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Synthesis and Evaluation of Bile acid-ribavirin Conjugates as Prodrugs to Target the Liver

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Abstract

Ribavirin is used to treat hepatitis C but causes serious hemolytic anemia. The objective of the study was to develop a ribavirin prodrug to achieve liver specific drug delivery and to reduce its off-target effect in red blood cells (RBC). The approach aimed to target the human sodium taurocholate cotransporting polypeptide (NTCP), which is a bile acid transporter predominately expressed in the liver. Six prodrugs with ribavirin conjugation at C-3 or C-24 of the bile acids were synthesized. *In vitro* uptake studies indicated that all six prodrugs were NTCP substrates. Metabolic studies *in vitro* indicated that ribavirin-L-Val-GCDCA was able to release ribavirin in the mouse liver S9 fraction. Additionally, *in vitro* studies showed that ribavirin in RBC was reduced by 16.7 fold from prodrug compared to parent drug incubation. Moreover, almost no prodrug was present in RBC. *In vivo* study in mice also showed that ribavirin-L-Val-GCDCA could provide almost the same ribavirin exposure in the liver as ribavirin administration, but with about 1.8-fold less exposure of ribavirin in RBC, plasma, and kidney. Overall, the study suggested that ribavirin-L-Val-GCDCA has the potential to achieve ribavirin specific liver delivery.

Keywords

bile acid transporters; hepatocytes; metabolism; NTCP; prodrugs; ribavirin; site-specific delivery

INTRODUCTION

Ribavirin is a guanosine analog for treating hepatitis C (HCV) which is the disease that primarily affects the liver and may eventually cause liver cirrhosis or liver cancer.^{1, 2} It is usually administered along with interferon alpha or a protease inhibitor (e.g. boceprevir) to enhance the sustained virological response (SVR), where a higher dose of ribavirin is more effective.³ Recent studies suggest that ribavirin may also be used to treat rhinovirus and acute myeloid leukemia.^{4, 5} However, its usefulness is limited by the dose dependent hemolytic anemia, which results in dose reduction or treatment termination in approximate 20% and 5% treated patients, respectively.⁶ This serious side effect is caused by ribavirin accumulation in the anucleate red blood cells (RBC), where ribavirin undergoes irreversible

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phosphorylation. Ribavirin phosphorylation in RBCs causes depletion of ATP in a competitive manner and exerts oxidative damage on the cell membrane.⁷ Targeted delivery of ribavirin to the liver may lower its accumulation in the red blood cells and reduce its dose-limiting side effects.

Human sodium taurocholate cotransporting polypeptide (NTCP) is a sodium dependent bile acid transporter that is involved in the enterohepatic circulation of bile acids. NTCP is predominantly expressed at the basolateral membrane of hepatocytes and probably responsible for transporting more than 80% of conjugated bile acids from portal blood into the liver.⁸ Because of its liver specificity and high capacity for transporting conjugated bile acids, bile acid conjugates have potential to serve as prodrugs to achieve liver specific drug delivery.⁹ Previously, bile acid conjugates were shown to deliver the drug of interest to the liver.^{10, 11} In the current study, we aim to develop novel bile acid ribavirin prodrugs in order to reduce ribavirin accumulation in red blood cells and hence alleviate ribavirin's most concerned side effect of hemolytic anemia.

Briefly, six bile acid-ribavirin prodrugs were synthesized by conjugating ribavirin to bile acids *via* an amino acid linker. These prodrugs were then evaluated for their *in vitro* NTCP uptake, metabolic stability and potential of ribavirin accumulation in red blood cells. After *in vitro* evaluation, a prodrug of ribavirin conjugated to glycochenodeoxycholic acid (GCDCA) *via* an L-valine linker was selected for *in vivo* assessment. The *in vivo* distribution of ribavirin-L-Val-GCDCA was examined in mice and compared to that of ribavirin itself. Results indicated that this bile acid-ribavirin conjugate has the potential to achieve liver specific delivery of ribavirin and reduce its accumulation in non-target organs.

EXPERIMENTAL SECTION

Figure 1 illustrates the overall approach and scope of compounds tested. Six bile acid-ribavirin prodrugs were collectively evaluated for liver specific delivery. It should be noted that ribavirin itself is a prodrug, with the phosphorylated form being the active moiety. Nevertheless, for simplicity, ribavirin is referred to as a drug and ribavirin conjugates as prodrugs.

Initially, five ribavirin conjugates were synthesized by conjugating the drug to chenodeoxycholic acid, ursodeoxycholic acid or cholic acid at the C-24 position, *via* a linker. Prodrug was then screened *in vitro* for human NTCP-mediated uptake across the cell membrane. All five were NTCP substrates. CDCA-L-Glu-ribavirin and CDCA-L-Val-ribavirin showed the higher normalized J_{max} and were thus subjected to ribavirin release assays in mouse S9 fraction. CDCA-L-Val-ribavirin released more than 60% of ribavirin in mouse S9 fraction, and was assessed for accumulation into human red blood cells as compared to ribavirin itself. Due to unsatisfying CDCA-L-Val-ribavirin accumulation into human red blood cells, ribavirin-L-Val-GCDCA was synthesized to reduce passive prodrug permeability. This sixth prodrug was also assessed for NTCP-mediated uptake, ribavirin release, and human red blood cells accumulation. The prodrug had the lowest RBC accumulation and highest stability in whole blood, and was thus intravenously administrated

to mice to evaluate the distribution of both released ribavirin and intact prodrug. Its pharmacokinetics was compared to that of intravenously administrated ribavirin.

Materials

Ribavirin was purchased from Carbosynth (Berkshire, England). Chenodeoxycholic acid (CDCA) was purchased from AK Scientific, Inc (Union City, CA). Ursodeoxycholic acid (UDCA) was purchased from Spectrum Chemical (New Brunswick, NJ). Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (Pybop) was purchased from EMD Millipore (Billerica, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Geneticin, fetal bovine serum (FBS), trypsin, DMEM and pooled balb-c mice s9 fraction were obtained from Invitrogen (Rockville, MD). Human whole blood was obtained from Innovative Research (Novi, MI).

Synthetic method overview

Schemes 1 and 2 show the synthesis of ribavirin prodrug conjugated to the C-24 position of bile acids. Briefly, the hydroxyl groups of ribavirin were initially protected with an acetonide group. Following protection, the intermediate was conjugated to an N-protected amino acid *via* an ester bond. After removal of the acetonide group and the protecting group at the N-terminal, the compound was conjugated to the amino acid's carboxylic acid at the C-24 position of CDCA, UDCA or cholic acid (CA) *via* amide bond. Hydrogenation was carried out when necessary to yield final prodrugs. Ribavirin was conjugated to the bile acids using either L-valine or L-glutamic acid, as shown in schemes 1 and 2.

