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Synthesis and *in Vitro* Evaluation of Bile Acid Prodrugs of Floxuridine to Target the Liver

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Abstract

Floxuridine is often used to treat metastatic liver disease and is given as an infusion directly into the hepatic artery to increase the amount of intact drug that reaches the liver. The objective of this work was to design and synthesize prodrugs of floxuridine through conjugation to chenodeoxycholic acid (CDCA) to target the liver via the bile acid liver uptake transporter Na⁺/taurocholate cotransporting polypeptide (NTCP, *SLC10A1*). Two isomeric prodrugs of floxuridine were synthesized: floxuridine 3' glutamic acid-CDCA and floxuridine 5'-glutamic acid-CDCA. Both were potent inhibitors and substrates of NTCP. Floxuridine 3' glutamic acid-CDCA showed $K_i = 6.86 \pm 1.37 \mu\text{M}$, $K_m = 10.7 \pm 2.1 \mu\text{M}$, and passive permeability = $0.663 (\pm 0.121) \times 10^{-7} \text{ cm/s}$ while floxuridine 5'-glutamic acid-CDCA showed $K_i = 0.397 \pm 0.038 \mu\text{M}$, $K_m = 40.4 \pm 15.2 \mu\text{M}$, and passive permeability = $1.72 (\pm 0.18) \times 10^{-7} \text{ cm/s}$. Floxuridine itself had a higher passively permeability of $7.54 (\pm 0.45) \times 10^{-7} \text{ cm/s}$ in the same cell line, indicating that both prodrugs have the potential for lower non-specific effects than the drug alone. Prodrugs were stable in rat plasma ($t = 3\text{h}$), but quickly released in rat liver s9 fraction, suggesting future *in vivo* evaluation.

Keywords

Prodrug; bile acid; targeted drug delivery; Na⁺/taurocholate cotransporting polypeptide (NTCP, *SLC10A1*); floxuridine

1. INTRODUCTION

Floxuridine (5-fluoro-2'-deoxyuridine) is a chemotherapeutic nucleoside analog often used for the treatment of colorectal cancer. It is reversibly converted into the well-known anti-cancer drug 5-fluorouracil (5-FU), which was rationally designed as an antimetabolite to uracil (1). 5-FU incorporates into RNA in place of uracil and inhibits DNA synthesis (2). It also exerts its anti-proliferative effect by incorporating into DNA in place of thymine (3), and by the binding of one of its metabolites, FdUMP, to thymidylate synthase (4, 5).

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Thymidylate synthase is an enzyme that catalyzes the production of thymidine monophosphate, which in turn is phosphorylated to form thymidine triphosphate, an essential component for DNA synthesis and repair. Thus, thymidylate synthase is an attractive inhibition target for chemotherapeutic agents (6). Floxuridine has been shown to have a 5,000-fold more potent anti-cancer activity than 5-FU, due to extended inhibition of thymidylate synthase (7). However, it has low and variable bioavailability, between 34–47% in orally dosed patients (8). Floxuridine converts rapidly to 5-FU by the enzyme thymidine phosphorylase (TP), which is abundant in many tissues, including the intestine and liver (9).

Several prodrug strategies of floxuridine have previously been explored. Floxuridine conjugates of peptides have been synthesized to target peptide transporter 1 (PEPT1, *SLC15A1*) with the objective of increasing oral bioavailability, lowering variability in uptake, and preventing conversion to 5-FU by TP (10, 11). Additionally, polymer conjugates of its active triphosphate form have been synthesized (12), as well as phosphoactivated (13), phosphoramidated (14), and alkyl ester (15, 16) forms of floxuridine prodrugs. These prodrugs aim to enhance floxuridine bioavailability and to promote its tumor-specific delivery, thus reducing non-specific toxicity.

Floxuridine is frequently administered by hepatic artery infusion (HAI) when colorectal cancer is advanced and has metastasized to the liver (17). Patients show improved outcomes when receiving both systemic treatment of 5-FU and HAI of floxuridine than with systemic administration of 5-FU alone (18). This treatment involves laparoscopic surgery to place an HAI pump directly into the abdomen (19).

