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Increased Acyclovir Oral Bioavailability via a Bile Acid Conjugate

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Abstract: The objective of this work was to design an acyclovir prodrug that would utilize the human apical sodium-dependent bile acid transporter (hASBT) and enhance acyclovir oral bioavailability. Using each chenodeoxycholate, deoxycholate, cholate, and ursodeoxycholate, four bile acid prodrugs of acyclovir were synthesized, where acyclovir was conjugated to a bile acid via a valine linker. The affinity of the prodrug for hASBT was determined through inhibition of taurocholate uptake by COS-7 cells transfected with hASBT (hASBT-COS). The prodrug with the highest inhibitory affinity was further evaluated in vitro and in vivo. The prodrug acyclovir valylchenodeoxycholate yielded the highest affinity for hASBT (K_i = 35 μM), showing that chenodeoxycholate is the free bile acid with the greatest affinity for hASBT. Acyclovir valylchenodeoxycholate's affinity was similar to that of cholic acid (K_i = 25 μM). Further characterization showed that acyclovir was catalytically liberated from acyclovir valylchenodeoxycholate by esterase. Relative to cellular uptake studies of acyclovir alone, the cellular uptake from the prodrug resulted in a 16-fold greater acyclovir accumulation within hASBT-COS cells, indicating enhanced permeation properties of the prodrug. Enhanced permeability was due to hASBT-mediated uptake and increased passive permeability. The extent of acyclovir uptake in the presence of sodium was 1.4-fold greater than the extent of passive prodrug uptake in the absence of sodium ($p = 0.02$), indicating translocation of the prodrug by hASBT. The prodrug also exhibited an almost 12-fold enhanced passive permeability, relative to acyclovir's passive permeability. Oral administration of acyclovir valylchenodeoxycholate to rats resulted in a 2-fold increase in the bioavailability of acyclovir, compared to the bioavailability after administration of acyclovir alone. Results indicate that a bile acid prodrug strategy may be useful in improving the oral bioavailability of intestinal permeability-limited compounds.

Keywords: Prodrug; acyclovir; bioavailability; bile acid

Introduction

The intestinal absorption of bile acids involves the human apical sodium-dependent bile acid transporter (hASBT) and Na^+/K^+-ATPase.[1,2] hASBT consists of 348 amino acids and efficiently recycles bile acids from the gastrointestinal lumen during enterohepatic recirculation.[3−6] Also known as SLC10A2 and as the human ileal (or intestinal) bile acid transporter (hIBAT), hASBT is 35% identical in sequence, with a similar predicted structure, to the human liver Na^+-taurocholate

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transporting polypeptide (NTCP).[7] hASBT is also expressed in cholangiocytes and the renal proximal tubules.[8]

hASBT appears to be a potential prodrug target for increasing the intestinal permeation and oral bioavailability of drugs with low intrinsic intestinal permeability. hASBT has a high transport capacity of more than 10 g/day.[9,10] Hence, the bile acid transporter appears to be a promising mechanism for improving oral drug absorption by linking a bile acid to a drug in a prodrug fashion. Such a bile acid prodrug approach to targeted bile acid transporters in various tissues has been investigated with peptides[9−12] and chlorambucil.[13] Other attempts have involved inhibiting the bile acid transporter to reduce the extent of cholesterol synthesis in the liver.[14]

Acyclovir is an antiviral drug that inhibits herpes virus proliferation. Typical treatment requires 200 mg doses administered five times daily, with an oral bioavailability of only 20% because of low acyclovir intestinal permeability.[15] Valacyclovir is a prodrug of acyclovir that has improved the oral bioavailability of acyclovir.[16] Valacyclovir, the L-valine ester prodrug of acyclovir, has an oral bioavailability of 54%.[17] This increased bioavailability for acyclovir allows for a more convenient dosing regimen of 1000 mg twice daily, with a clinical efficacy similar to that of the acyclovir parent compound.[18] Valacyclovir is a substrate for the PepT1 intestinal transporter, with a K_i of 4.08 mM in PepT1-expressing *Xenopus laevis* oocytes[19] and a K_i of 1.10 mM in stable lines of CHO/PepT1.[20] In comparison to PepT1, hASBT has potential advantages of higher capacity and micromolar affinity. Therefore, it may be possible to further enhance the oral bioavailability of acyclovir beyond that afforded by valacyclovir by synthesizing a bile acid prodrug to target the hASBT. The objective of this work was to design an acyclovir prodrug that would utilize the human apical sodium-dependent bile acid transporter (hASBT) and enhance acyclovir oral bioavailability.

Experimental Section

Materials. The pCMV5-hASBT expression plasmid was a generous gift from P. A. Dawson (Wake Forest University, Winston-Salem, NC). COS-7 and Caco-2 cells were obtained from ATCC (Manassas, VA). Subcloning efficiency DH5α competent cells and LipofectAMINE 2000 transfection reagent were obtained from Life Technologies (Grand Island, NY). Biocoat polylysine-coated cluster dishes were obtained from Beckton-Dickinson (Bedford, MA). The DNA Plasmid Maxi Kit (no. 12162) was purchased from Qiagen (Valencia, CA). Radiolabeled [^{3}H]taurocholic acid (specific activity of 3.14 Ci/mmol) was obtained from DuPont NEN (Boston, MA). The following were purchased from Sigma Chemical Co. (St. Louis, MO): DMEM, ampicillin, uptake buffer components, unlabeled bile acids, acyclovir, and esterase from porcine liver.