Scheme 3 shows the synthesis of ribavirin prodrugs conjugated at the C-3 position of bile acids. Briefly, the hydroxyl groups of ribavirin were initially protected by benzylidene acetals. The resulting intermediate was then conjugated to L-valine *via* an ester bond. The compound was then coupled to the hydroxyl group at the C-3 position of glycochenodeoxycholic acid (GCDCA) benzyl ester *via* a carbamate bond. Hydrogenation was carried out to remove the benzylidene acetals and the benzyl ester protection group, yielding the final prodrug.

The identity and purity of all prodrugs were confirmed by thin layer chromatography, mass spectrometry (MS) and NMR (Table S1).

Synthesis of ribavirin acetonide (compound II)

To the suspension of ribavirin (compound I in Scheme 1, 2.0 g, 8.2 mmol) in acetone (40 mL), *p*-toluenesulfonic acid monohydrate (*p*-TsOH, 0.30 g, 1.58 mmol) and 2, 2-dimethoxypropane (DMP, 2 mL, 16 mmol) were added. The reaction was heated to 60°C and stirred for 6 hours, then cooled to room temperature. The acetone was evaporated under vacuum and purified by silica gel column chromatography using a mobile phase of dichloromethane (DCM) and methanol (v/v=14:1) to afford the intermediate II as a white solid with 73% yield. MS showed an appropriate peak of [M+Na] 307.2.

Synthesis of L-valine-ribavirin conjugates of bile acids (compounds 1 and 2)

Intermediate II (1.2 g, 4.1 mmol) was coupled to N-Cbz-L-valine (1.0 g, 4.1 mmol) using Pybop (2.1 g, 4.1 mmol) as the coupling reagent in 10 mL dimethyl formamide (DMF) along with N,N-diisopropylethylamine (DIPEA, 1.4 mL, 8.1 mmol). The reaction was stirred at room temperature overnight. The reaction was then quenched by adding 50 mL water followed by extraction with ethyl acetate (EtOAc) (3x). The combined organic layer was washed with saturated sodium bicarbonate solution (1x), water (3x), brine (1x) and dried over anhydrous sodium sulfate. The solvent was evaporated to yield a white solid intermediate (compound III, Scheme 1) with 99% yield. MS showed an appropriate peak of [M+H] 518.9.

The intermediate III was dissolved in 5 mL DCM and trifluoroacetic acid (TFA, v/v=1:9) and stirred at room temperature for 48 hours. After excess solvent evaporation under vacuum, saturated sodium bicarbonate solution was added, followed by extraction with EtOAc (3x). The combined organic layer was washed with water (3x), brine (1x) and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was purified with silica gel column chromatography using a mobile phase of DCM and methanol (v/v=14:1) to yield a white solid intermediate (compound IV, Scheme 1) with 20% yield.

The intermediate IV was subjected to catalytic hydrogenation to remove the Cbz protecting group using 10% palladium (Pd)/charcoal in methanol for 4 hours. The solution was filtered through Celite and the solvent was evaporated yielding a white solid intermediate (compound V, Scheme 1) with 97% yield. MS showed an appropriate peak of [M+H] 344.1.

To the solution of the intermediate V (0.40 g, 1.2 mmol) in 10 mL DMF, CDCA (0.46 g, 1.2 mmol) was added along with diethyl cyanophosphonate (DEPC, 0.2 mL, 1.3 mmol) and triethylamine (TEA, 1.7 mL, 12 mmol). The reaction was stirred at room temperature overnight. The reaction was quenched by adding 50 mL of water followed by extraction with EtOAc (3x). The combined organic layer was washed with saturated sodium bicarbonate solution (1x), water (3x), brine (1x) and dried over anhydrous sodium sulfate. The solvent was evaporated to yield a white solid. The crude product was further purified by silica gel column chromatography using a mobile phase of DCM/methanol/acetic acid (v/v=10:1:0.001) which yielded a final white solid product (compound 1, Scheme 1). MS of compound 1 showed appropriate peaks of [M+Na] 740.2 with 78% yield. Compound 2 was synthesized in the same manner, but with UDCA substituted for CDCA. MS of compound 2 showed appropriate peaks of [M+Na] 740.2 with 81% yield.

Synthesis of L-Glutamate-ribavirin amide conjugates of bile acids (compound 3, 4, and 5)

Intermediate II in Scheme 1 (1 g, 3.52 mmol) was coupled to Boc-L-glutamic acid 5-benzyl ester (1.19 g, 3.52 mmol) using Pybop (1.83 g, 3.52 mmol) as the coupling reagent in 10 mL DMF along with DIPEA (1.22 mL, 7.04 mmol). The reaction was stirred at room temperature overnight. The reaction was quenched by adding 50 mL water followed by extraction with EtOAc (3x). The combined organic layer was washed with saturated sodium bicarbonate solution (1x), water (3x), brine (1x) and dried over anhydrous sodium sulfate.

The solvent was evaporated to yield a white solid intermediate (compound VI, Scheme 2) with 47% yield. MS showed an appropriate peak of [M+Na] 626.1.

The intermediate VI was dissolved in 5 mL DCM and TFA (v/v=1:9) and stirred at room temperature for 48 hours to remove both the acetonide and Boc protecting groups. Excess DCM and TFA were evaporated under vacuum to yield an orange oily intermediate (compound VII, Scheme 2) which was used without further purification with 44% yield. MS showed an appropriate peak of [M+H] 464.1.

To the solution of intermediate VII (0.13 g, 0.28 mmol) in 4 mL DMF, CDCA (0.11 g, 0.28 mmol) was added along with DEPC (47 μ L, 0.31 mmol) and TEA (390 μ L, 2.8 mmol). The reaction was stirred at room temperature overnight. The reaction was quenched by adding 50 mL water followed by extraction with EtOAc (3x). The combined organic layer was washed with saturated sodium bicarbonate solution (1x), water (3x), brine (1x) and dried over the anhydrous sodium sulfate. The solvent was evaporated to yield a white solid intermediate (compound VIII, Scheme 2) with 76% yield. MS showed an appropriate peak of [M+H] 838.3.