Bile acids are steroidal molecules that aid in the digestion of lipids, among other roles. They are synthesized in the liver from cholesterol and secreted into the gallbladder. In response to a meal, they empty into the duodenum, where they solubilize lipids and lipid-soluble vitamins through the formation of mixed micelles. Bile acids are absorbed passively and actively into portal circulation, and subsequently into the liver to complete their enterohepatic circulation. The human bile acid pool is 2–4 g, and cycles multiple times daily for a total of up to 30 g/day absorbed and less than 10% lost to feces (20). Thus, the enterohepatic circulation of bile acids is a high capacity, high efficiency system.

The objective of the present study is to synthesize bile acid prodrugs of floxuridine and test them *in vitro* for the Na⁺/taurocholate cotransporting polypeptide (NTCP, *SLC10A1*) affinity. NTCP is responsible for over 80% of conjugated bile acid uptake into hepatocytes (21). We hypothesize that through conjugation to bile acids, floxuridine prodrugs have the potential to non-invasively target the liver by mimicking the hepatic uptake of native bile acids.

2. MATERIALS AND METHODS

2.1. Materials

Taurocholate, chenodeoxycholic acid (CDCA), Hank's balanced salt solution, rat liver s9 fraction, and di-tert-butyl dicarbonate were purchased from Sigma-Aldrich (St. Louis, Missouri). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were

obtained from Invitrogen (Rockville, Maryland), [^3H]-taurocholate (10 $\mu\text{Ci}/\text{mM}$) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, Missouri). Benzyl-protected glutamic acid was purchased from Aroz Technologies, LLC (Cincinnati, Ohio) and floxuridine was purchased from Carbosynth (Berkshire, United Kingdom). Other reagents were purchased at the highest purity commercially available.

2.2. Synthesis of two floxuridine prodrugs of chenodeoxycholic acid

Two prodrugs of floxuridine were separately synthesized: floxuridine-5'-glutamic acid-CDCA and floxuridine-3'-glutamic acid-CDCA. Each is a conjugate of the native bile acid chenodeoxycholic acid and employ a glutamic acid linker between the drug and the bile acid. However, they differ in that they are positional isomers of one another. Prodrug synthesis of both targets is collectively described in Figures 1 and 2. Briefly, benzyl protected glutamic acid was N-boc protected, then reacted to form an ester with floxuridine, yielding two intermediates of interest (i.e. compounds 2 and 3 in Figure 1). These intermediates are positional isomers and were purified to subsequently yield the two final target prodrugs. For each purified intermediate, their N-boc groups were deprotected, and they were reacted with an activated OB ester of chenodeoxycholic acid. After purification, the benzyl protecting group was removed by hydrogenation, resulting in final floxuridine prodrugs. All reactions were performed at room temperature.

Specifically, 2 g (8.4 mmol) of benzyl-protected glutamic acid (H-Glu-OBzl) was stirred overnight with 1 eq. (8.4 mmol) triethylamine and 1.2 eq. (10.1 mmol) di-tert-butyl dicarbonate (Boc_2O) in 10 mL 1:1 tetrahydrofuran (THF):methanol (MeOH). Resultant mixture was dried under vacuum, reconstituted in ethyl acetate, washed three times with water, once with 0.1 M hydrochloric acid (HCl), once with saturated sodium chloride solution, and dried over sodium sulfate before evaporation. This common intermediate showed an appropriate mass spectrometry (MS) peak of $[\text{M} + 23]$ 360.1 (compound 1 in Figure 1).

Next, 2.74 g (8.1 mmol) compound 1 was stirred with 1 eq. (8.1 mmol) N,N'-dicyclohexylcarbodiimide (DCC) and 0.1 eq. 4-dimethylaminopyridine (DMAP) in dimethyl formamide (DMF). 1 eq. (8.1 mmol) floxuridine was added to this mixture and stirred overnight. The resultant mixture was diluted with 80 mL ethyl acetate and washed with 20 mL water (3x) and brine. The organic layer was then dried over sodium sulfate, and solvent was evaporated. Product contained esters of floxuridine at the 3' position, the 5' position, and a double ester at both the 3' and 5' positions.

