Targeted delivery to PEPT1-overexpressing cells: Acidic, basic, and secondary floxuridine amino acid ester prodrugs

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Abstract

Floxuridine is a clinically proven anticancer agent in the treatment of metastatic colon carcinomas and hepatic metastases. However, prodrug strategies may be necessary to improve its physiochemical properties and selectivity and to reduce undesirable toxicity effects. Previous studies with amino acid ester prodrugs of nucleoside drugs targeted to the PEPT1 transporter coupled with recent findings of the functional expression of the PEPT1 oligopeptide transporter in pancreatic adenocarcinoma cell lines suggest the potential of PEPT1 as therapeutic targets for cancer treatment. In this report, we show the feasibility of achieving enhanced transport and selective antiproliferative action of amino acid ester prodrugs of floxuridine in cell systems overexpressing PEPT1. All prodrugs exhibited affinity for PEPT1 (IC_{50}, 1.1 – 2.3 mmol/L). However, only the prolyl and lysyl prodrugs exhibited enhanced uptake (2- to 8-fold) with HeLa/PEPT1 cells compared with HeLa cells, suggesting that the aspartyl prodrugs are PEPT1 inhibitors. The selective growth inhibition of Madine-Darby canine kidney (MDCK)/PEPT1 cells over MDCK cells by the prodrugs was consistent with the extent of their PEPT1-mediated transport. All ester prodrugs hydrolyzed to floxuridine fastest in Caco-2 cell and MDCK homogenates and slower in human plasma and were most chemically stable in pH 6.0 buffer. Prolyl and lysyl prodrugs were relatively less stable compared with aspartyl prodrugs in buffers and in cell homogenates. The results suggest that optimal design for targeted delivery would be possible by combining both stability and transport characteristics afforded by the promoiety. [Mol Cancer Ther 2005;4(4):659 – 67]

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Introduction

Floxuridine (5-fluoro-2′-deoxyuridine, FUdR) has been used for >40 years in the treatment of colon carcinoma and hepatic metastases. The mechanism of action and clinical pharmacology of floxuridine are well understood (1). Although it is a clinically effective drug, prodrugs of floxuridine have been synthesized to improve its physiochemical properties and to reduce toxicity. Thus, a variety of alkyl ester prodrugs (2, 3), phosphoramidate prodrugs (4), photoactivated prodrugs (5), and amino acid ester prodrugs (6) have been examined as potential alternatives to floxuridine.

Amino acid ester prodrugs of nucleoside agents have been shown to be substrates for the PEPT1 peptide transporter (7). PEPT1 has wide substrate specificity and transports dipeptides and tripeptides as well as many peptidomimetic drugs (7) and is an attractive transporter target for prodrug design. Amino acid prodrugs of the acyclic nucleoside analogues acyclovir and ganciclovir have been shown to be substrates of peptide transporters (8, 9). Although the ability of amino acid ester prodrugs of antiviral agents to inhibit uptake of dipeptide substrates by PEPT1 have been widely reported (9, 10), there are less data on uptake of these prodrugs by PEPT1. Thus, structure-transport relationship data for nucleoside amino acid ester substrates of PEPT1 are limited.

The peptide transporters have a relatively limited expression profile in healthy tissues. PEPT1 is predominantly expressed in the upper small intestine, whereas a related transporter PEPT2 is most abundant in the kidney (7). PEPT1 has also been described to be highly expressed in two human malignant ductal pancreatic cancer cell lines, AsPc-1 and Capan-2, although its expression in noncancerous pancreatic tissue is much lower (11). It has been reported that >90% of pancreatic cancers originate from the epithelial duct cells (ref. 12; http://www.cancer.gov/). In addition, PEPT1 was also found to be present in the human fibrosarcoma cell line HT-1080, although it was not found to be active in the normal fibroblast cell line IMR-90 (13). The authors showed in mouse models that the differential expression of PEPT1 in these cancer cell lines can be used for targeted and selective antiproliferative activity of anticancer agents, such as bestatin, which are known substrates of PEPT1 (14).

Although prodrug uptake via peptide transporters has been studied in some detail, the activation of the prodrug, also an essential step for prodrug activity, has not received much attention. Enzymatic stability involving acyl-floxuridine esters has been studied in a limited manner using tissue homogenates containing esterase enzymes (3, 15). Yet, the specific enzymes involved in the activation process of prodrugs have been rarely studied and have often been termed ubiquitous esterases and

proteases (16, 17). An esterase enzyme involved in the conversion of valacyclovir to its parent drug acyclovir has been identified and may be involved in the activation of other amino acid prodrugs (18). Because the enzymatic activity toward these prodrugs could be influenced by the structure of the promoiety and drug molecule, a variety of prodrugs need to be investigated. Recently, the influence of stereochemistry and site of esterification on the activation rate of nucleoside analogue prodrugs was reported (6, 19). Beyond these studies, there are very limited structure-activation studies evaluating the influence of the promoiety on the prodrug activation rate. Understanding how the promoiety structure influences the prodrug activation rate is important to design prodrugs with optimum stability and activation profiles.