Intermediate VIII was subjected to catalytic hydrogenation to remove the 5-benzyl ester group using 10% Pd/charcoal in methanol for 4 hours. The solution was filtered through Celite and the solvent was evaporated to give a white solid product. The crude product was further purified by preparative high performance liquid chromatography (HPLC) using a ZORBAX 300SB C18 PrepHT column (7 μ m, 21.2 $\times$ 250 mm; Agilent, Santa Clara, CA). The mobile phase was composed of 68% methanol and 32% water with a flow rate of 5 mL/min. UV absorbance was monitored at 210 nm. The fractions containing the product were collected and freeze dried to yield a final white fluffy solid product (compound 3, Scheme 2). Compounds 4 and 5 were synthesized in the same manner, but with ursodeoxycholic acid and cholic acid substituted for chenodeoxycholic acid, respectively. MS showed appropriate peaks of [M-H] 746.3 with 88% yield, [M-H] 746.3 with 87% yield, and [M-H] 762.3 with 75% yield.

Synthesis of ribavirin benzylidene acetal (compound IX)

To the suspension of ribavirin (compound I in Scheme 3, 2 g, 8.2 mmol) in benzaldehyde (20 mL), ZnCl₂ (2 g, 14.8 mmol) was added. The reaction was stirred at room temperature for six hours and poured into 250 mL diethyl ether, yielding a white precipitate. After suction filtration, the precipitate was purified by silica gel column chromatography using a mobile phase of DCM and methanol (14:1) to give a white solid intermediate (compound IX, Scheme 3) with 55% yield. MS showed an appropriate peak of [M+Na] 355.1.

Synthesis of L-valine-ribavirin carbamate conjugate of CDCA (compound 6)

Intermediate IX (1.49 g, 4.50 mmol) was then coupled to Fmoc-L-valine (1.53 g, 4.50 mmol) using Pybop (2.34 g, 4.50 mmol) in 15 mL DMF along with DIPEA (1.57 mL, 9.00 mmol). The reaction was stirred at room temperature overnight. The reaction was quenched by adding 50 mL water followed by extraction with EtOAc (3x). The combined organic layer was washed with water (3x), brine (1x) and dried over anhydrous sodium sulfate. The

solvent was evaporated to yield a white solid intermediate (compound X, Scheme 3) with 84% yield.

The intermediate X was dissolved in 10 mL of DMF with 5% piperidine and reacted for 2 hours to remove the Fmoc protecting group. The resulting product was acidified with 50 mL 2M HCl and extracted with 50 mL EtOAc. The aqueous layer was immediately alkalized with saturated sodium bicarbonate solution followed by extraction with EtOAc (3x). The combined organic layer was washed with water (3x), brine (1x) and dried over anhydrous sodium sulfate. The solvent was evaporated to yield a white solid intermediate (compound XI, Scheme 3) with 55% yield.

Glycine benzyl ester was conjugated to CDCA with DEPC as the coupling reagent, as described above with 72% yield. To the solution of glycine benzyl ester, protected CDCA (1 g, 1.86 mmol) in 80 mL anhydrous DCM in the presence of the 0.5 mL pyridine (6.21 mmol), triphosgene (0.275 g, 0.93 mmol) in anhydrous DCM was added dropwise. The reaction was stirred at room temperature for 4 hours and intermediate XI (0.80 g, 1.86 mmol) was subsequently added. The reaction was stirred for 48 hours. After the solvent was evaporated, extraction was conducted with water and EtOAc (3x). The combined organic layer was washed with brine (1x) and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to further purification by silica gel column chromatography using a mobile phase of DCM and methanol (v/v=33:1) to afford the intermediate XII (Scheme 3) as a white solid. MS showed an appropriate peak of [M+Na] 1019.3 with 32% yield.

The intermediate XII was subjected to catalytic hydrogenation to remove the benzylidene acetal and benzyl ester protecting groups using 20% palladium hydroxide (PdOH)/charcoal in methanol overnight. The solution was filtered through Celite and solvent was evaporated. The crude product was purified by C18 column chromatography using a mobile phase of water and methanol (v/v=1.85:1), yielding a final white solid product (compound 6, Scheme 3) with 58% yield. MS showed an appropriate peak of [M-H] 817.4.

Cell culture

Stably transfected human NTCP-HEK293 cells were grown in a 37 °C, 5% CO₂ atmosphere with 90% relative humidity and fed every 2 days as previously described.¹² The medium was composed of DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and geneticin (1 mg/mL). Cells were passaged when they reach 80% confluence.

Uptake of prodrugs by NTCP into cells

Uptake studies were conducted on NTCP-HEK293 cells. Cells were seeded at the density of 300,000 cells/well in 24-well Biocoat plates. On day 2 after seeding, cells were exposed to various concentrations of prodrugs (0–500 µM) which were dissolved in either Hank's Balanced Salts Solution (HBSS) with 137 mM sodium chloride (pH=6.8) or sodium free buffer (SFB) where sodium chloride was replaced with tetraethylammonium chloride (pH=6.8). Cells were exposed to the incubation buffer solutions for 5 minutes. The uptake was terminated by adding ice-cold SFB buffer after incubation buffer was removed. Cells

were lysed using acetonitrile. After acetonitrile was evaporated, the lysate were then dissolved in the acetonitrile/H₂O (1:1) with 500 nM internal standard (i.e. taurocholate or carbamazepine). The samples were stored at -80°C until further analysis.

In order to determine the prodrug kinetic parameters K_t and $J_{\max}$, uptake data in HBSS buffer was fit to equation 1 where K_t is prodrug Michaelis-Menten constant; J is prodrug flux; $J_{\max}$ is the maximal flux of prodrug; S is the prodrug concentration; and P_p is the prodrug passive permeability which is estimated from the uptake data in the SFB buffer since NTCP is sodium dependent transporter where no active transport takes place in the absence of sodium. As a positive control, $J_{\max}$ of taurocholate, a prototypical native bile acid, was estimated from taurocholate uptake studies at high taurocholate concentrations where transporter was saturated (i.e. 200 μ M) on each occasion. K_t , $J_{\max}$ and P_p were calculated through nonlinear regression using WinNonlin (Pharsight; Sunnyvale, CA). $J_{\max}$ of each prodrug was normalized to the $J_{\max}$ of taurocholate yielding normalized $J_{\max}$ in order to accommodate the variation in NTCP expression level across the studies.

$$J = \frac{J_{\max} S}{K_t + S} + P_p S \quad \text{eqn 1}$$

***In vitro* stability study**

Metabolic stability of prodrug was assessed in mouse liver S9 fraction. Briefly 5 μ M of prodrug was incubated with 1 mg/mL mouse liver S9 fraction supplemented with 1 mM nicotinamide adenine dinucleotide phosphate in Dulbecco's phosphate buffered saline at 37°C. At designated time points (i.e. 0, 0.5, 1, 3, 5 hr), 100 μ L samples were collected and quenched immediately with 400 μ L acetonitrile containing 1 μ M internal standard. Samples were vortexed for 30 seconds and centrifuged at 15,000 rpm for 10 min. Supernatant was dried overnight and reconstituted in 200 μ L water and store at -80 °C until further analysis.