Prodrug Synthesis. Valacyclovir was prepared through previously described methods.[21] Coupling of valacyclovir to

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bile acids was achieved through the formation of the mixed anhydride of the bile acid, to yield four acyclovir bile acid prodrugs. Briefly, acyclovir valylchenodeoxycholate, acyclovir valylcholate, acyclovir valyldeoxycholate, and acyclovir valylursodeoxycholate were synthesized as follows. Isobutyl chloroformate (*i*-BuOCOCl, 130 μL, 1 mmol) was added dropwise to a cooled (−15 °C) solution of the appropriate bile acid (1 mmol) and triethylamine (140 μL, 1 mmol) in *N*,*N*-dimethylformamide (DMF, 10 mL). After 1.5 min, valacyclovir (0.42 g, 1.3 mmol) and triethylamine (NEt_3, 280 μL, 2 mmol) were added to the reaction mixture as a solution in DMF (5 mL). The reaction mixture was stirred at −15 °C for 0.5 h and allowed to warm to room temperature for 1 h. The triethylammonium chloride formed during the reaction was removed by filtration, and the resulting solution was concentrated. The crude material was purified using silica gel flash chromatography with MeOH and $CHCl_3$ (1:4, 250 mL) and recovered from the eluent, eliminating bile acid as an impurity.

All reactions were performed under a nitrogen atmosphere, and solvents were removed on a rotary evaporator under reduced pressure. TLC was performed on plates coated with silica gel GHLF 0.25 mm plates (60 F_{254}) (Analtech, Inc., Newark, DE). Fast-atom bombardment mass spectrometry (FAB-MS) and high-resolution mass spectrometry (HRMS) spectra were obtained on a JEOL SX 102 double-focus mass spectrometer in the positive ion mode (JEOL, Peabody, MA). The purity of the bile acid conjugate was determined by analysis on a Beckman (Fullerton, CA) Gold HPLC system consisting of a model 126 solvent module, a model 168 detector, and a model 507 autosampler. A Vydac analytical column (C18, 300 Å, 5 μm, 4.6 mm × 250 mm) equipped with a guard cartridge was employed (Grace Vydac, Columbia, MD). Solvent A was aqueous 0.1% trifluoroacetic acid (TFA), and solvent B was acetonitrile containing 0.1% TFA. The conjugates were eluted using a linear gradient of 5 to 75% B over 50 min at a flow rate of 1.0 mL/min and detected at 214 nm. NMR spectra were obtained in d_6-DMSO on a 300 MHz Aquerius model spectrometer (General Electric, Fremont, CA).

Initial efforts to conjugate acyclovir directly with bile acids were made. This approach resulted in a low yield due to the low acyclovir solubility in the reaction solvent. Valacyclovir was subsequently chosen since a bile acid conjugate of it will also hydrolyze to acyclovir, and was found to possess acceptable solubility in DMF.

Transient Transfection of COS-7 Cells with hASBT cDNA. The pCMV5-hASBT expression plasmid was amplified in *Escherichia coli*[8] and isolated using a Qiagen Maxi Kit, according to the supplier's protocol. The cDNA stock solution used for transfection contained 1.14 μg/μL cDNA in TEA buffer (pH 7.5) and was stored at −80 °C until it was used; the cDNA purity ratio was 1.9 (260 nm/280 nm). Transiently hASBT-transfected COS-7 (hASBT-COS) cells were grown as previously described by Craddock et al.,[8] and transfected using LipofectAMINE2000 reagent according to the supplier's protocol.

hASBT-COS Uptake and Taurocholate Inhibition Studies. Taurocholate uptake and inhibition studies in hASBT-COS cells were performed 24 h post-transfection. Uptake buffer consisted of either Hank's Balanced Salts Solution (HBSS) containing 137 mM NaCl or modified HBSS (MHBSS) that replaced the sodium chloride with 137 mM tetraethylammonium chloride. Because bile acid transport is sodium-dependent, MHBSS allowed for the simple modification of the uptake buffer to exclude all sources of sodium, and thus enabled measurement of the extent of passive diffusional uptake of bile acid. Control uptake studies were also performed in untransfected COS-7 cells and in COS-7 cells transfected with the antibiotic resistant vector, pcDNA3.

Taurocholate uptake at concentrations between 0.1 and 125 μM, each containing 0.2% [^{3}H]taurocholate, was investigated in both HBSS and MHBSS to determine V_{max}, K_m, and the passive uptake rate constant. Cells were incubated at 37 °C and 100 rpm for 10 min. The uptake solution was removed; cells were washed three times with ice-cold HBSS or MHBSS. Cells were lysed, neutralized, and counted for associated radioactivity. Each well was analyzed for protein content using the Lowry method. Taurocholate uptake data were fit to

$$\frac{dM}{dt} = \frac{V_{max}S}{K_m + S} + k_p S \qquad (1)$$

where V_{max} and K_m represent the Michaelis−Menten constants, k_p is the passive uptake rate constant, S is the concentration of taurocholate, and dM/dt is the rate of uptake of taurocholate into the hASBT-COS cells.