In this report, we describe the synthesis and characterization of amino acid ester prodrugs of floxuridine synthesized using acidic (aspartic acid), basic (lysine), and imino (proline) amino acids designed for targeted delivery to the PEPT1 transporter. In addition to uptake studies in PEPT1-overexpressing cells, the chemical and enzymatic stability of these prodrugs were also evaluated to determine the effects of the amino acid promoiety structure and esterification site on enzyme-mediated activation. Finally, the feasibility of selective antiproliferative action of amino acid floxuridine prodrugs is shown using cancer cells that overexpress PEPT1.

Materials and Methods

Materials

Floxuridine was obtained from Lancaster (Windham, NH). The *tert*-butyloxycarbonyl (Boc) protected amino acids Boc-L-Lys, Boc-L-Pro, and Boc-L-Asp were obtained from Calbiochem-Novabiochem (San Diego, CA). High-performance liquid chromatography (HPLC) grade acetonitrile was obtained from Fisher Scientific (St. Louis, MO). *N*,*N*-dicyclohexylcarbodiimide, *N*,*N*-dimethylaminopyridine, trifluoroacetic acid (TFA), and all other reagents and solvents were purchased form Aldrich Chemical Co. (Milwaukee, WI). Valacyclovir was a gift from Glaxo-SmithKline, Inc. (Research Triangle Park, NC). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA) and cell culture supplies were obtained from Corning (Corning, NY) and Falcon (Lincoln Park, NJ). All chemicals were either analytic or HPLC grade.

FUdR Prodrug Synthesis

The synthesis and characterization of prolyl prodrugs of floxuridine has been reported previously (6). Lysyl and aspartyl prodrugs of floxuridine were synthesized in a similar manner (Fig. 1). Briefly, Boc-protected amino acids (Boc-L-Asp and Boc-L-Lys; 1 mmol), *N*,*N*-dicyclohexylcarbodiimide (1 mmol), and *N*,*N*-dimethylaminopyridine (0.1 mmol) were allowed to react with FUdR (1 mmol) in 7 mL of dry DMF for 24 hours. The reaction progress was monitored by TLC (ethyl acetate). The reaction mixture was filtered and DMF was removed under vacuum at 50°C to 55°C. The residue was extracted with ethyl acetate (30 mL) and washed with water (2 × 20 mL), saturated $NaHCO_3$ (2 × 20 mL), and saturated NaCl (20 mL). The organic layer was dried over $MgSO_4$ and concentrated under vacuum. The reaction yielded a mixture of 3′-monoester, 5′-monoester, and 3′,5′-diester FUdR prodrugs. The three spots observed on TLC were separated and purified using column chromatography (ethyl acetate/hexanes, 1:1–1:10). Fractions from each spot were concentrated under vacuum separately. The Boc group was cleaved by treating the residues with 4 mL TFA:DCM:H_2O (6:3:1). After 4 hours, the solvent was removed and the residues were reconstituted with water and lyophilized. The TFA salts of amino acid prodrugs of FUdR were obtained as white fluffy solids. The combined yield of FUdR prodrugs was ~60%.

HPLC was used to evaluate the prodrug purity. Prodrugs were between 90% and 99% pure. These prodrugs were easily separated from parent drug by HPLC. Electrospray ionization mass spectra (ESI-MS) were obtained on a Thermoquest LCQ ESI-MS. The observed molecular weights of all prodrugs were found to be consistent with that required by their structure. The structural identity of the prodrugs was then confirmed using proton nuclear magnetic resonance spectra (^{1}H NMR). ^{1}H NMR spectra were obtained on a 300 MHz Bruker DPX-300 NMR spectrometer.

3′-Aspartyl-FUdR: yield, 15%; percent purity, 90%; ^{1}H NMR (DMSO-d_6) δ: 2.33-2.35 (2H, m, C2′); 2.81-3.01 (2H, m, $^{\beta}CH_2$); 3.65-4.38 (4H, m, C4′, C5′, $^{\alpha}CH$); 5.24-5.32 (1H, m, C3′); 6.27 (1H, t, J = 6.8 Hz, C1′); 8.22 (1H, d, J = 6.9 Hz, CHF); ESI-MS, 362.3 $(M + H)^+$.

5′-Aspartyl-FUdR: yield, 15%; percent purity, 91%; ^{1}H NMR (DMSO-d_6) δ: 2.08-2.25 (2H, m, C2′); 2.81-2.89 (2H, m, $^{\beta}CH_2$); 3.92-4.42 (5H, m, C3′, C4′, C5′, $^{\alpha}CH$); 6.25 (1H, t, J = 6.5 Hz, C1′); 7.91 (1H, d, J = 6.9 Hz, CHF); ESI-MS, 362.3 $(M + H)^+$.

3′,5′-Aspartyl-FUdR: yield, 30%; percent purity, 96%; ^{1}H NMR (DMSO-d_6) δ: 2.35 (2H, m, C2′); 2.89-2.95 [4H, m, $(^{\beta}CH_2)_2$]; 4.22-4.42 [5H, m, C4′, C5′ $(^{\alpha}CH)_2$]; 5.57-5.59 (1H, m, C3′); 6.18-6.23 (1H, m, C1′); 7.96 (1H, d, J = 6.9 Hz, CHF); ESI-MS, 477.4 $(M + H)^+$.