The two target intermediates were separated using flash column chromatography with an eluent mixture of 50% ethyl acetate, 50% hexane. Both the 3' and 5' esters (compounds 3 and 2 in Figure 1, respectively) showed appropriate MS peaks of $[\text{M} + 23]$ 588.1 and $[\text{M} - 1]$ 564.3. Purity was confirmed by thin layer chromatography (TLC) and C-13 nuclear magnetic resonance (NMR) spectroscopy. TLC showed single spots when stained with 10% w/v phosphomolybdic acid in ethanol (compound 2: $R_f = 0.25$, compound 3: $R_f = 0.07$; 50% ethyl acetate, 50% hexane). Assignment of 3' and 5' esters was made from 2 dimensional heteronuclear single quantum coherence (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC) NMR analysis. NMR spectrums were acquired from a

Varian INOVA 500MHz machine (Agilent Technologies, Santa Clara, California). Compound 2, ^{13}C NMR (dimethyl sulfoxide (DMSO)-d₆): δ 25.66, 28.12, 28.12, 28.12, 29.89, 36.82, 52.70, 61.23, 65.91, 74.80, 78.31, 84.40, 84.90, 124.44, 127.79, 127.79, 128.03, 128.38, 128.38, 135.93, 140.08, 149.02, 155.53, 156.86, 157.07, 171.79. Compound 3, ^{13}C NMR (DMSO-d₆): δ 25.70, 28.10, 28.10, 28.10, 29.77, 38.59, 52.77, 63.95, 65.87, 69.94, 78.31, 83.76, 84.46, 124.63, 127.70, 127.70, 127.98, 128.34, 128.34, 135.92, 140.05, 148.95, 155.53, 156.86, 157.07, 172.00.

Separately for each compound 2 and 3, the intermediate was mixed for 15 min in a 10 mL mixture of 1:1 trifluoroacetic acid (TFA): dichloromethane (DCM) to remove the N-boc protecting group. Excess solvent was evaporated, yielding products 4 and 5 in Figure 1 from compounds 2 and 3, respectively.

The carboxylic acid of chenodeoxycholic acid was activated by forming a benzotriazole ester as previously described (21). Briefly, 2 g (5.1 mmol) chenodeoxycholic acid was stirred with 1 eq. (5.1 mmol) O-benzotriazol-1-yloxytris-1,1,3,3 tetra methyl uranium hexafluorophosphate (HBTU) and 1 eq. (5.1 mmol) triethylamine (TEA) in DMF for 15 min, then 1 eq. (5.1 mmol) hydroxybenzotriazole (HOBt) was added and the mixture was allowed to react overnight. DMF was diluted with 80 mL ethyl acetate, washed with 20 mL water (3x), 20 mL brine, dried over sodium sulfate, and evaporated under vacuum. CDCA-OBt was used without further purification, and showed an MS peak of $[\text{M} + 1]$ 510.4.

Each compound 4 and 5 was reacted overnight in parallel with 1 eq. of CDCA-OBt in DMF, shown in Figure 2. After each reaction, DMF was diluted with 80 mL ethyl acetate, washed with 20 mL water (3x), 20 mL brine, dried over sodium sulfate, and solvent was evaporated under vacuum. Compounds 6 and 7 were each purified using flash column chromatography with a solvent of ethyl acetate. They each showed an appropriate MS peak of $[\text{M} + 23]$ 862.3 and $[\text{M} - 1]$ 838.5, and TLC confirmed purity, as single spots were seen for each compound when stained with 10% w/v phosphomolybdic acid in ethanol (compound 6: $R_f = 0.22$, compound 7: $R_f = 0.12$; ethyl acetate).