Competitive inhibition studies were performed in HBSS with varying concentrations of the bile acid or acyclovir prodrug. Glycine, taurine, valacyclovir, and acyclovir inhibition studies were performed as negative controls. The following equation was used to estimate the competitive inhibitor coefficient K_i for the bile acid or acyclovir prodrug:

$$\frac{dM}{dt} = \frac{V_{max}(S/K_m)}{1 + \frac{S}{K_m} + \frac{I}{K_i}} \qquad (2)$$

where V_{max} and K_m are the Michaelis−Menten parameters for taurocholate uptake, S equals 0.25 μM ([^{3}H]taurocholate), dM/dt is the rate of uptake of taurocholate, and I is the concentration of competitive inhibitor applied to the cells.

Uptake of Acyclovir Valylchenodeoxycholate into hASBT-COS Cells. Since acyclovir valylchenodeoxycholate yielded the greatest affinity for hASBT, as determined from the inhibition studies, this prodrug was further investigated. The accumulation of the prodrug and acyclovir in hASBT-COS cells from a 15 μM acyclovir valylchenodeoxycholate solution was assessed. Uptake studies were performed in HBSS and MHBSS. Identical studies were performed to measure the rate of uptake of acyclovir into hASBT-COS cells from 15 μM acyclovir solutions. The rate of taurocho-

late uptake at 1 μM from a taurocholate solution was determined under identical conditions, and indicated functional expression of hASBT. Acyclovir valylchenodeoxycholate was found to be stable during the duration of the uptake study.

Hydrolysis of Acyclovir Valylchenodeoxycholate to Acyclovir. Hydrolysis of the acyclovir prodrug to acyclovir was evaluated in sodium hydroxide, esterase-containing buffer, and Caco-2 cell lysate. Briefly, in the sodium hydroxide studies, separate prodrug and acyclovir solutions in PBS were prepared in triplicate, to which concentrated sodium hydroxide was quantitatively added. Samples were incubated at 37 °C for 35 min and then neutralized for analysis. To evaluate the esterase effect, the prodrug solution in HBSS and MHBSS with 50 IU of esterase/mL was incubated at 37 °C for 2 h. Solutions were analyzed by HPLC for acyclovir and prodrug.

For acyclovir formation in Caco-2 cell lysate, confluent Caco-2 cells were washed twice with PBS and harvested in sterile, prewarmed PBS at pH 7.4. The cell suspension was homogenized on ice with an ultrasound apparatus for 20 s. Homogenate was spiked with acyclovir valylchenodeoxycholate or acyclovir. Aliquots were removed at various times up to 60 min at 37 °C. To estimate the loss of prodrug not due to cell lysate, parallel studies containing no cell lysate were performed. HPLC was used to quantify prodrug and acyclovir, after protein precipitation.

Oral Bioavailability of Acyclovir Valylchenodeoxycholate in Rats. A study to compare acyclovir valylchenodeoxycholate and acyclovir oral bioavailabilities in rats was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health (Bethesda, MD). The study was approved by the University of Maryland Institutional Animal Care and Use Committee.

In rats and humans, acyclovir is largely excreted unchanged in the urine (>75% of the absorbed oral dose).[15] Hence, urine collection was chosen as the method for estimating and comparing the oral bioavailabilities from acyclovir valylchenodeoxycholate and acyclovir. Acyclovir was administered at a dose of 10 mg of acyclovir/kg. With the anticipation of a bioavailability 3-fold greater than that of acyclovir, the acyclovir prodrug was administered at a dose of 3.33 mg of acyclovir equivalent/kg (10.4 mg of acyclovir valylchenodeoxycholate/kg). In the dose range that was studied, acyclovir absorption is linear.[15,22]

Male Sprague-Dawley rats between 175 and 235 g (n = 8) were dosed by oral gavage with solutions of acyclovir prodrug or acyclovir after a 12 h fasting period. The rats were randomly divided into two groups, to conduct the study in a two-way crossover fashion. The acyclovir half-life is 1−3.6 h.[15,23,24] The second treatment of prodrug or acyclovir was administered after a washout period of at least 72 h. Urine samples were collected up to 48 h and analyzed according to a previously published and validated method.[24]

Acyclovir Quantification. In the HPLC quantification of acyclovir for in vitro studies, acyclovir was analyzed using a Zorbax eclipse C8 column (250 mm × 4.6 mm) (Agilent Technologies, Palo Alto, CA). The mobile phase contained phosphate buffer (40 mM, pH 2.0), 2.5% acetonitrile, and 0.2% heptanesulfonic acid ion pair reagent. The flow rate was 1.0 mL/min. The wavelength was 254 nm. In the HPLC quantification of acyclovir in rat urine, a Supelco (Bellefonte, PA) Discovery C18 column (250 mm × 4.6 mm) was used. The mobile phase contained phosphate buffer (20 mM, pH 6.8) and acetonitrile in a 99:1 ratio. The flow rate was 1.0 mL/min. The wavelength was 254 nm. Assay validation results were in agreement with previous results.[24]

Prodrug Quantification. Acyclovir valylchenodeoxycholate was quantified by HPLC using a C18 Supelco Discovery column. The mobile phase contained acetonitrile and water in a 1:1 ratio with 0.1% TFA. The flow rate was 1.0 mL/min. The wavelength was 254 nm. The prodrug was stable during the time course of the analysis.