3′-Lysyl-FUdR: yield, 15%; percent purity, 95%; ^{1}H NMR (DMSO-d_6) δ: 1.38-1.77 (6H, m, $^{\beta}CH_2$, $^{\gamma}CH_2$, $^{\delta}CH_2$); 2.28-2.33 (2H, m, C2′); 3.33-4.08 (4H, m, $^{\varepsilon}CH_2$ C5′); 4.26-4.56 (2H, m, $^{\alpha}CH$, C4′); 5.34-5.43 (1H, m, C3′); 6.20 (1H, t, J = 6.5 Hz, C1′), 8.22 (1H, d, J = 7.0 Hz, CHF); ESI-MS, 375.4 $(M + H)^+$.

5′-Lysyl-FUdR: yield, 15%; percent purity, 95%; ^{1}H NMR (DMSO-d_6) δ: 1.36-1.79 (6H, m, $^{\beta}CH_2$, $^{\gamma}CH_2$, $^{\delta}CH_2$); 2.07-2.24 (2H, m, C2′); 3.90-4.03 (4H, m, C3′, $^{\alpha}CH$, $^{\varepsilon}CH_2$); 4.2-4.26 (1H, m, C4′); 4.36-4.41 (1H, m, C5′); 6.14 (1H, t, J = 6.6 Hz, C1′), 7.92 (1H, d, J = 6.9 Hz, CHF); ESI-MS, 375.4 $(M + H)^+$.

3′,5′-Lysyl-FUdR: yield, 30%; percent purity, 90%; ^{1}H NMR (DMSO-d_6) δ: 1.21-2.01 [12H, m, $(^{\beta}CH_2)_2$, $(^{\gamma}CH_2)_2$, $(^{\delta}CH_2)_2$]; 2.01 (2H, m, C2′); 3.66-3.74 [4H, m, $(^{\varepsilon}CH_2)_2$]; 3.95-3.97 (2H, m, C5′); 4.28-4.44 (3H, m, $(^{\alpha}CH)_2$, C4′); 5.32 (1H, m, C3′); 6.20 (1H, t, J = 6.5 Hz, C1′); 7.87 (1H, d, J = 7.0 Hz, CHF); ESI-MS, 503.5 $(M + H)^+$.

Cell Culture

HeLa cells (passages 50–54), Madine-Darby canine kidney (MDCK) cells (passages 30–40), and Caco-2 cells (passages 30–35) from American Type Culture Collection (Rockville, MD) were routinely maintained in DMEM containing 10% fetal bovine serum, 1% nonessential amino acids, 1 mmol/L sodium pyruvate, and 1% L-glutamine. Cells were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C.

A PEPT1 stably expressing cell line was created using the canine kidney cell line MDCK. PEPT1 cDNA was subcloned into the pcDNA3.0 selection/expression vector (Invitrogen) between *Xho*I and *Xba*I sites in the multicloning region. The construct pcDNA3.0/PEPT1 was generated and used to create a stably expressing MDCK line. The PEPT1 construct was transfected into MDCK cells via FuGene reagent (Roche, Indianapolis, IN) and stably expressing clones were selected using the antibiotic G418 (Life Technologies, Grand Island, NY). Positive clones were screened for functional uptake ([^{3}H]Gly-Sar) and reverse transcription-PCR determinations of PEPT1 expression were conducted as described previously (20). MDCK cells with vector control only, MDCK/pcDNA3.0, were created in a similar fashion.

Hydrolysis Studies

Enzymatic Stability. Confluent Caco-2 cells were washed with PBS (pH 7.4) and then harvested with 0.05% trypsin-EDTA at 37°C for 5 to 10 minutes. Trypsin was neutralized by adding DMEM. The cells were washed off the plate and spun down by centrifugation. The pelleted cells were washed twice with pH 7.4 phosphate buffer (10 mmol/L) and resuspended in pH 7.4 phosphate buffer (10 mmol/L) to obtain a final concentration of 4.7×10^6 cells/mL. The cells were then lysed with 1 volume of 0.5% Triton X-100 solution. The cells were then homogenized by vigorous pipetting and total protein was quantified with the Bio-Rad (Hercules, CA) DC Protein Assay using bovine serum albumin as a standard. The hydrolysis reactions were carried out in 96-well plates (Corning). Caco-2 cell suspensions (230 μL) were placed in triplicate wells, the reactions were started with the addition of substrate, and cells were incubated at 37°C for 30 to 60 minutes. At each time point, aliquot sample (40 μL) was removed and added to 2 volumes of 10% ice-cold TFA. The mixtures were centrifuged for 10 minutes at 1,800 rcf and 4°C. The supernatant was then filtered with a 0.45 μm filter and analyzed via reverse-phase HPLC.

Stability in Human Plasma. The stability of the prodrugs in human plasma was determined using the procedure below. Undiluted plasma (230 μL) was added to each well in triplicate and substrate (40 μL) was added to initiate the reactions that were conducted at 37°C for up to 3 hours. At various time points, aliquots (40 μL) were removed and added to 2 volumes of 10% TFA. The mixtures were centrifuged for 10 minutes at 1,800 rcf and 4°C. The supernatant was then filtered with a 0.45 μm filter and analyzed via reverse-phase HPLC.

Chemical Stability. The degradation profiles of the prodrugs were determined in pH 7.4 phosphate buffer (10 mmol/L) and pH 6.0 MES (10 mmol/L) buffer alone to obtain the contribution of nonenzymatic hydrolysis. The experiments were carried out in triplicate as described above, except that each well contained buffer instead of cell homogenate.