After purification, compounds 6 and 7 were hydrogenated independently at atmospheric pressure for 4 h, stirred in methanol with 10 weight % palladium/carbon catalyst. Both final compounds 8 (floxuridine 5'-glutamic acid-CDCA) and 9 (floxuridine 3'-glutamic acid-CDCA) showed an appropriate MS peak of $[\text{M} + 23]$ 772.3 and $[\text{M} - 1]$ 748.4. MS confirmed that no CDCA was present in the final products. Purity was analyzed by high performance liquid chromatography (HPLC) using a Waters (Milford, Massachusetts) system (1525 binary HPLC pump, 717 plus autosampler, and 486 tunable absorbance detector) with an ultra phenyl column (5 μm , 250 x 4.6 mm, Restek Corporation, Bellefonte, Pennsylvania). A 1.0 mL flow rate and absorbance wavelength of 218 nm were employed. Method one used an isocratic solvent of 30% ACN and 70% water with 0.1% formic acid, while method two employed a 67:33 v/v mixture of methanol and [20 mM ammonium formate, 0.5% formic acid, 0.2% TEA (pH 3)] (23). For each prodrug, the methods were linear from 25 to 200 μM (floxuridine 3'-glutamic acid-CDCA: method one $R^2 = 0.9971$, $R_T = 2.82$ min, method two $R^2 = 0.9957$, $R_T = 3.43$ min, purity = 98.6%; floxuridine 5'-glutamic

acid-CDCA: method one $R^2 = 0.9998$, $R_T = 3.12$ min, method two $R^2 = 0.9993$, $R_T = 3.64$ min, purity = 96.9%).

2.3. Cell Culture

NTCP- human embryonic kidney (HEK) cells were cultured as previously described (24), at 37 °C, 90% humidity, and 5% CO₂. Cells were fed every two days with media consisting of DMEM with 10% FBS, 50 units/mL penicillin, 50 µg/mL streptomycin, 1 mg/mL geneticin to maintain selection pressure, and 1% nonessential amino acids. Cells were passaged approximately every 4 days, when 90% confluent. Cells were seeded at a density of 0.6 million cells/well (24-well plates, 2 cm² wells) and studies were performed on day two after seeding.

2.4. Inhibition of Taurocholate Uptake into NTCP-HEK cells

NTCP-HEK cells were washed three times with Hank's balanced salt solution (HBSS) at pH 6.8, then exposed to donor solution and incubated at 37 °C for 5 min. Donor solutions consisted of 0–200 µM prodrug or floxuridine, 2.5 µM taurocholate, and 2.5% DMSO as a co-solvent (25) in HBSS. Donor solutions were spiked with 0.5 µCi/mL [³H] taurocholate. After incubation, cells were washed three times with cold sodium-free buffer (SFB), wherein sodium chloride is replaced with 137 mM tetraethylammonium chloride. Cells were lysed with 150 µL acetonitrile and allowed to dry. Lysates were reconstituted in either 1:1 acetonitrile (prodrugs) or 4:1 water: 70% acetonitrile, 30% methanol (floxuridine). Lysates were counted for radioactivity with an LS6500 liquid scintillation counter (Beckmann Instruments, Inc., Fullerton, California).

Inhibition data was fitted using a modified Michaelis-Menten competitive inhibition equation that accounts for aqueous boundary layer permeability, as previously described (26) (eqn. 1).

$$V = \frac{P_{ABL} \cdot \left(\frac{V_{max}}{K_m (1 + I/K_i) + S} + P_p \right)}{P_{ABL} + \frac{V_{max}}{K_m (1 + I/K_i) + S} + P_p} \cdot S \quad (\text{eqn. 1})$$

In eqn. 1, V is taurocholate flux, P_{ABL} is aqueous boundary layer permeability (previously measured to be 1.5×10^{-4} cm/s (27)), P_p is passive permeability, and S is taurocholate concentration (i.e. 2.5 µM). K_m was set as 5.03 µM, determined from historic data (28), and P_p was determined by taurocholate uptake in SFB (measured using saturating concentration of 200 µM taurocholate on each study occasion).

2.5. Uptake into NTCP-HEK cells

Donor solutions consisted of 0–200 µM prodrug or floxuridine in either HBSS (to measure total uptake) or SFB (to measure sodium-independent, passive uptake), as NTCP is a sodium-dependent active transporter. Cells were washed three times with either HBSS or SFB (consistent with donor) and incubated for 5 min at 37 °C. Cells were lysed, dried, and reconstituted as in inhibition studies. Lysates were centrifuged at 12,000 g for 10 min in

silanized centrifuge tubes and stored at -20°C until analysis by liquid chromatography/tandem mass spectrometry (LC/MS/MS).