Statistical Analysis. Results were analyzed with a Student's t test or by ANOVA with post hoc analysis, using SPSS version 10 (SPSS, Chicago, IL). A p value of <0.05 was considered significant. SEMs of ratios were calculated by the delta method.[25]

Results

Prodrug Synthesis. The four acyclovir prodrug conjugates were identified via the NMR spectra which contained peaks consistent with both the bile acid and valacyclovir portions, as listed below. Coupling through the amino acid amine (and not the aniline) was confirmed through the presence of the aromatic NH_2 signals at ~5.3 ppm. Mass spectrometry data were consistent with the desired structures. Microanalysis of each synthesized prodrug was consistent with calculated amounts (see the Supporting Information).

Synthesis of acyclovir valylchenodeoxycholate gave a yield of 89%. The structure of acyclovir valylchenodexycholate is illustrated in Figure 1: TLC ($MeOH/CHCl_3$, 1:4) $R_f = 0.46$; HPLC $t_R = 32.1$ min (99.3% pure); FAB-MS [M + H] = 700.4; HRMS calcd for $C_{37}H_{59}O_7N_6$ 699.4445, found 699.4454; ^{1}H NMR δ 0.55 (s, 1H), 0.70−0.90 (m, 3H), 1.05−2.20 (m, 5H), 2.46 (s, 2H), 2.71 (s, 2H), 2.84 (s, 2H), 3.01−3.2 (m, 1H), 3.55−3.65 (m, 1H), 4.0−4.22 (m, 1H),

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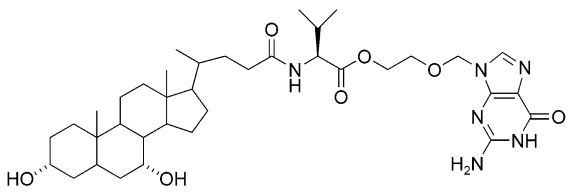

Figure 1. Structure of the prodrug acyclovir valylchenodexycholate. Acyclovir is linked to the bile acid chenodeoxycholate via valine.

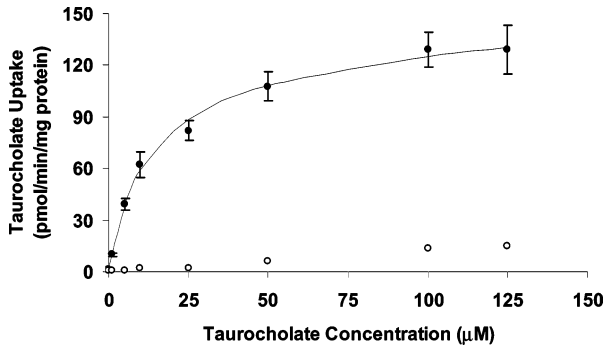

Figure 2. Concentration dependence of taurocholate uptake. Uptake of taurocholate into COS-hASBT cells in HBSS (●). Uptake of taurocholate into COS-hASBT cells in MHBSS (without sodium) (○). The best fit line from nonlinear regression is shown for combined carrier-mediated uptake and passive uptake (i.e., eq 1). Each point is the mean ± SEM of three measurements.

5.41 (s, 2H), 6.49 (s, 1H, br), 7.77 (s, 1H), 7.9−8.1 (m, 1H). Anal. ($C_{37}H_{58}O_7N_6 \cdot 2H_2O$) C, H, N.

Synthesis of acyclovir valylcholate gave a yield of 70%: TLC (MeOH/$CHCl_3$, 1:4) $R_f = 0.58$; HPLC $t_R = 28.3$ min (97.4% pure); FAB-MS [M + H] = 715.4; HRMS calcd for $C_{37}H_{59}O_8N_6$ 715.4394, found 715.4401. Anal. ($C_{37}H_{58}O_8N_6 \cdot 0.25H_2O$) C, H, N.

Synthesis of acyclovir valyldeoxycholate gave a yield of 85%: TLC (MeOH/$CHCl_3$, 1:4) $R_f = 0.47$; HPLC $t_R = 33.0$ min (98.1% pure); FAB-MS $[M + H]^+ = 699.54$; HRMS calcd for $C_{37}H_{59}O_6N_7$ 699.4445, found 699.4448. Anal. ($C_{37}H_{58}O_7N_6 \cdot 4H_2O$) C, H, N.

Synthesis of acyclovir valylursodeoxycholate gave a yield of 92%: TLC (MeOH/$CHCl_3$, 1:4) $R_f = 0.59$; HPLC $t_R = 28.5$ min (97.3% pure); FAB-MS $[M + H]^+ = 699.54$; HRMS calcd for $C_{37}H_{59}O_6N_7$ 699.4445, found 699.4428. Anal. ($C_{37}H_{58}O_7N_6 \cdot 3H_2O$) C, H, N.

hASBT-COS Uptake and Taurocholate Inhibition Studies. Figure 2 shows both the sodium dependence and saturation of taurocholate uptake. The uptake rate was measured in HBSS with 137 mM NaCl and in MHBSS which contained no sodium. As expected, hASBT-mediated taurocholate uptake did not occur in the absence of sodium, thus establishing the passive uptake of taurocholate into the cells. A passive uptake rate constant (k_p) of 0.123 ± 0.005 pmol min^{-1} (mg of protein)$^{-1}$ μM^{-1} was estimated using linear regression. Control uptake studies were also performed in untransfected COS-7 cells and in COS-7 cells transfected with the antibiotic resistant vector, pcDNA3. The passive uptake of [^{3}H]taurocholate from these experiments was the same as that obtained under sodium-free conditions (see the Supporting Information). The resulting kinetic parameters were as follows: $K_m = 12.0 \pm 2.2$ μM and $V_{max} = 126 \pm 6$ pmol min^{-1} (mg of protein)$^{-1}$ (in agreement with previous findings).[8,24]