Hydrolysis Data Analysis. The initial rates of hydrolysis were used to obtain the apparent first-order rate constants and estimate the half-lives. The apparent first-order degradation rate constants of various floxuridine prodrugs at 37°C were determined by plotting the logarithm of prodrug remaining as a function of time. The slopes of these plots are related to the rate constant, k, and given by

$$k = 2.303 \times \text{slope}(\log C \text{ vs. time}) \quad (1)$$

The degradation half-lives were then estimated by the equation,

$$t_{1/2} = 0.693/k \quad (2)$$

Statistical significance was evaluated with GraphPad Prism version 3.0 by performing one-way ANOVA with *post hoc* Tukey's test to compare means.

[^{3}H]Gly-Sar Uptake Inhibition

HeLa cells were infected with adenovirus containing PEPT1 as described previously (21). Two days after viral infection, the HeLa/PEPT1 cells were incubated with 10 μmol/L Gly-Sar (9.98 μmol/L Gly-Sar and 0.02 μmol/L [^{3}H]Gly-Sar) along with various prodrug concentrations (5–0.01 mmol/L) for 30 minutes. The cells were washed thrice with ice-cold PBS and solubilized with 0.1% Triton X-100/0.1 N NaOH. The suspension was then counted by scintillation counting (Beckman LS-9000, Beckman Instruments, Fullerton, CA). IC_{50} values were determined using nonlinear data fitting (GraphPad Prism version 3.0).

Uptake Studies

Carrier-mediated prodrugs transport was screened in HeLa/PEPT1 cells as described previously (22). Briefly, 2 days postinfection, the cells were incubated with 0.5 mL freshly prepared drug solution (1 mmol/L) in uptake buffer. After 45 minutes, cells were washed with ice-cold uptake buffer and water (0.3 mL) containing 0.1% SDS was added in to each well. The protein was precipitated from the cell lysates by 7% TFA (final concentration) and pelleted by centrifugation. Filtered supernatant was analyzed by HPLC to determine prodrug/drug concentrations. Control experiments were done in normal HeLa cells.

Gly-Sar uptake studies were conducted with MDCK/PEPT1 and MDCK/pcDNA3.0 cells. MDCK cells were incubated with freshly prepared 10 μmol/L Gly-Sar (9.98 μmol/L Gly-Sar and 0.02 μmol/L [^{3}H]Gly-Sar) in pH 6.0 uptake buffer. After 20 minutes, the cells were washed thrice with ice-cold PBS and solubilized with 0.1% Triton X-100/0.1 N NaOH. The suspension was then counted by scintillation counting (Beckman LS-9000).

HPLC Analysis

The concentrations of prodrugs and their parent drugs were determined on a Waters HPLC system (Waters, Inc., Milford, MA). The HPLC system consisted of two Waters pumps (model 515), a Waters autosampler (WISP model 712), and a Waters UV detector (996 photodiode array detector). The system was controlled by Waters Millennium 32 software (version 3.0.1). Samples were injected onto a Waters Xterra C_{18} reverse-phase column (5 μm, 4.6 × 250 mm) equipped with a guard column. The compounds were eluted using gradient and isocratic methods. The prolyl prodrug and valacyclovir HPLC analysis was done as described previously (6). The Asp and Lys monoesters were run with isocratic TFA/water mobile phase for 12 minutes. The diesters were analyzed using 1% HFBA/water (solvent A) and 1% HFBA/acetonitrile (solvent B), with the solvent B gradient changing 4% to 56% at a rate of 2%/min during a 30-minute run. Standard curves generated for each prodrug and their parent drugs were used for quantitation of integrated area under peaks.

Cell Proliferation Assays

Cell proliferation studies were conducted with PEPT1 stably overexpressing MDCK cell lines and compared with MDCK/pcDNA3.0 control cells. The cells were seeded into 96-well plates at 4,000 cells per well and allowed to attach/grow for 12 hours before drug solutions were added. The culture medium (DMEM + 10% fetal bovine serum) was removed and the cells were gently washed once with sterile pH 6.0 uptake buffer. Floxuridine and floxuridine prodrugs were diluted in pH 6.0 uptake buffer to 500 to 0.78 μmol/L using no drug as a 100% viability control. The wash buffer was removed and 50 μL drug solution per well were added and incubated at 37°C for 3 hours in the cell incubator. After this time, the drug solutions were removed and the cells were again gently washed twice with sterile uptake buffer. Phenol-free DMEM (100 μL; 5% fetal bovine serum) were then added to each well after washing. The cells were allowed to recover for 30 hours before evaluating cell viability via 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt assays. A mixture (50 μL) containing 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt in sterile DMEM without phenol red (1 mg/mL) and phenazine methosulfate (*N*-methyl dibenzopyrazine methyl sulfate in sterile PBS, 0.383 mg/mL) reagents were added to the cells and incubated at 37°C for the color to develop. Several absorbance readings at 490 nm were recorded every 30 minutes for 3 hours. The data were plotted as percent viability and GI_{50} values were calculated using GraphPad Prism version 3.0 by nonlinear data fitting.

Results

Floxuridine Prodrug Synthesis

Amino acid ester prodrugs of floxuridine were synthesized as described in Fig. 1. The TFA salts of the 3′-monoester (***5a–5c***), 5′-monoester (***6a–6c***), and 3′,5′-diester (***7a–7c***) floxuridine prodrugs were obtained as white fluffy powders. The total prodrug yield for each amino acid was >60% and the purity for all prodrugs was ≥90% as determined by HPLC. The impurities were the other isomers and/or the parent drug. The prodrug identities were confirmed by ESI-MS and ^{1}H NMR. The purity and mass data are shown in Table 1.