Total uptake data were regressed to eqn. 2, where V is substrate flux, P_{ABL} is 1.5×10^{-4} cm/s, defined above, and P_p is passive substrate permeability fitted by sodium-independent uptake to eqn. 3. Data were regressed to define substrate Michaelis-Menten constants K_m and V_{max} . V_{max} is expressed as $\text{norm}V_{max}$, where substrate V_{max} is normalized to taurocholate V_{max} measured on the same occasion, estimated by a saturating taurocholate concentration of $200\text{ }\mu\text{M}$. Normalizing V_{max} accounts for variation in transporter expression levels across study days.

$$V = \frac{P_{ABL} \cdot \left(\frac{V_{max}}{K_m + S} + P_p \right)}{P_{ABL} + \frac{V_{max}}{K_m + S} + P_p} \cdot S \quad (\text{eqn. 2})$$

$$V = \frac{P_{ABL} \cdot P_p}{P_{ABL} + P_p} \cdot S \quad (\text{eqn. 3})$$

2.6. In Vitro Stability

Stability of floxuridine 3'-glutamic acid-CDCA, floxuridine 5'-glutamic acid-CDCA, and floxuridine was determined in rat liver s9 fraction and rat plasma. To evaluate liver stability, $5\text{ }\mu\text{M}$ of each compound was incubated with 2 mL of 1 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 2 mg/mL rat liver s9 fraction in Dulbecco's phosphate-buffered saline (DPBS). Samples were incubated at 37°C ($n = 3$), and $200\text{ }\mu\text{L}$ aliquots were taken out at time 0 min , 15 min , 30 min , 45 min , 1 h , 2 h , and 3 h . To evaluate plasma stability, $5\text{ }\mu\text{M}$ of each compound was incubated with rat plasma at 37°C , and $200\text{ }\mu\text{L}$ aliquots were removed at 0 h , 1 h , 2 h , and 3 h . All aliquots were immediately diluted with four parts cold acetonitrile and stored at -20°C until after the final incubation time, then centrifuged at $12,000\text{ g}$ for 10 min . $500\text{ }\mu\text{L}$ aliquots of the prodrug and floxuridine samples at each time point were removed, dried, and reconstituted in 4:1 water: 70% acetonitrile, 30% methanol for floxuridine quantification by LC/MS/MS. Samples were analyzed for prodrug concentration without reconstitution.

2.7. LC/MS/MS Quantification

Prodrug and floxuridine concentrations were quantified by LC/MS/MS. A Waters Acquity UPLC system with triple quadrupole detector (Waters Corporation, Milford, Massachusetts) and a Waters Acquity UPLC Ethylene Bridged Hybrid C8 $1.7\text{ }\mu\text{m}$ $2.1 \times 50\text{ mm}$ column were used. The flow rate was 0.4 mL/min and the injection volume was $10\text{ }\mu\text{L}$. Prodrugs were quantified with positive ion electrospray mode and a multiple reaction monitoring (MRM) method. For floxuridine 3'-glutamic acid-CDCA, the transition 750.61 Da to 81.00 Da was monitored (dwell time 0.1 s , cone voltage 30 V , collision energy 68 V). For floxuridine 5'-glutamic acid-CDCA, the transition 750.61 Da to 504.44 Da was monitored (dwell time 0.1 s , cone voltage 35 V , collision energy 22 V). For both prodrug methods, the gradient was as follows (expressed as % ACN in water, including 0.1% formic acid): 50% from 0 to 0.5

min, increased to 95% until 1.5 min, decreased to 50% at 1.7 min, and finally held at 50% until 2.0 min. For floxuridine quantification, a negative electrospray ionization MRM method was used that monitored the transition 245.10 Da to 154.99 Da (dwell time 0.1 s, cone voltage 35 V, collision energy 15 V). Solvents used for floxuridine detection were water (A) and 70% acetonitrile:30% methanol (B), with the following gradient: 80% A until 0.5 min, then increased to 80% B at 1.5 min, and back down to 80% A at 1.7 min and held until 2.0 min. The methods were linear over the range of 10 to 2000 nM (floxuridine 3'-glutamic acid-CDCA, $R^2 = 0.9998$; floxuridine 5'-glutamic acid-CDCA, $R^2 = 0.9989$; floxuridine, $R^2 = 0.9968$).