Table 1. Inhibition of [^{3}H]Taurocholate Uptake by Bile Acids and Acyclovir Conjugates in COS-hASBT Cells[a]

inhibitor	K_i (μM)	SEM
cholate	24.8	5.1
glycocholate	20.6	7.3
taurocholate	9.54	1.19
deoxycholate	5.16	1.09
glycodeoxycholate	2.38	0.36
taurodeoxycholate	4.54	1.40
chenodeoxycholate	1.28	0.31
glycochenodeoxycholate	2.86	0.17
taurochenodeoxycholate	0.99	0.19
ursodeoxycholate	66.1	10.9
lithocholate	>100	N/A
glycine	N/A	N/A
taurine	N/A	N/A
acyclovir valylcholate	N/A (>600)	N/A
acyclovir valyldeoxycholate	401	50
acyclovir valylchenodeoxycholate	35.6	4.4
acyclovir valylursodeoxycholate	N/A (>600)	N/A

[a] In all studies, [^{3}H]taurocholate concentrations were held constant at 0.25 μM. The inhibitory bile acid concentration was varied between 1 and 100 μM; the glycine and taurine concentration ranged from 50 to 200 μM. The concentration of acyclovir bile acid conjugates varied between 1 and 400 or 600 μM. Inhibition studies were performed in triplicate. N/A denotes no evidence of inhibition, or the fact that a numerical value could not be calculated.

The effect of bile acids on the uptake of taurocholate was determined. Inhibition studies using glycine and taurine alone were performed as negative controls. In Table 1, all bile acids inhibited the uptake of taurocholate. Among the free, unconjugated bile acids, chenodeoxycholic acid was the most potent, followed by deoxycholic acid, cholic acid, and ursodeoxycholic acid. Lithocholate concentrations of up to 100 μM did not reduce the rate of taurocholate uptake beyond 50% maximal velocity. These results suggest that hASBT-COS was a suitable model for evaluating the affinity of prodrugs for hASBT.

The K_i value for inhibition of taurocholate uptake of the four acyclovir prodrug conjugates is listed in Table 1. The rank order of potency on inhibition followed that of the free bile acids. The conjugate of chenodeoxycholic acid exhibited the greatest potency with a K_i of 35.6 μM. The K_i value for the deoxycholic acid conjugate was 401 μM. The K_i values for the cholic acid and ursodeoxycholic acid conjugates were >600 μM. It appeared that the acyclovir prodrug employing chenodeoxycholic acid was recognized with relatively high affinity by the transporter, and may have the potential to be employed to increase the bioavailability of acyclovir.

Figure 3 illustrates the taurocholate uptake inhibition profiles of acyclovir valylchenodeoxycholate, valacyclovir,

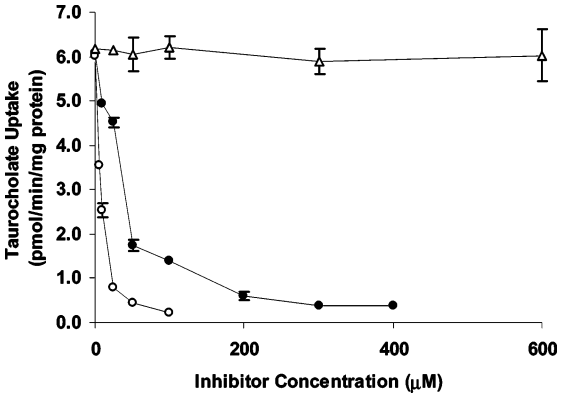

Figure 3. Inhibition of taurocholate uptake by acyclovir valylchenodeoxycholate. Inhibition profile by acyclovir valylchenodeoxycholate (●). Inhibition profile by chenodeoxycholate (○). Inhibition profile by valacyclovir (△). Acyclovir valylchenodeoxycholate inhibited taurocholate uptake, resulting in a K_i of 35.6 ± 4.4 μM. Hence, valacyclovir chenodeoxycholate strongly interacted with hASBT. Chenodeoxycholate inhibited taurocholate uptake, yielding a K_i of 5.4 ± 0.4 μM. Valacyclovir did not inhibit taurocholate uptake. Each point is the mean ± SEM of three measurements.