MDCK/PEPT1 Cell Line Characterization

The mRNA PEPT1 expression was evaluated by reverse transcription-PCR in both MDCK and MDCK/PEPT1 cells. The vector-only control cell line did not express PEPT1 mRNA, whereas the stably expressing PEPT1 cells exhibited higher mRNA levels. This expression difference was confirmed by functional [^{3}H]Gly-Sar uptake, which indicated that MDCK/PEPT1 cells transported over 10 times more [^{3}H]Gly-Sar than the parent cell line (Fig. 2).

Stability Studies

The half-lives of degradation of prodrugs, $t_{1/2}$, determined in pH 6.0 phosphate buffer and pH 7.4 Tris buffer at 37°C are shown in Table 2. The mass balance for prodrug disappearance and parent drug appearance was excellent under our HPLC analysis conditions (100 ± 2%). The reference prodrug, valacyclovir, was stable ($t_{1/2}$ = 1,029.1 ± 11.2 minutes) at near neutral buffer conditions and even more stable under acidic conditions ($t_{1/2}$ > 2,880.0 minutes). Valacyclovir ester chemical stability was greater than all the floxuridine prodrugs tested. The order of stability of the floxuridine prodrugs at both pH 6.0 and 7.4 was aspartyl > lysyl ⋙ prolyl.

Enzymatic ester bond stability was evaluated in Caco-2 and MDCK cell homogenates as well as in human plasma at 37°C (Table 2). In Caco-2 homogenates, the aspartyl prodrugs were considerably more stable than either lysyl or prolyl prodrugs. Valacyclovir was enzymatically more stable than the lysyl and prolyl prodrugs but *more* labile than aspartyl drugs. In comparison with hydrolysis in Caco-2 homogenates, the prolyl and lysyl prodrugs as well as valacyclovir were more stable in MDCK homogenates. On average, the aspartyl prodrugs were 5- to 52-fold less stable in the MDCK homogenates compared with Caco-2. The floxuridine prodrugs esters were severalfold less stable compared with valacyclovir in human plasma. Overall, the prodrugs were significantly more stable in human plasma compared with their stability in Caco-2 homogenates (Table 2).

Uptake Inhibition Studies

IC_{50} values of the amino acid ester prodrugs of floxuridine for PEPT1 determined using inhibition of Gly-Sar uptake in HeLa/PEPT1 cells are summarized in Table 3. All amino acid ester prodrugs displayed increased affinity for PEPT1 compared with the parent drug floxuridine, which exhibited no apparent affinity in the concentration range tested. IC_{50} values for the floxuridine prodrugs were similar to those obtained with valacyclovir (IC_{50}, 1.46 ± 0.36 mmol/L). Aspartyl prodrugs exhibited the highest overall average affinity (IC_{50}, 1.16 ± 0.09 mmol/L),