***In vitro* accumulation of ribavirin and prodrug in red blood cells**

Accumulation of the ribavirin, CDCA-L-Val-ribavirin and ribavirin-L-Val-GCDCA in RBC was determined as previous described with minor changes.¹³ Briefly, human RBCs were separated from plasma by centrifugation at 2,000 rpm at room temperature for 10 minutes. Plasma was spiked with ribavirin or prodrug and gently mixed with RBC and incubated at 37 °C. The final concentration of initial ribavirin or initial prodrug in the whole blood was 100 μ M. At designated time point (i.e. 0 and 5 hr), 50 μ L aliquots of whole blood were collected and centrifuged at 6,000 rpm at 4 °C for 2 minutes to separate RBCs from plasma. RBCs were lysed by adding 100 μ L 0.1% triton X-100. Protein was precipitated with the addition of 300 μ L and 400 μ L of acetonitrile containing 1 μ M internal standard to the lysed RBC and plasma, respectively. After vortexing for 30 seconds, the samples were centrifuged at 15,000 rpm for 10 minutes.

After centrifugation, 300 μ L supernatant was transferred to 24 well plates and evaporated overnight at room temperature. Dry extracts were reconstituted in 200 μ L 5 mM ammonium acetate buffer (pH = 4.8). RBC samples were treated with 1 unit of phosphatase and

incubated at 37°C overnight to convert phosphorylated ribavirin to ribavirin, which was measured. Both plasma and RBC samples were stored at -80°C until further analysis.

***In vivo* distribution of ribavirin and prodrug in mice**

The study 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) and was approved by the University of Maryland Institutional Animal Care and Use Committee.

Normal non-fasted Balb/c mice (male, 12 weeks) were tail injected (IV bolus) with a dose of 20 mg/kg (0.08 mmol/kg) ribavirin and 67 mg/kg (0.08mmol/kg) ribavirin-L-Val-GCDCA respectively, to yield an equimolar amount of drug or prodrug. The compounds were dissolved in 60% saline and 40% polyethylene glycol (PEG 400). At designated time point (10min, 30 min, 1 hr, 2 hr, 4 hr and 8 hr), mice (n=3 per time point) were euthanized by CO₂ (carbon dioxide) asphyxiation, followed by cervical dislocation. Blood was drawn immediately by cardiac puncture and collected in heparinized vials. Samples were then centrifuged at 6,000 rpm and 4 °C for 2 minutes to separate plasma and RBC. Liver, small intestine, and kidney were isolated and weighed before homogenization in 2 mL water using Digital SLP cell disruptor (Branson Ultrasonics, Danbury, CT).

400 µL acetonitrile containing 1 µM internal standard was added to 50 µL tissue homogenate or plasma to precipitate protein. Samples were vortexed for 30 seconds and centrifuged at 15,000 rpm for 10 minutes. After centrifugation, 300 µL supernatant was transferred to 24 well plates and evaporated overnight at room temperature. Dry extracts were reconstituted in 300 µL water and stored at -80°C until further analysis.

For RBC, 25 µL RBC were firstly lysed by 100 µL 0.1% triton X-100. 300 µL acetonitrile containing 1 µM internal standard was then added to the lysate and vortexed for 30 seconds. Samples were centrifuged at 15,000 rpm for 10 minutes. After centrifugation, 300 µL supernatant from each sample was transferred to 24 well plates and evaporated overnight at room temperature. Dry extracts were reconstituted in 300 µL 5 mM ammonium acetate buffer (pH = 4.8). Samples were treated with 1 unit of phosphatase at 37°C overnight and stored at -80°C until further analysis.

In order to assess if the drug achieved liver targeting, liver targeting index (LTI) was calculated using equation 2

$$\text{Liver targeting index} = \frac{AUC_{0-t} \text{ in Liver}}{AUC_{0-t} \text{ in Plasma}} \quad \text{eqn 2}$$

The LTI of ribavirin employed the AUC of ribavirin in the liver and plasma over 8 hr (t=8hr).

Analytical method

Both ribavirin and prodrugs were quantified by liquid chromatography/mass spectrometry (LC/MS) which was performed on Waters ACQUITY UPLC/TQD system. All the samples

were centrifuged at 15,000 rpm for 5 minutes and 5 μ L supernatant was injected into LC/MS. Taurocholate was used as the internal standard for CA-L-Glu-ribavirin while carbamazepine was used as the internal standard for all other compounds.

To determine the prodrug concentration in the cell, a Luna C8 (2) column (3 μ m, 2.0 $\times$ 50 mm; Torrance, CA) was used. A gradient mobile phase employed methanol and water (v/v) with 0.1% formic acid, respectively. The flow rate was 0.7 ml/min and the compounds were detected under positive electrospray ionization mode $[M+H]^+$, except for CA-L-Glu-ribavirin which was detected under negative mode $[M-H]^-$. Multiple reaction monitoring (MRM) was used for all methods. The lower limit of quantification for all compounds was between 1 and 10 nM. Mass transitions and parameter of mass spectrometer for all six prodrugs and internal standards were detailed in Table 1.

Quantification of ribavirin and prodrug concentrations in liver S9 fraction, RBC and mice tissues was conducted using the same mass transition as in Table 1. A Xterra RP18 column (5 μ m, 4.6 $\times$ 150 mm; Waters, Milford, MA) was used in order to separate endogenous compound (most likely uridine) from the compounds of interest.¹⁴ The mobile phase was composed of water and methanol with 0.1% acetic acid. The flow rate was 1 mL/min. The limit of quantification for all compounds was between 100 nM and 500 nM.

As ribavirin-L-Val-GCDCA was studied *in vivo*, the extraction efficiency of the ribavirin-L-Val-GCDCA from plasma, red blood cell, liver, kidney and small intestine was 98%, 99%, 100%, 98% and 93% respectively. The accuracy of quantification of ribavirin-L-Val-GCDCA in plasma, red blood cell, liver, kidney and small intestine was 1.96%, 7.77%, -6.92%, 8.37% and 0.121% respectively. Ribavirin-L-Val-GCDCA stability in plasma, red blood cell, liver, kidney and small intestine for 30 min on ice showed greater than 80% stability. Similarly, a freeze-thaw cycle (-80°C and subsequent room temperature for 48 hr) of ribavirin-L-Val-GCDCA in plasma, red blood cell, liver, kidney and small intestine showed greater than 80% stability.