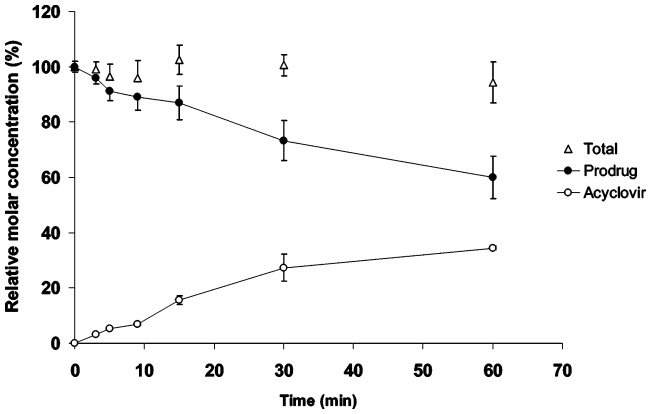

Figure 4. Hydrolysis of acyclovir valylchenodeoxycholate in Caco-2 cell lysates at pH 7.4 in PBS at 37 °C. Prodrug loss profile (●). Acyclovir formation profile (○). Molar sum profile of prodrug and acyclovir (△). After 60 min, 60.0 ± 7.8% of the acyclovir valylchenodeoxycholate remained, with the balance converted to acyclovir (34.4 ± 0.7%). For comparison, in parallel studies without cell lysate after 60 min in PBS, 83.8 ± 2.5% of the acyclovir valylchenodeoxycholate remained.

and chenodeoxycholate. Chenodeoxycholate inhibited taurocholate uptake, consistent with chenodeoxycholate being a substrate for hASBT that competitively inhibits taurocholate uptake via hASBT. Acyclovir valylchenodeoxycholate inhibited taurocholate uptake, although not as strongly as chenodeoxycholate. There was no inhibition of the bile acid transporter in hASBT-COS cells after application of up to 600 μM valacyclovir, indicating that valacyclovir is not a substrate for hASBT. Additionally, acyclovir did not inhibit taurocholate uptake (see the Supporting Information).

Hydrolysis of Acyclovir Valylchenodeoxycholate to Acyclovir. Liberation of acyclovir from acyclovir valylchenodeoxycholate was observed in sodium hydroxide solution, esterase-containing buffer, and Caco-2 cell lysate. After incubation with sodium hydroxide, 86.8 ± 0.1% of the initial prodrug was recovered as acyclovir, indicating hydrolysis of the prodrug to acyclovir. No parent prodrug was recovered. Recovery of acyclovir alone after the same sodium hydroxide treatment was 96.0 ± 0.1%, an indication of the stability of acyclovir in sodium hydroxide.

In vitro treatment of the acyclovir prodrug with esterase in HBSS for 2 h resulted in the majority of the prodrug being converted to acyclovir (71.4 ± 1.9%). Similarly, 68.0 ± 0.9% of the prodrug was converted to acyclovir in MHBSS in the presence of esterase. The mass balance of the formed acyclovir and the remaining prodrug was 88.4 ± 3.0%. In comparison to parallel studies without esterase, where only 15% of the prodrug was lost, these results indicated that esterase catalyzed the formation of acyclovir from the prodrug.

Acyclovir was also formed from acyclovir valylchenodeoxycholate in Caco-2 cell lysate. In Figure 4, the prodrug concentration decreased with time, in a nearly linear fashion at a rate of ~0.7%/min. The acyclovir concentration increased at approximately the same rate and to approximately the same extent as prodrug loss. The molar sum of acyclovir and prodrug was equal to the initial prodrug amount at all time points (ranging between 94.4 and 103%). After 1 h, 40.0 ± 7.5% of the prodrug was lost, with acyclovir being formed, equal to 34.4 ± 0.7% of the original prodrug. Under identical conditions but without Caco-2 cell lysate, only 16.2 ± 2.5% of the prodrug was lost, indicating that the cellular lysate accelerated the conversion of the prodrug to acyclovir ($p = 0.04$).

Uptake of Acyclovir Valylchenodeoxycholate into hASBT-COS Cells. In the taurocholate inhibition studies described above, acyclovir valylchenodeoxycholate exhibited an affinity for hASBT. To assess whether the prodrug was also translocated by hASBT, accumulation of the prodrug and acyclovir in hASBT-COS cells from prodrug solution was quantified in the presence and absence of sodium (i.e., in HBSS and MHBSS). An increased level of accumulation due to sodium would be attributed to translocation of the prodrug via hASBT, since hASBT is sodium-dependent. Accumulation in the absence of sodium reflected passive uptake.

In Figure 5, acyclovir appeared in hASBT-COS cells after incubation with either the acyclovir prodrug or acyclovir. No prodrug, but only acyclovir, was detected in the cells after incubation with the prodrug. Hence, Figure 5 describes uptake in terms of acyclovir, from both the prodrug and acyclovir, in the presence and absence of sodium. It should be noted that the detection of acyclovir from prodrug studies does not indicate that acyclovir was formed from the prodrug in these cell uptake studies, since sample preparation involved sodium hydroxide, which was shown to convert the prodrug to acyclovir.

Acyclovir uptake from the acyclovir prodrug was 202 ± 9 and 143 ± 2 pmol min^{-1} (mg of protein)$^{-1}$ in the presence

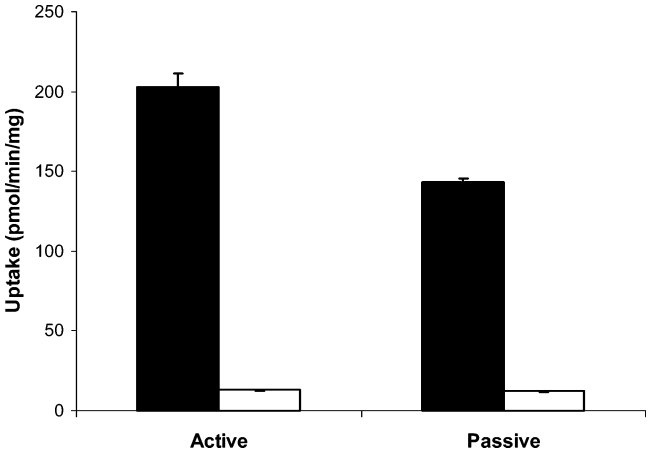

Figure 5. Sodium-dependent uptake into COS-hASBT cells from the prodrug. Uptake from acyclovir valylchenodeoxycholate prodrug (black bar). Uptake from acyclovir (white bar). Uptake from acyclovir valylchenodeoxycholate prodrug was greater than uptake from acyclovir under both active and passive uptake conditions. Active uptake used the sodium-containing buffer HBSS. The passive uptake condition used the sodium-free buffer MHBSS. Uptake was assessed by quantification of acyclovir in the cell lysate. No intact prodrug was detected in the cell lysate. Error bars represent SEMs ($n = 3$).