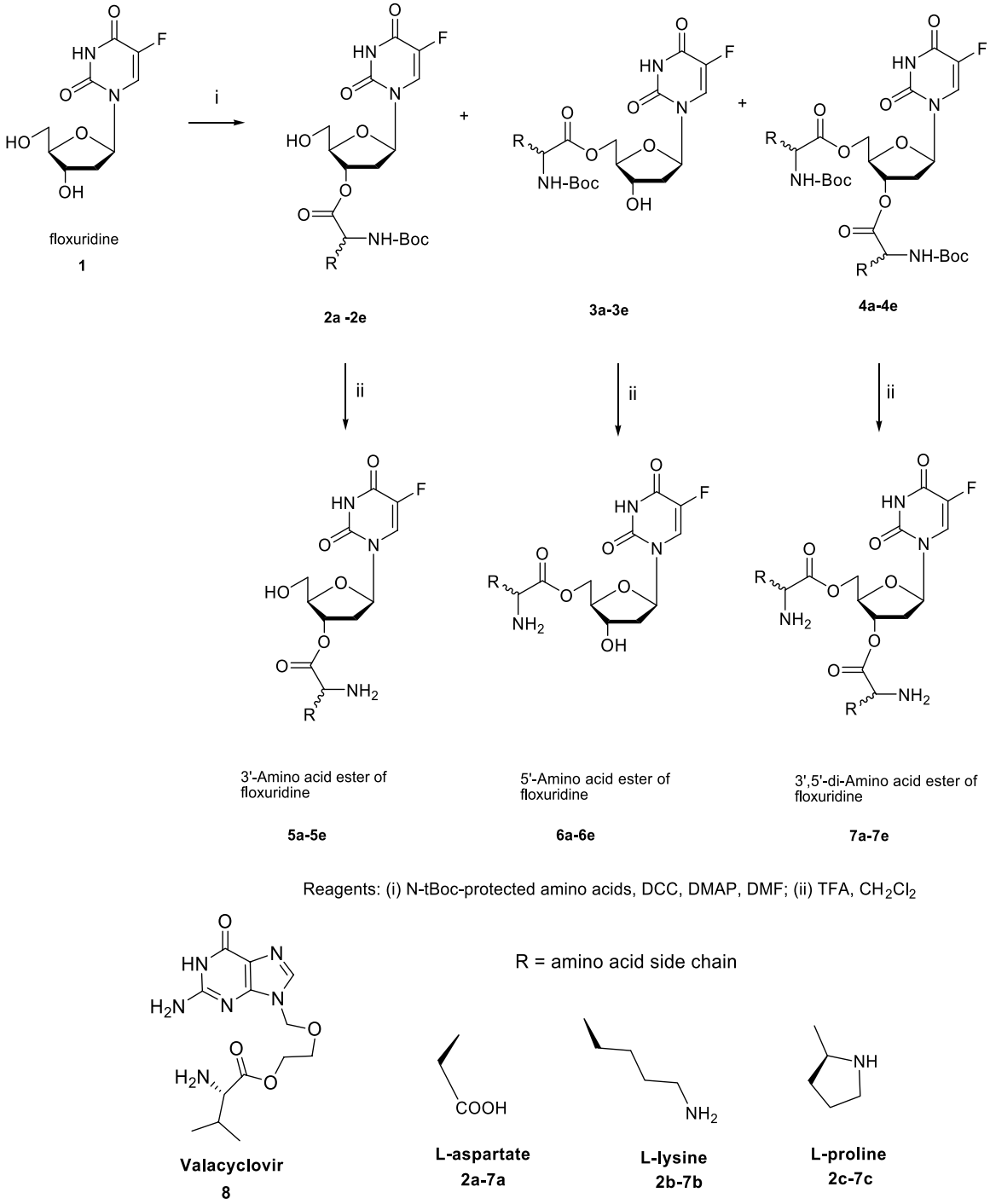

Figure 1. Synthesis of amino acid ester prodrugs of floxuridine.

whereas the lysyl prodrugs exhibited lowest overall average affinity (IC_{50}, 1.97 ± 0.41 mmol/L). The 5′ aspartyl and lysyl esters were found to exhibit the highest affinity for PEPT1, whereas the 3′ esters displayed the lowest affinity. Among the prolyl prodrugs, the diester exhibited the highest PEPT1 affinity (IC_{50}, 1.26 ± 0.17 mmol/L).

Direct Uptake Studies

Enhanced PEPT1-mediated uptake in HeLa/PEPT1 cells was observed with prolyl and lysyl floxuridine prodrugs and with valacyclovir compared with that in control HeLa cells but not with the aspartyl prodrugs or floxuridine itself (Table 3). The enhancement in uptake ranged from 1.9- to

Table 1. Analytic data for amino acid ester prodrugs of floxuridine

Prodrugs	% Purity (HPLC)	ESI-MS (M + H)$^+$		Molecular weight (TFA salt)	*C* log *P**
		Required	Observed		
3′,5′-Di-*O*-prolyl (**7c**)	94	441.5	441.1	668.5	−0.09
3′-*O*-prolyl (**5c**)	99	344.3	344.0	457.3	−1.07
5′-*O*-prolyl (**6c**)	98	344.3	344.1	457.3	−1.07
3′,5′-Di-*O*-aspartyl (**7a**)	96	477.4	477.4	704.4	−3.36
3′-*O*-aspartyl (**5a**)	91	362.3	362.3	475.3	−2.71
5′-*O*-aspartyl (**6a**)	90	362.3	362.3	475.3	−2.71
3′,5′-Di-*O*-lysyl (**7b**)	90	503.6	503.5	958.6	−2.02
3′-*O*-lysyl (**5b**)	95	375.4	375.4	602.4	−2.04
5′-*O*-lysyl (**6b**)	92	375.4	375.4	602.4	−2.04

*Calculated using Chem Draw 7.0.

8.2-fold for the prolyl and lysyl prodrugs. Valacyclovir was transported 4.7-fold higher in HeLa/PEPT1 compared with that in HeLa cells and is consistent with previous findings (22). For both amino acids, the diester prodrugs exhibited lower PEPT1 carrier-mediated transport compared with the monoesters.

Cell Growth Inhibition

GI_{50} values for floxuridine and its amino acid ester prodrugs determined in cell proliferation studies with MDCK/PEPT1 cells and control MDCK/pcDNA3.0 cells are shown in Table 4. The results indicate that the antiproliferative activity of prolyl and lysyl prodrugs was enhanced up to 8-fold in MDCK/PEPT1 cells compared with controls. Little or no enhancement in antiproliferative activity in MDCK/PEPT1 cells compared with controls was evident with aspartyl prodrugs or the parent floxuridine. The parent drug was 4- to 17-fold more potent in MDCK/pcDNA3.0 cells than the prodrugs. GI_{50} values for the 3′-L-prolyl and 5′-L-lysyl floxuridine prodrugs were ~2-fold higher than that for floxuridine in MDCK/PEPT1; however, these two prodrugs displayed the highest growth inhibition enhancement, 7.0- and 7.8-fold, respectively, compared with GI_{50} values in MDCK/pcDNA3.0 cells. The 3′,5′-prolyl diester was as potent as the parent floxuridine in MDCK/PEPT1 cells. Although aspartyl prodrugs did not exhibit preferential antiproliferative activity in MDCK/PEPT1 cells, they were still effective in both cell lines with GI_{50} values in the range of 20 to 50 μmol/L (Table 4).