Statistical Analysis

Student's t-test was used to evaluate difference of prodrug uptake in the presence and absence of sodium. It was also used to assess different ribavirin accumulation in RBCs and plasma when ribavirin or prodrug (e.g. CDCA-L-Val-ribavirin or ribavirin-L-Val-GCDCA) were incubated with human whole blood. A p-value less than 0.05 denoted statistical significance.

RESULTS

NTCP uptake of prodrugs with C-24 conjugation

Ribavirin was first conjugated to carboxylic acid at the C-24 position of CDCA, UDCA, or CA using either L-valine or L-glutamic acid as the linker to yield five neutral or monoanionic compounds: CDCA-L-Val-ribavirin, UDCA-L-Val-ribavirin, CDCA-Glu-ribavirin, UDCA-Glu-ribavirin, and CA-L-Glu-ribavirin. Concentration-dependent uptake of all five compounds using NTCP-HEK293 stably transfected cells are shown in Figure 2.

Studies were conducted in the presence and absence of sodium, where uptake in the absence of sodium served as the negative control since NTCP is a sodium-dependent transporter.

CDCA-L-Val-ribavirin and UDCA-L-Val-ribavirin are neutral compounds while CDCA-L-Glu-ribavirin, UDCA-L-Glu-ribavirin, and CA-L-Glu-ribavirin each possess a single negative charge at the C-24 side chain. Despite these charge differences, all five compounds were NTCP substrates, where flux differed (*Student's t-test*, $p < 0.05$) between passive and total uptake at each concentration, except for the lowest concentration (2.5 μM) of CA-L-Val-ribavirin.

Prodrug uptake kinetic parameters are listed in Table 2. The transport capacities (i.e. normalized J_{max}) of all prodrugs were significantly lower than that of the native substrate taurocholate, with the normalized J_{max} values ranging from 0.011 to 0.302. CDCA-L-Val-ribavirin provided the highest normalized J_{max} (i.e. 0.302, or 30% that of taurocholate), followed by CDCA-L-Glu-ribavirin (normalized $J_{\text{max}} = 0.075$). The two prodrugs using L-valine as the linker yielded higher NTCP affinity (i.e. lower K_t) than their corresponding L-glutamic acid-linked prodrugs. UDCA-L-Val-ribavirin had the highest affinity toward NTCP, with a K_t value of 11.6 μM . The L-glutamic acid conjugates had much lower passive permeability than the neutral L-valine conjugates, possibly due to the negative charge on the linker.

CDCA-L-Val-ribavirin and CDCA-L-Glu-ribavirin were the two compounds with the highest normalized J_{max} values and hence were subjected to further evaluation for ribavirin release in mouse liver S9 fraction.

Release of ribavirin from prodrugs with C-24 conjugation

In order to possess efficacy in the liver to treat hepatitis C, ribavirin needs to be released from the prodrugs after hepatic uptake by NTCP. Thus, mouse liver S9 fraction, which contains a wide variety of liver enzymes, was used to assess ribavirin release from the prodrugs. CDCA-L-Val-ribavirin and CDCA-L-Glu-ribavirin were incubated with mouse liver S9 fraction over five hours. As shown in Figure 3, by five hours, 63% of ribavirin was released from the neutral CDCA-L-Val-ribavirin, while the prodrug itself was largely degraded by one hour. By contrast, very little ribavirin (4%) was released from the anion CDCA-L-Glu-ribavirin and 96% of the prodrug remained intact after five hours. The results suggest that CDCA-L-Val-ribavirin has the potential to release the drug in the liver, while CDCA-L-Glu-ribavirin showed little promise.

Reduced ribavirin accumulation in red blood cells with CDCA-L-Val-ribavirin

Given its susceptibility to release drug, CDCA-L-Val-ribavirin was incubated with human whole blood to assess ribavirin and prodrug distribution in plasma and RBC. The RBC is an off-target of ribavirin therapy, with hemolytic anemia limiting ribavirin dosing. By five hours, 42% of initial prodrug remained intact in plasma (Figure 4). Twenty-two percent of initial prodrug distributed into RBC and remained intact. Relative to initial prodrug, 13% of prodrug was converted to ribavirin and present in plasma and RBC, respectively. By contrast, the incubation of same molar amount of ribavirin itself with human whole blood

resulted in 25% of ribavirin in plasma and 50% ribavirin accumulation in RBC. The comparison can be translated that CDCA-L-Val-ribavirin was able to reduce ribavirin accumulation in RBC by 3.8-fold (i.e. 13% vs. 50%). However, 22% of the prodrug permeated into RBC, reflecting the high passive permeability of CDCA-L-Val-ribavirin. This high passive permeability may result in the passive diffusion of the prodrug into organs or tissues other than the liver, as observed here in RBC. This potential wide distribution may promote prodrug hydrolysis before it enters the liver, thus reducing the amount of ribavirin delivered into the liver.

***In vitro* characterization of ribavirin-L-Val-GCDCA**

In order to reduce a prodrug's passive permeability as observed for CDCA-L-Val-ribavirin and preserve its ability to release ribavirin in the liver, ribavirin was conjugated to the C-3 position of GCDCA using L-valine as the linker. GCDCA was selected over CDCA as GCDCA has higher capacity to be taken up by NTCP than CDCA.¹⁵ This strategy retains the ester bond between L-valine and ribavirin and introduces one negative charge at the C-24 region to reduce the passive permeability, as observed from the above for L-glutamic linked prodrugs.

Ribavirin-L-Val-GCDCA was assessed for NTCP uptake, ribavirin release in mouse liver S9 fraction, and drug distribution in human whole blood. As shown in Figure 5a, the passive permeability across HEK293 cells was reduced by 5.7-fold as compared to CDCA-L-Val-ribavirin. Interestingly, the transport capacity also decreased significantly (normalized $J_{\max}$ = 0.0538). However, the prodrug had much higher affinity toward NTCP than CDCA-L-Val-ribavirin, with a K_t value of 1.63 μ M vs. 35.0 μ M for CDCA-L-Val-ribavirin. Metabolic stability in mouse liver S9 fraction (Figure 5b) suggests that ribavirin-L-Val-GCDCA has the potential to release ribavirin in the liver. After five hours, 34% of ribavirin was released from the prodrug. The prodrug itself largely degraded within one hour. Prodrug distribution in human whole blood demonstrated that 85% of the prodrug remained intact in the plasma while almost no prodrug accumulated in the RBC (1%). Meanwhile, 3% of the released ribavirin was present in both the plasma and RBC (Figure 5c), which was 16.7-fold lower in RBC than when ribavirin alone was tested. Based on these *in vitro* studies, ribavirin-L-Val-GCDCA possessed overall greater potential to achieve *in vivo* liver targeting with reduced ribavirin accumulation in RBC.