and absence of sodium, respectively. Active uptake in the presence of sodium resulted in a 1.4-fold enhancement in the rate of prodrug uptake ($p = 0.02$), relative to passive conditions (i.e., without sodium). This sodium-dependent uptake indicates the prodrug is translocated by hASBT. In Figure 5, acyclovir uptake from acyclovir solution was 12.9 $\pm$ 0.3 and 12.1 $\pm$ 0.4 pmol min^{-1} (mg of protein)$^{-1}$ in the presence and absence of sodium, respectively. Uptake of acyclovir from acyclovir solution was not sodium-dependent ($p = 0.2$), indicating acyclovir uptake occurs via passive diffusion (i.e., not via hASBT).

In Figure 5, the uptake of prodrug was more than 10-fold larger than the corresponding acyclovir uptake. The ratio of the prodrug to acyclovir uptake was 11.8 $\pm$ 0.4 in the absence of sodium, implicating that the prodrug possesses more than 10-fold higher passive permeability, compared to that of acyclovir ($p = 0.002$). Additionally, the ratio of prodrug versus acyclovir uptake was 16.2 $\pm$ 0.8 in the presence of sodium, indicating that the prodrug's translocation via hASBT further enhances prodrug uptake, relative to acyclovir.

Oral Bioavailability in Rats. Table 2 gives the urinary acyclovir excretion, which indicates the systemic bioavailability of acyclovir after oral administration of acyclovir valylchenodeoxycholate and acyclovir. Each value is equal to the percent of the oral dose that was collected as acyclovir from rat urine after 48 h. The mean percent acyclovir recovered $\pm$ the standard error of the mean (SEM) after oral administration of acyclovir was 24.6 $\pm$ 2.5%. The mean percent acyclovir recovered ($\pm$SEM) after oral administration of the prodrug was 48.0 $\pm$ 5.6%, which reflected a 2-fold enhancement in acyclovir oral bioavailability ($p < 0.007$). Bioavailability was increased via the prodrug in all rats.

Table 2. Urinary Acyclovir Excretion after Oral Administration of Acyclovir and Acyclovir Valylchenodeoxycholate to Tats in a Crossover Design[a]

rat	percent acyclovir recovered after acyclovir dose	percent acyclovir recovered after acyclovir valylchenodeoxycholate dose[b]	recovery ratio of acyclovir from prodrug vs acyclovir
A	15.5	34.9	2.25
B	21.6	38.5	1.78
C	24.4	42.7	1.75
D	20.6	24.3	1.18
E	26.3	69.0	2.63
F	22.9	67.3	2.94
G	24.1	43.1	1.79
H	41.3	63.9	1.55
mean	24.6	48.0[c]	1.98
SEM	2.5	5.6	0.19

[a] Rats A–D were first given acyclovir, and then the prodrug after a washout period. Rats E–H were first given the prodrug, and then acyclovir after a washout period. [b] The percent recovery of acyclovir from the prodrug dose is based upon the molar acyclovir equivalents from the prodrug. [c] The level of acyclovir excretion after prodrug administration was higher than after acyclovir administration ($p = 0.005$).

Discussion

Enhanced intestinal permeability via a prodrug approach is exemplified with valacyclovir, which increases acyclovir oral bioavailability 3-fold to 54% through valacyclovir being taken up by the PepT1 intestinal transporter.[20] The physiologic function of PepT1 is the active absorption of di- and tripeptides. L-Valine is conjugated to acyclovir to yield valacyclovir, which is recognized by PepT1. After absorption, valacyclovir is de-esterified to liberate acyclovir.

An alternative to a peptide prodrug approach was explored here by synthesizing bile acid prodrugs to target hASBT. In comparison to PepT1, hASBT has potential advantages of higher capacity and micromolar affinity.

Prodrug Design. The structural requirements necessary for recognition at hASBT are generally considered to be a steroid nucleus and a hydrogen bond acceptor at C-24.[26] We considered the possibility that coupling acyclovir through its alcohol function via a valine linker to the acidic group of C-24 would give a metabolically labile ester that functions as a hydrogen bond acceptor such that the prodrug may possess affinity for hASBT. In addition, the fact that the transporter possesses a high capacity implicates that even a prodrug with low affinity may be transported to a significant extent.

Two of the four acyclovir prodrugs exhibited affinity for hASBT by inhibition of taurocholate uptake into hASBT-

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COS cells. While prodrug potency was at least 25-fold lower than that of the free bile acids, prodrug affinity reflected the rank-order potency of their corresponding bile acid. The acyclovir conjugate of chenodeoxycholic acid possessed the greatest affinity ($K_i = 36\ \mu M$), which was comparable to that of cholic acid ($K_i = 25\ \mu M$). Acyclovir valylchenodeoxycholate was selected for further evaluation, because of its highest apparent affinity for hASBT.