Discussion

Prodrug strategies are generally adopted to improve the undesirable properties of therapeutic drugs to overcome barriers, such as poor oral absorption, chemical instability, and toxicity. The design of amino acid ester prodrugs offers a high degree of flexibility, because there are a large variety of amino acids available for optimization of prodrug characteristics. This flexibility allows modulation of stability characteristics as well as the mode of cellular uptake of these prodrugs. A good prodrug should be chemically stable but must be enzymatically converted to the active parent drug once it enters the cell. The optimal chemical stability and activation characteristics depend on the route of administration and the cellular target.

Of the three amino acids, the positively charged lysine, the negatively charged aspartic acid, and the secondary amino acid proline examined as promoieties, the prolyl prodrugs of floxuridine were found to be the least stable in buffers, exhibiting half-lives in the range of 10 to 20 minutes. The presence of a highly ionized α-amine group in proline (pK_a ~ 10.61) in the acyl portion of the esters can increase the hydrolysis of ester bonds (23). These results suggest that the prolyl prodrugs are unsuitable candidates for further development. The aspartyl prodrugs were more chemically stable potentially due to the negatively charged side chain that may hinder hydroxide ion catalysis of the ester bond. The greater stability of the prodrugs at pH 6.0 can be attributed to a lower hydroxide ion concentration and is consistent with previous findings

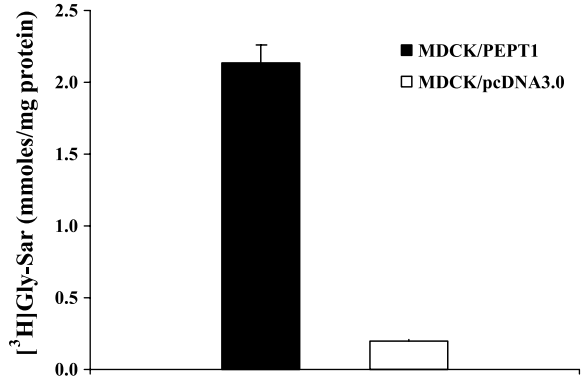

Figure 2. [^{3}H]Gly-Sar uptake in MDCK/PEPT1 and MDCK/pcDNA3.0 cells (n = 3). MDCK cells were incubated with freshly prepared 10 μmol/L Gly-Sar (9.98 μmol/L Gly-Sar and 0.02 μmol/L [^{3}H]Gly-Sar) in pH 6.0 uptake buffer. After 20 min, the cells were washed thrice with ice-cold PBS and solubilized with 0.1% Triton X-100/0.1 N NaOH. The suspension was then assayed by scintillation counting. *Columns,* mean; *bars,* SD.

Table 2. Floxuridine prodrugs and valacyclovir chemical and enzymatic stability (mean ± SD, *n* = 3)

Prodrugs	pH 6.0 $t_{1/2}$ (min)	pH 7.4 $t_{1/2}$ (min)	Plasma $t_{1/2}$ (min)	Caco-2 $t_{1/2}$ (min)	MDCK $t_{1/2}$ (min)
3′,5′-Di-*O*-prolyl (**7c**)	55.4 ± 4.8	9.5 ± 0.4	6.0 ± 0.4	0.9 ± 0.0	5.8 ± 0.1
3′-*O*-prolyl (**5c**)	150.9 ± 2.1	16.7 ± 1.4	13.8 ± 1.6	3.7 ± 0.1	11.7 ± 0.5
5′-*O*-prolyl (**6c**)	166.1 ± 5.7	19.2 ± 1.2	5.7 ± 0.2	1.1 ± 0.0	11.2 ± 0.2
3′,5′-Di-*O*-aspartyl (**7a**)	353.4 ± 21.2	75.0 ± 3.1	65.0 ± 6.3	42.6 ± 0.3	36.0 ± 1.9
3′-*O*-aspartyl (**5a**)	512.0 ± 33.3	148.9 ± 6.6	53.9 ± 4.3	44.1 ± 6.6	38.6 ± 3.7
5′-*O*-aspartyl (**6a**)	302.9 ± 11.8	141.6 ± 3.9	38.6 ± 2.9	42.1 ± 0.5	30.2 ± 2.5
3′,5′ -Di-*O*-lysyl (**7b**)	122.7 ± 2.1	52.1 ± 2.3	29.5 ± 3.8	4.6 ± 0.2	21.4 ± 0.4
3′-*O*-lysyl (**5b**)	137.3 ± 9.4	97.1 ± 5.5	51.0 ± 1.8	4.3 ± 0.2	22.4 ± 0.5
5′-*O*-lysyl (**6b**)	133.8 ± 10.2	95.0 ± 3.2	38.8 ± 2.0	5.6 ± 0.0	12.9 ± 0.7
Valacyclovir (**8**)	5,700*	1,029.1 ± 11.2	312.0 ± 24.6	10.3 ± 0.3	42.2 ± 0.3

*From ref. 25.

with acyl floxuridine esters (24) and with valacyclovir (25, 26). These results also suggest that degradation of the prodrugs is not substantial (<20% for monoesters) over the time course of the uptake experiments conducted at pH 6.0 to provide the proton gradient necessary for PEPT1-mediated uptake. The greater stability in slightly acidic conditions may also be advantageous for antitumor activity considering that the extracellular pH in tumors has been reported to be acidic (27, 28). The amino acid promoiety influenced the chemical stability of the prodrug in a profound manner, whereas the esterification site seems to be quite nominal in determining the ester bond chemical stability. Thus, the stability of 3′-monoesters was comparable with that of 5′-monoesters and is consistent with previous observations (6, 24). However, the diester prodrugs were consistently less stable than either monoester. These results are again similar to that observed previously with floxuridine prodrugs (24).