2.8. Statistical Analysis

Stability data was evaluated using the Student's paired *t*-test, with a *p*-value of 0.05 or greater considered significant. Throughout the text and figures, error is presented as standard error of the mean.

3. RESULTS

3.1. Inhibition of Taurocholate Uptake into NTCP-HEK cells

Both floxuridine 3'-glutamic acid-CDCA and floxuridine 5'-glutamic acid-CDCA were potent inhibitors of taurocholate uptake by NTCP. Results are shown in Figure 3. Floxuridine 3'-glutamic acid-CDCA had a fitted K_i value of $6.86 \pm 1.37 \mu\text{M}$, while floxuridine 5'-glutamic acid-CDCA had a K_i value of $0.397 \pm 0.038 \mu\text{M}$. Conversely, floxuridine itself showed no inhibition of taurocholate uptake by NTCP.

3.2. Uptake into NTCP-HEK cells

Floxuridine 3'-glutamic acid-CDCA and floxuridine 5'-glutamic acid-CDCA were potent substrates of NTCP, as shown in Figure 4. Floxuridine 3'-glutamic acid-CDCA had a K_m value of $10.7 \pm 2.1 \mu\text{M}$, P_p of $0.663 (\pm 0.121) \times 10^{-7} \text{ cm/s}$, and normVmax of 0.0187. Floxuridine 5'-glutamic acid-CDCA had a K_m value of $40.4 \pm 15.2 \mu\text{M}$, P_p of $1.72 (\pm 0.18) \times 10^{-7} \text{ cm/s}$, and normVmax of 0.0185. For comparison, taurocholate has a K_m value of $5.31 \mu\text{M}$ (29) in this same stably transfected cell line. Results indicate that the K_m values of each prodrug is near the potency of the native bile acid taurocholate.

Floxuridine showed no sodium-dependent uptake into NTCP-HEK cells. Passive permeability was calculated as $7.54 (\pm 0.45) \times 10^{-7} \text{ cm/s}$, which is higher than the passive permeability of both floxuridine 3'-glutamic acid-CDCA and floxuridine 5'-glutamic acid-CDCA. These results suggest that each prodrug has the potential for lower non-specific toxicity than floxuridine, since each has lower passive permeability compared to floxuridine.

In Figure 5, the apparent permeabilities of floxuridine 3'-glutamic acid-CDCA, floxuridine 5'-glutamic acid-CDCA, and floxuridine are presented. Apparent permeability is flux divided by concentration. At low concentrations ($< 20 \mu\text{M}$), apparent permeability of the prodrugs was much higher than that of floxuridine, due to favorable active uptake. At concentrations $50 \mu\text{M}$ and above, transporter saturation caused the two prodrugs to favorably exhibit lower passive permeabilities than that of floxuridine. This apparent permeability data

suggests that floxuridine 3'-glutamic acid-CDCA and floxuridine 5'-glutamic acid-CDCA have the potential to actively target NTCP-containing cells (i.e. hepatocytes) and reduce off-target disposition of floxuridine.

3.3. *In Vitro* Stability

Stability of floxuridine and its novel chenodeoxycholic acid prodrugs were evaluated in rat liver s9 fraction and rat plasma. Overall, the synthesized chenodeoxycholic acid prodrugs of floxuridine released floxuridine when exposed to rat liver s9 fraction and showed favorable stability in rat plasma. These stability results indicate that the prodrugs have potential for *in vivo* liver targeting after intravenous administration.

Figure 6 shows the disappearance of floxuridine 3'-glutamic acid-CDCA, floxuridine 5'-glutamic acid-CDCA, and floxuridine in the presence of rat liver s9 fraction, as well as the appearance of floxuridine in response to the degradation of both prodrugs. Each prodrug (initial 5 μ M concentration) exhibited complete degradation from 2 mg/mL rat liver s9 fraction with 1 mM NADPH. Floxuridine 3'-glutamic acid-CDCA degraded more rapidly than floxuridine 5'-glutamic acid-CDCA, with 6.8% and 24.7% remaining after 1 h, respectively. For each prodrug, the amount of formed floxuridine peaked after 45 min, and then diminishes at subsequent time points, consistent with the rapid degradation of floxuridine itself in Figure 6, Panel C.