Pharmacokinetics of ribavirin-L-Val-GCDCA and ribavirin in mice

A pharmacokinetic study of ribavirin-L-Val-GCDCA and ribavirin was conducted in mice in order to assess the liver targeting potential of the prodrug *in vivo*. Administration entailed 0.08 mmol/kg intravenous (IV) tail vein injection in mice. After prodrug or ribavirin administration, prodrug and/or ribavirin concentrations were measured in the plasma, RBC, liver, kidney and small intestine over eight hours.

Previous studies suggested that ribavirin was present in RBCs in the form phosphorylated ribavirin.⁶ Thus, RBC samples were treated with phosphatase, which converted phosphorylated ribavirin to ribavirin; total ribavirin concentration measurement reflects the sum of ribavirin and phosphorylated ribavirin. Meanwhile, pilot studies here indicated that

non-phosphorylated ribavirin was the predominant form in the liver sample (Figure S1). Hence, for liver samples, no treatment of phosphatase was needed, yet ribavirin concentration is interpreted to reflect the sum of ribavirin and phosphorylated ribavirin.

As shown in Figure 6, in comparison to ribavirin, administration of ribavirin-L-Val-GCDCA resulted in less ribavirin exposure in the plasma, RBC and kidney, increased exposure in the intestine, and similar exposure in the liver. The T_{max} of ribavirin in the liver was shifted from 30 min to 120 min when prodrug was administered, likely due to the time required for ribavirin to be released from the prodrug. The AUC of both ribavirin and ribavirin-L-Val-GCDCA was calculated for the plasma, RBC, liver, kidney and intestine, as shown in Table 3. The liver targeting index (LTI) for ribavirin was 0.0125 from ribavirin-L-Val-GCDCA administration and 0.00695 from ribavirin administration, indicating that the prodrug has achieved better ribavirin liver targeting by 1.80-fold. This enhancement was largely due to prodrug administration yielding about a 1.73-fold reduction in ribavirin exposure in plasma, compared to parent drug administration. Prodrug and drug administration afforded about the same ribavirin exposure in the liver.

DISCUSSION

In the current study, we have developed and characterized six ribavirin prodrugs with the goal of achieving liver specific delivery of ribavirin *via* targeting NTCP, thereby reducing toxic ribavirin accumulation in the red blood cells. Bile acid-ribavirin prodrugs were synthesized with either L-valine or L-glutamic acid as the linker. Bile acids are native substrates of NTCP, and both L-valine and L-glutamic acid are endogenous compounds to impart either neutral or anionic character. Both *in vitro* and *in vivo* tests were conducted to characterize the six prodrugs among which ribavirin-L-Val-GCDCA was shown to achieve ribavirin liver specific delivery in mice.

NTCP uptake

Human NTCP uptake studies were conducted to screen the synthesized prodrugs for potential liver targeting. A previous study suggested that a negative charge at the C-24 side chain of bile acids is essential for NTCP transport.¹⁶ Bile acid conjugates with monoanionic side chains (i.e. CDCA-L-Glu-ribavirin, UDCA-L-Glu-ribavirin, CA-L-Glu-ribavirin and ribavirin-L-Val-GCDCA) were substrates of NTCP. Interestingly, conjugates with no charge on the C-24 side chain (i.e. CDCA-L-Val-ribavirin and UDCA-L-Val-ribavirin) also showed favorable uptake, with K_t values comparable to that of taurocholate (i.e. 22.7 μ M),¹² suggesting that a monoanion at the C-24 side chain may not be necessary for the transport by human NTCP. Potentially, there may be unknown physicochemical or structural variables other than charge that affect NTCP transport, such as hydrophobicity which has been shown to impact ASBT transport activity.¹⁷

A previous study from our laboratory showed that bile acid conjugates coupled at the C-3 position afford translocation by NTCP.¹⁸ This C-3 coupling was also observed in the current study where the C-3 conjugated prodrug (i.e. ribavirin-L-Val-GCDCA) was a potent substrate with K_t of 1.63 μ M. In terms of transport capacity, all six prodrugs possess a significantly reduced NTCP capacity compared with taurocholate. Of the six prodrugs

investigated, ribavirin-L-Val-GCDCA had the lowest K_t value and a $J_{\max}$ of and about 75% transport efficiency ($J_{\max}/K_t$) of taurocholate, suggesting moderately efficient uptake by NTCP.¹² In addition, CDCA conjugates were shown to have higher transport capacity (i.e. normalized $J_{\max}$) than their corresponding UDCA or CA conjugates.

Prodrug metabolism in mouse liver S9 fraction

Metabolic stability studies were conducted to assess ribavirin release when exposed to mouse liver S9 fraction *in vitro*. All of the synthesized prodrugs possessed an ester bond between the amino acid linker and ribavirin. However, only CDCA-L-Val-ribavirin and ribavirin-L-Val-GCDCA showed favorable drug release when exposed to mouse liver S9 fraction, while CDCA-L-Glu-ribavirin was stable, suggesting that L-valine is more susceptible to enzymatic hydrolysis than L-glutamic acid. Although about 94% of CDCA-L-Val-ribavirin and ribavirin-L-Val-GCDCA were degraded after five hours, only 63% and 34% of ribavirin was recovered, respectively. It has been reported that there are two metabolic pathways for ribavirin.¹⁹ One is the reversible phosphorylation pathway where ribavirin is phosphorylated to mono-, di-, and tri-phosphorylated ribavirin. The other pathway is the degradation involving deribosylation and amide hydrolysis to yield a triazole carboxylic acid metabolite. Our results suggest that ribavirin might undergo an irreversible metabolism by five hours, resulting in incomplete recovery of ribavirin (Figure S1).

Prodrug distribution in whole blood

Both prodrug and ribavirin were incubated in human whole blood to assess ribavirin accumulation in RBC and stability of the prodrug in whole blood. It has been shown that ribavirin is phosphorylated in RBC where triphosphorylated ribavirin is the predominant metabolite.²⁰ Phosphatase was used to convert phosphorylated ribavirin to ribavirin so that ribavirin concentration measured in RBC by LC/MS represents all of the ribavirin accumulated in cells.²⁰ Ribavirin is actively transported into RBCs by equilibrative nucleoside transporter 1 (ENT1).²¹ Likely, the coupling of a bulky bile acid would hamper ribavirin's uptake into RBC which represents an off-target in clinical use of ribavirin. This reduced uptake was observed in our study where both CDCA-L-Val-ribavirin and ribavirin-L-Val-GCDCA caused significantly reduced ribavirin accumulation in RBC. However, due to the high passive permeability, 22% of CDCA-L-Val-ribavirin was also present in RBC. The subsequent introduction of a single negative charge (i.e. ribavirin-L-Val-GCDCA) decreased the prodrug's passive permeability and reduced the accumulation of prodrug to only 1%. Meanwhile, the presence of prodrug in the plasma increased from 42% to 85% (CDCA-L-Val-ribavirin vs. ribavirin-L-Val-GCDCA).