Prodrug Properties of Acyclovir Valylchenodeoxycholate. In addition to exhibiting affinity for hASBT, acyclovir valylchenodeoxycholate was shown in vitro and in vivo studies to qualitatively possess three other necessary prodrug properties, i.e., the translocation of the prodrug by hASBT, the liberation of parent acyclovir, and increased acyclovir bioavailability from the bile acid prodrug. Further studies are required to optimize each of these characteristics.

Using a hASBT-COS cell model, acyclovir valylchenodeoxycholate was actively translocated by hASBT. In the presence of sodium, uptake from prodrug solution was 16-fold greater than uptake from an acyclovir solution, due to both active uptake of prodrug by hASBT and a greater passive membrane permeability of the prodrug, relative to acyclovir. In the absence of sodium, uptake from prodrug solution was 12-fold greater than uptake from acyclovir solution, reflecting a significantly larger passive uptake of prodrug, relative to acyclovir. Uptake from the prodrug in the presence of sodium was 1.4-fold higher than uptake from the prodrug in the absence of sodium ($p = 0.02$). Hence, hASBT-mediated uptake was achieved, as well as the substantial enhancement of passive drug uptake.

Given that the prodrug has a more than 10-fold higher passive permeability than acyclovir, the prodrug's enhanced passive permeability may be the major contributor to the prodrug's enhanced oral bioavailability. However, as we have seen previously with other transporters,[27] we speculate that the high passive membrane permeability of the prodrug overwhelms hASBT-mediated uptake such that the prodrug's favorable affinity for hASBT ($K_i = 35\ \mu M$) is not fully evident. Our current research efforts include the design of prodrugs with high affinity for hASBT but with low passive membrane permeability, since such an approach may better enable the measurement of active prodrug uptake by hASBT, with perhaps a more marked sodium dependence.

Second, acyclovir resulted from hydrolysis of the prodrug. Liberation of acyclovir from the acyclovir prodrug was demonstrated by chemical hydrolysis. Caco-2 cellular contents hydrolyzed acyclovir valylchenodeoxycholate to acyclovir to a greater extent than buffer alone ($p = 0.04$), which appears to be attributed to the presence of esterases in the cell lysate. The esterase solution catalyzed the hydrolysis of the prodrug such that the majority of the prodrug was converted to acyclovir in 2 h. It would appear that the prodrug can be hydrolyzed intracellularly by enterocytes during the absorption process. Additionally, plasma esterase would expectedly hydrolyze the prodrug.

Third, the bile acid prodrug increased the oral bioavailability of acyclovir. Rats were selected to evaluate this prodrug approach since acyclovir's bioavailability in rats is low (i.e., 15%)[28] and similar to the low human acyclovir bioavailability. Additionally, a bile acid transporter is expressed in the rat intestine.[29] Urine collection was chosen to estimate systemic acyclovir bioavailability since more than 75% of orally absorbed acyclovir is recovered unchanged in urine in rats.[15,30] On the basis of the recovery of acyclovir in urine, the prodrug doubled the oral bioavailability of acyclovir, from 24.6% after administration of acyclovir to 48.0% after administration of the prodrug ($p < 0.007$). Acyclovir bioavailability was increased via the prodrug in all rats. Given the ability of the prodrug to enhance acyclovir bioavailability, acyclovir recovery in the urine suggests the in vivo conversion of the prodrug to acyclovir, after enhanced absorption of the prodrug. While a 2-fold enhancement in acyclovir bioavailability from 25 to 48% in rats is notable, the PepT1 prodrug approach of valacyclovir increases acyclovir bioavailability 3-fold to 54% in humans.[23]

ASBT is located in the distal segment of the small intestine (i.e., ileum) such that a bile acid conjugate must exhibit a degree of hydrolytic stability to reach ASBT intact. A possible explanation for the only 2-fold increase in oral acyclovir bioavailability by acyclovir valylchenodeoxycholate, despite the prodrug's more favorable in vitro uptake properties, is prodrug hydrolysis in the stomach and proximal intestine. Future studies will aim to better optimize prodrug stability.

In summary, four bile acid prodrugs of acyclovir were synthesized, with the aim of utilizing hASBT and enhanced acyclovir oral bioavailability. In a taurocholate uptake inhibition assay, acyclovir valylchenodeoxycholate exhibited the highest affinity for hASBT, and was chosen for further investigation. Liberation of acyclovir from acyclovir valylchenodeoxycholate was demonstrated by chemical hydrolysis; acyclovir liberation was enhanced by esterase and Caco-2 cell lysate. In the transiently hASBT-transfected COS-7 cell model, acyclovir valylchenodeoxycholate's permeability was increased by both hASBT-mediated uptake and enhanced passive membrane permeability. In rats, the oral bioavail-

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ability of acyclovir was 2-fold greater after administration of the prodrug than after administration of acyclovir, based upon urinary excretion of acyclovir.

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Supporting Information Available: Microanalysis of acyclovir conjugates (Table 1), concentration dependence of taurocholate uptake (Figure 1), and inhibition of taurocholate uptake by acyclovir (Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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