Floxuridine prodrugs showed no significant degradation in rat plasma. Floxuridine 3'-glutamic acid-CDCA provided $96.7 \pm 3.7\%$ remaining after 3 h (Student's paired *t*-test, one-tailed *p* = 0.19), while floxuridine 5'-glutamic acid-CDCA resulted in $95.1 \pm 4.2\%$ remaining (Student's paired *t*-test, one-tailed *P* = 0.21). No time points showed any appearance of floxuridine, further evidence that the prodrugs did not degrade in rat plasma. This plasma stability could indicate that a liver-specific esterase is involved in the release of floxuridine in rat liver s9 fraction.

4. DISCUSSION

Bile acid transporters have previously been identified as potential prodrug targets because of the highly efficient and specific nature of enterohepatic circulation (30). An example prodrug is valylchenodeoxycholate, previously developed in our group as a conjugate of valacyclovir and chenodeoxycholic acid (31). Acyclovir is an antiviral drug, and valacyclovir is a prodrug of acyclovir conjugated to valine that is a substrate for PEPT1. Valacyclovir increases the oral bioavailability of acyclovir from 20% (32) to 54% (33). When orally administered to rats, valylchenodeoxycholate increased the bioavailability of acyclovir from 25% to 48% (31), showing the potential for increased oral drug bioavailability by exploitation of the enterohepatic circulation of bile acids. Additionally, a 5-FU conjugate of cholic acid was shown to have increased mouse liver concentrations of 5-FU, compared with when 5-FU itself was dosed (34).

NTCP, located on the basolateral membrane of hepatocytes, is a potential target for increasing drug exposure to the liver. Although down-regulated in cases of cholestatic liver disease (35), NTCP is expressed on the membrane of hepatocellular carcinoma cells, and has

been shown to mediate the uptake of a taurocholic acid prodrug of the chemotherapeutic chlorambucil into NTCP-expressing *Xenopus laevis* oocytes (36). These findings suggest that NTCP may be used to transport chemotherapeutics into liver tumors, to reduce non-specific chemotherapy toxicity. Potentially in this case, floxuridine would be delivered to both hepatocellular carcinoma cells and normal hepatocytes. Floxuridine HAI treatment sometimes presents local toxicity such as sclerosing cholangitis and chemical hepatitis (37, 38, 39), which could also occur with the current prodrug approach. However, delivering intact floxuridine to the liver would provide the increased efficacy of floxuridine over its metabolites and reduce non-specific bone marrow and gastrointestinal toxicity (40) of 5-FU resulting from rapid TP conversion from floxuridine.

Previous floxuridine prodrug studies have shown that esterification of the 3' or 5' free hydroxyl group of floxuridine leads to resistance to TP conversion of floxuridine to 5-FU (41). Thus, with the aim of delivering intact floxuridine to the liver, an ester bond was used to conjugate floxuridine to the primary native bile acid chenodeoxycholic acid, through the application of a glutamic acid linker. Two prodrugs were synthesized: floxuridine 3'-glutamic acid-CDCA and floxuridine 5'-glutamic acid-CDCA. They are identical except in the position of glutamic acid conjugation to the free hydroxyl of floxuridine (i.e. either the 3' or 5' hydroxyl was esterified). Both prodrugs were inhibitors and substrates of NTCP. The NTCP K_m and K_i values for floxuridine 3'-glutamic acid-CDCA were 10.7 μM and 6.86 μM , respectively, reflecting similar K_i and K_m values, which implicate competitive inhibition for this compound (42). Meanwhile, the NTCP K_m and K_i values for floxuridine 5'-glutamic acid-CDCA were 40.4 μM and 0.397 μM , respectively, reflecting more potent inhibition than uptake. Potentially, these values for floxuridine 5'-glutamic acid-CDCA may suggest a different mechanism for inhibition than for uptake.