In vivo study in mice

The potential advantage of targeting NTCP to achieve liver specific drug delivery motivated the *in vivo* evaluation of ribavirin-L-Val-GCDCA. A prodrug of ribavirin that targets NTCP may allow for preferential uptake into the liver. Hence, with subsequent release of ribavirin from prodrug, ribavirin may preferentially accumulate in the liver, which is the site of drug action in treating hepatitis C. Correspondingly, liver targeting has promise to minimize

ribavirin accumulation in the RBC. Of course, all released ribavirin in the liver may not be retained in the liver.

A pharmacokinetic study was conducted to evaluate the liver targeting potential of ribavirin-L-Val-GCDCA *in vivo*. The study here showed that ribavirin exposure was reduced in the plasma, RBC and kidney by about 40%, while an approximately equal amount of ribavirin was seen in the liver as compared the administration of ribavirin itself. It should be noted that where in the liver the ribavirin was exposed (e.g. hepatocyte, bile canaliculi) was not specifically evaluated. Ribavirin exposure was increased in the intestine, perhaps due to the enterohepatic circulation of bile acids where the apical sodium-dependent bile acid transporter (ASBT) may be responsible for reabsorbing unmetabolized prodrug from the lumen of the small intestine. Prodrug enterohepatic circulation could potentially increase the amount of prodrug delivered to the liver, if the prodrug is not quickly released in hepatocytes and instead is circulated. However, prodrug hydrolysis in the intestine may result in increased intestinal ribavirin exposure. Thus, a prodrug with faster hydrolysis in the liver may reduce ribavirin exposure in the intestine.

CONCLUSIONS

Six bile acid-ribavirin prodrugs were synthesized. All six demonstrated NTCP uptake, but had low transport capacity compared to that of taurocholate. Of these six prodrugs, ribavirin-L-Val-GCDCA had the highest affinity toward NTCP while CDCA-L-Val-ribavirin had the highest transport capacity (i.e. normalized $J_{\max}$). Metabolic stability in mouse liver S9 fraction indicated that CDCA-L-Val-ribavirin and ribavirin-L-Val-GCDCA efficiently released ribavirin in the liver. Of the two, ribavirin-L-Val-GCDCA had more favorable drug distribution in human whole blood, with a high amount of prodrug present in the plasma but a favorably low ribavirin accumulation in RBC. The *in vivo* pharmacokinetic study in mice showed that IV administration of ribavirin-L-Val-GCDCA allowed for greater liver targeting of ribavirin while reducing ribavirin accumulation in RBC. Results indicate that a study of oral administration of this prodrug for liver targeting is merited. Overall, the current study suggests that ribavirin-L-Val-GCDCA has the potential to achieve liver-specific delivery of ribavirin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ASBT	Sodium-dependent bile acid transporter
CA	cholic acid

CDCA	chenodeoxycholic acid
DCM	dichloromethane
DEPC	diethyl cyanophosphonate
DIPEA	N,N-diisopropylethylamine
DMF	dimethyl formamide
DMP	2, 2-dimethoxypropane
ENT1	equilibrative nucleoside transporter 1
EtOAc	ethyl acetate
GCDCA	glycochenodeoxycholic acid
HBSS	Hank's Balanced Salts Solution
HCV	hepatitis C
LTI	liver targeting index
NTCP	Sodium taurocholate cotransporting polypeptide
Pd	palladium
PdOH	palladium hydroxide
p-TsOH	p-toluenesulfonic acid monohydrate
Pybop	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RBC	red blood cells
SFB	sodium free buffer
TEA	triethylamine
TFA	trifluoroacetic acid
UDCA	ursodeoxycholic acid

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Figure 1. Flow diagram of approach to synthesize and characterize six bile acid-ribavirin prodrugs for liver specific delivery.

Figure 2.

Concentration-dependent uptake of (a) CDCA-L-Val-ribavirin, (b) UDCA-L-Val-ribavirin, (c) CDCA-L-Glu-ribavirin, (d) UDCA-L-Glu-ribavirin, and (e) CA-L-Glu-ribavirin into NTCP-HEK293 cells for 5 min. Closed circles indicate total prodrug uptake by NTCP in the presence of sodium. Open circles indicate passive uptake of the prodrug in the absence of sodium. The solid line indicates model fit of the total uptake. Data are presented as mean (±SEM) of three measurements at each concentration.

Figure 3. Hydrolysis of (a) CDCA-L-Val-ribavirin and (b) CDCA-L-Glu-ribavirin in mouse liver S9 fraction. For each prodrug, prodrug (5 μ M) was incubated with mouse liver S9 fraction (1mg/mL) at 37°C for 0, 0.5, 1, 3 and 5 hr. Open circles indicate percentage of prodrug remaining intact. Closed circles indicate percentage of ribavirin appearing in mouse S9 fraction, relative to initial prodrug. Data are presented as mean ($\pm$ SEM) of three measurements at each time point.

Figure 4.

Distribution of ribavirin and CDCA-L-Val-ribavirin in human whole blood at 37°C after 5 hr. Ribavirin or CDCA-L-Val-ribavirin (100μM) was incubated with human whole blood for 0 and 5 hr, followed by quantification of ribavirin (if ribavirin incubated) or released ribavirin and intact prodrug (if prodrug incubated) in plasma and red blood cells (RBC). Closed bars indicate ribavirin incubation. The open bars indicate prodrug incubation (and hence prodrug or release ribavirin quantified). Data are presented as mean ($\pm$ SEM) of three measurements at each time point. * denotes that relative amount was reduced for CDCA-L-Val-ribavirin incubation versus ribavirin incubation [Student's t-test; p value = 9.08×10^{-3} (plasma) and p value = 4.25×10^{-5} (RBC)].

Figure 5.

In vitro characterization of ribavirin-L-Val-GCDCA. (a) From the NTCP uptake of ribavirin-L-Val-GCDCA, $K_t=1.63(+0.70)$ μM, $J_{max}=0.100(+0.004)$ (pmol/sec/cm²), Normalized $J_{max}=0.0538(+0.0038)$ and Passive permeability= $0.655(+0.025) \times 10^{-6}$ cm/s. (b) Hydrolysis of ribavirin-L-Val-GCDCA in mouse liver S9 fraction over five hours. After five hours, 34% of ribavirin was released from the prodrug (c) Distribution of released ribavirin and intact ribavirin-L-Val-GCDCA in human whole blood at 37°C after 5 hours. 16.7-fold lower amounts of ribavirin accumulated in RBC when tested as prodrug compared with ribavirin itself. Data are presented as mean ($\pm$ SEM) of three measurements at each time

point. * denotes that relative amount was reduced for ribavirin-L-Val-GCDCA incubation versus ribavirin incubation [Student's t-test, p value= 1.24×10^{-3} (plasma), p value= 1.22×10^{-5} (RBC)].

