydrophobic moieties that enhance interaction with ASBT. To achieve optimal recognition by hASBT, we employed a "bile acid-linker drug" strategy to design gabapentin prodrugs **2** and **5**. A negative charge near C-24 was afforded by the linker glutamic acid. The negative charge on the drug was concealed by methyl ester protection on **2**. Alternatively, for **4**, bile acid was directly coupled to gabapentin and a negative charge was afforded by drug.

Among five analogues, two were found to be potential prodrugs that may increase gabapentin absorption via hASBT uptake: CDCA-glu-gabapentin methyl ester and CDCA-gabapentin. hASBT translocates several grams of bile acids daily with little loss to feces. CDCA-glu-gabapentin methyl ester and CDCA-gabapentin was each designed to be a monoanion, with the negative charge within the vicinity of C-24 of the bile acid. The native bile acid CDCA possesses a negative charge at C-24, while its glycine conjugated form, glycochenodeoxycolate possess a negative charge at C-25. For CDCA-glu-gabapentin methyl ester, a negative charge was maintained at a position favorable for uptake by hASBT (i.e., C-25). For CDCA-gabapentin, gabapentin is a small molecule possessing a carboxylate, such that directly coupling it to CDCA maintained a negative charge, but at a slightly distal location to C-24 (i.e., C-28).

Results from inhibition studies with taurocholate demonstrated that the gabapentin conjugates are potent inhibitors, with strong interaction with the transporter. For definitive evidence of transport, uptake studies were performed in a stably transfected cell line and quantified using LC/MS/MS. The K_m and V_{max} values for **2** and **4** indicate high transporter affinity and capacity. The normalized V_{max} showed hASBT to possess higher transporter capacity for these two conjugates than even for the native bile acid taurocholate. Additionally, each was chemically stable but catalytically degraded, and yielded drug from prodrug. Overall, these two conjugates are novel prodrugs of gabapentin and illustrate that prodrugs can be designed to target hASBT.

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