The stability and activation of the prodrugs appeared favorable. Floxuridine 3'-glutamic acid-CDCA and floxuridine 5'-glutamic acid-CDCA were stable in rat plasma, but showed rapid release of floxuridine in rat liver s9 fraction. This stability profile suggests that glutamic acid is a promising linker to conjugate bile acids with liver-targeted drugs, as the ester bond remains stable in plasma and degrades in liver s9 fraction. Overall, *in vitro* results show that floxuridine 3'-glutamic acid-CDCA and floxuridine 5'-glutamic acid-CDCA properties are favorable for subsequent *in vivo* testing of their liver targeting capabilities.

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ABBREVIATIONS

5-FU	5-fluorouracil
Boc₂O	di-tert-butyl dicarbonate
CDCA	chenodeoxycholic acid

DCC	N,N'-dicyclohexylcarbodiimide
DCM	dichloromethane
DMAP	4-dimethylaminopyridine
DMEM	Dulbecco's modified Eagle medium
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
FBS	fetal bovine serum
HAI	hepatic artery infusion
HBSS	Hank's balanced salt solution
HBTU	O-benzotriazol-1-yloxytris-1,1,3,3 tetra methyl uranium hexafluorophosphate
HCl	hydrochloric acid
HEK	human embryonic kidney
HMBC	heteronuclear multiple-bond correlation spectroscopy
HOBt	hydroxybenzotriazole
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
LC/MS/MS	liquid chromatography/tandem mass spectrometry
MeOH	methanol
MRM	multiple reaction monitoring
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
NTCP	the Na ⁺ /taurocholate cotransporting polypeptide (<i>SLC10A1</i>)
P_{ABL}	aqueous boundary layer permeability
PEPT1	peptide transporter 1 (<i>SLC15A1</i>)
P_p	passive permeability
SFB	sodium-free buffer
THF	tetrahydrofuran
TEA	triethylamine
TFA	trifluoroacetic acid

TLC	thin layer chromatography
TP	thymidine phosphorylase

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Figure 1.

Synthesis of floxuridine prodrug intermediates, compound 4 and compound 5. Benzyl-protected glutamic acid was boc-protected at the amine group, then esterified with floxuridine, yielding compounds 2 and 3. From these two compounds, the boc groups were then removed to form compounds 4 and 5, respectively.

Figure 2.

Synthesis of two floxuridine prodrugs of chenodeoxycholic acid. Compounds 4 and 5 were reacted with activated CDCA-obt to form compounds 6 and 7, respectively. Prodrug synthesis was completed by catalytic hydrogenation of compounds 6 and 7 to floxuridine-5'-glutamic acid-CDCA (compound 8) and floxuridine-3'-glutamic acid-CDCA (compound 9), respectively.

Figure 3.

Prodrug inhibition of taurocholic acid uptake by NTCP. In Panel A, floxuridine 3'-glutamic acid-CDCA inhibited taurocholic uptake into NTCP-HEK cells with $K_i = 6.86 \pm 1.37 \mu\text{M}$. In Panel B, floxuridine 5'-glutamic acid-CDCA inhibited with $K_i = 0.397 \pm 0.038 \mu\text{M}$ affinity.

Figure 4.

Uptake of floxuridine prodrugs into NTCP-HEK cells. In Panel A, floxuridine 3'-glutamic acid-CDCA was a potent substrate with $K_m = 10.7 \pm 2.1 \mu\text{M}$, while in Panel B, floxuridine 5'-glutamic acid-CDCA had a K_m value of $40.41 \pm 15.20 \mu\text{M}$.

Figure 5.

Prodrug and drug apparent permeability. For the two prodrugs, active uptake provided a high apparent permeability compared to that of floxuridine at concentrations less than or equal to 20 μM. At concentrations greater than or equal to 50 μM, transporter saturation caused floxuridine prodrugs to have a lower apparent permeability than that of floxuridine.

Figure 6.

Prodrug and drug stability in rat liver s9 fraction. In Panel A, the disappearance of 5 μ M floxuridine 3'-glutamic acid-CDCA is shown, as well as the appearance of released floxuridine. In Panel B, the disappearance of 5 μ M floxuridine 5'-glutamic acid-CDCA and subsequent release of floxuridine is shown. Finally, Panel C shows the degradation of 5 μ M floxuridine in rat liver s9 conditions. In each panel, each time point represents n = 3 samples.