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Figure 6.

Pharmacokinetics of ribavirin and prodrug ribavirin-L-Val-GCDCA in mice after IV administration. Each data point represents data from three mice IV injected with either 0.08 mmol/kg ribavirin or 0.08 mmol/kg ribavirin-L-Val-GCDCA. Plasma, red blood cell, liver, kidney and small intestine were collected at 0, 10, 30, 60, 120, 240, 480 min. Panels a-e are concentrations of ribavirin and prodrug in the (a) plasma, (b) red blood cell, (c) liver, (d) kidney and (e) small intestine respectively. Closed squares are the ribavirin concentration after ribavirin IV injection. Closed circles are the concentration of released ribavirin from ribavirin-L-Val-GCDCA after IV prodrug injection. Open circles are the concentration of

intact ribavirin-L-Val-GCDCA after IV prodrug injection. Open circles and close circles represent data from the same mouse. Liver, kidney and small intestine concentrations were normalized by its tissue weight. Data are presented as mean ($\pm$ SEM) of three measurements at each time point.

Scheme 1.

Synthesis of CDCA-L-Val-ribavirin (compound 1). a. p-TsOH, DMP, acetone, 60 °C, 6 hrs
 b. N-Cbz-L-valine, Pybop, DIPEA, DMF, RT, overnight c. DCM/TEA(v/v=1:9), RT, 48 hrs
 d. H₂, Pd/C, RT, 4 hrs e. CDCA, TEA, DEPC, DMF, RT, overnight.

Scheme 2.

Synthesis of CDCA-L-Glu-ribavirin (compound 3). f. Boc-Glu(OBzl)-OH, Pybop, DIPEA, DMF, RT, overnight g. DCM/TFA(v/v=1:9), RT, 48 hrs h. CDCA, TEA, DEPC, DMF, RT, overnight i. H₂, Pd/C, RT, 4 hrs.

Scheme 3.

Synthesis of ribavirin-L-Val-GCDCA (compound 6). j. benzaldehyde, ZnCl_2 , RT, 6 hrs k. Fmoc-L-valine, Pybop, DIPEA, DMF, RT, overnight l. 5% piperidine, DMF, RT, 2 hrs m. CDCA-Gly-benzyl ester, triphosgene, pyridine, DCM, RT, overnight n. H_2 , PdOH/C, RT, overnight.

Table 1

Parameters of mass spectrometer and monitored ion transitions for ribavirin prodrugs quantification.

Compound	MRM transition	Collision energy(V)
Ribavirin	245.13->112.87	16
Carbamazepine (internal standard)	237.13->193.99	26
Taurocholate (internal standard)	514.26->79.96	100
CDCA-L-Val-ribavirin ^a	718.67->97.00	35
UDCA-L-Val-ribavirin ^a	718.67->97.00	35
CDCA-L-Glu-ribavirin ^a	748.54->97.06	72
UDCA-L-Glu-ribavirin ^a	748.54->97.06	72
CA-L-Glu-ribavirin ^a	762.33->128.01	68
Ribavirin-L-Val-GCDCA ^a	819.48->96.93	74

All compounds were detected under positive electrospray ionization mode $[M+H]^+$ except CA-L-Glu-ribavirin which was detected under negative mode $[M-H]^-$.

^a Accuracy for CDCA-L-Val-ribavirin, UDCA-L-Val-ribavirin, CDCA-L-Glu-ribavirin, UDCA-L-Glu-ribavirin, CA-L-Glu-ribavirin and ribavirin-L-Val-GCDCA were 1.83%, 0.318%, -3.58%, 1.99%, 3.87%, 0.508% respectively.

Table 2

NTCP uptake parameters of CDCA-L-Glu-ribavirin, UDCA-L-Glu-ribavirin, CA-L-Val-ribavirin, CDCA-L-Val-ribavirin and UDCA-L-Val-ribavirin.

Prodrug	K_t (μM)	$J_{\max}$ (pmol/sec/cm ²)	Normalized $J_{\max}$ ^a	$P_{\text{px}}10^6$ (cm/sec)
CDCA-L-Glu-ribavirin	182±26	0.145±0.009	0.0752±0.047	0.0567±0.0026
UDCA-L-Glu-ribavirin	244±41	0.0330±0.0026	0.0171±0.0013	0.0154±0.0004
CA-L-Glu-ribavirin	659±261	0.0208±0.0053	0.0108±0.0028	0.0274±0.0007
CDCA-L-Val-ribavirin	35.0±7.4	0.592±0.044	0.302±0.031	3.76±0.05
UDCA-L-Val-ribavirin	11.6±1.4	0.0654±0.0020	0.033±0.002	0.376±0.019

Data are summarized as mean ($\pm$ SEM) of three measurements.

^a $J_{\max}$ of each prodrug was normalized to $J_{\max}$ of taurocholate that was measured in parallel to each study in order to accommodate the variation in NTCP expression level across the studies.

Table 3

Exposure of ribavirin and prodrug ribavirin-L-Val-GCDCA to various compartments and tissues after IV administration of each ribavirin and prodrug in mice over 8 hours.

Compartment or tissue	AUC _{0-8hr} of ribavirin after ribavirin administration ($\mu\text{M} \times \text{min}$ or $\mu\text{mol} \times \text{min}$ per g tissue) ^a	AUC _{0-8hr} of ribavirin after ribavirin-L-Val-GCDCA administration ($\mu\text{M} \times \text{min}$ or $\mu\text{mol} \times \text{min}$ per g tissue) ^a	AUC _{0-8hr} of ribavirin-L-Val-GCDCA after its administration ($\mu\text{M} \times \text{min}$ or $\mu\text{mol} \times \text{min}$ per g tissue) ^a
Plasma	3857	2221	600
Red blood cell	2940	1788	547
Liver	53.6	55.6	3.28
Kidney	10.4	5.43	0.476
Intestine	8.47	16.6	7.03

Six time points (n=3 mice per time point) were employed in measuring AUC.

^aUnits are $\mu\text{M} \times \text{min}$ for plasma and red blood cell. Units are $\mu\text{mol} \times \text{min}$ per g tissue for liver, intestine, and kidney.