The hydrolysis of the prodrugs was more rapid in both Caco-2 and MDCK cell homogenates compared with that in pH 7.4 buffers. Significant ester prodrug converting activity has been reported with Caco-2 cells (6, 29). Individual prodrug-converting enzymes expressed in Caco-2 cells, such as biphenyl hydrolase-like protein, have been reported to hydrolyze the amino acid ester prodrugs valacyclovir and valganciclovir (18). In Caco-2 cell homogenates, the prolyl prodrugs were most rapidly converted to floxuridine potentially due to hydrolysis by biphenyl hydrolase-like protein or by other hydrolase enzymes (19). Lysyl prodrugs were also rapidly hydrolyzed; however, because they are not good substrates for biphenyl hydrolase-like protein, other enzymes may be responsible (19). A free lysyl α-amino group could render these esters more prone to potential aminopeptidase activity as observed with other lysyl prodrugs (30). Biphenyl hydrolase-like protein seems to prefer hydrophobic amino acids and is less active against lysyl and aspartyl ester prodrugs (19). Aspartyl esters were significantly more stable in Caco-2 homogenates than lysyl and prolyl ester prodrugs, consistent with previous enzymatic stability data for Asp-acyclovir prodrugs (31). Because their stability in cell homogenates and plasma is lower than that in pH 7.4 buffer, it is likely that enzymes, such as aspartyl aminopeptidases, in the homogenates or plasma may hydrolyze them at a relatively slow rate (32). Degradation half-lives of 30 minutes or higher in plasma suggest a greater probability of the prodrug being intact for targeted action (Table 2).

The esterification site also seems to have an effect on the enzymatic stability of the prodrugs. An example of this effect is evident with the 3′-prolyl and 5′-prolyl monoesters

Table 3. Uptake and [^{3}H]Gly-Sar uptake inhibition in HeLa and HeLa/PEPT1 cells (mean ± SD, *n* = 3)

Prodrugs	Uptake inhibition IC_{50} (mmol/L)	Uptake HeLa/PEPT1 (nmol/mg/45 min)	Uptake HeLa control (nmol/mg/45 min)	PEPT1/control
3′,5′-Di-*O*-prolyl (**7c**)	1.26 ± 0.17	1.66 ± 0.07	0.88 ± 0.05	1.9 ± 0.0
3′-*O*-prolyl (**5c**)	1.99 ± 0.14	2.40 ± 0.09	0.82 ± 0.04	2.9 ± 0.1
5′-*O*-prolyl (**6c**)	1.71 ± 0.07	3.71 ± 0.03	0.49 ± 0.03	7.5 ± 0.6
3′,5′-Di-*O*-aspartyl (**7a**)	1.13 ± 0.05	<0.02	<0.02	1.0
3′-*O*-aspartyl (**5a**)	1.27 ± 0.01	<0.02	<0.02	1.0
5′-*O*-aspartyl (**6a**)	1.09 ± 0.01	<0.02	<0.02	1.0
3′,5′-Di-*O*-lysyl (**7b**)	1.93 ± 0.14	1.80 ± 0.13	0.94 ± 0.03	1.9 ± 0.1
3′-*O*-lysyl (**5b**)	2.40 ± 0.06	1.10 ± 0.12	0.13 ± 0.01	8.2 ± 0.4
5′-*O*-lysyl (**6b**)	1.59 ± 0.03	1.48 ± 0.15	0.27 ± 0.02	5.5 ± 0.2
Valacyclovir (**8**)	1.46 ± 0.36	2.83 ± 0.30	0.60 ± 0.05	4.7 ± 0.2

Table 4. Cell growth inhibition in MDCK/PEPT1 and MDCK/pcDNA3.0 cells (mean ± SD, *n* = 3)

Prodrugs	GI_{50} values (μmol/L)		Selectivity enhancement
	MDCK/PEPT1	MDCK/pcDNA3.0	
3′,5′-Di-*O*-prolyl (**7c**)	5.4 ± 2.6	18.1 ± 5.3	3.4
3′-*O*-prolyl (**5c**)	11.1 ± 3.6	77.5 ± 6.2	7.0
5′-*O*-prolyl (**6c**)	31.1 ± 4.6	66.4 ± 4.4	2.1
3′,5′-Di-*O*-aspartyl (**7a**)	22.4 ± 4.0	39.8 ± 4.4	1.8
3′-*O*-aspartyl (**5a**)	28.8 ± 5.4	34.4 ± 5.6	1.2
5′-*O*-aspartyl (**6a**)	48.9 ± 2.2	52.9 ± 2.9	1.1
3′,5′-Di-*O*-lysyl (**7b**)	10.6 ± 2.5	33.8 ± 1.9	3.2
3′-*O*-lysyl (**5b**)	9.0 ± 3.1	20.8 ± 2.2	2.3
5′-*O*-lysyl (**6b**)	10.8 ± 6.3	84.2 ± 6.0	7.8
Floxuridine (**1**)	5.1 ± 0.1	5.1 ± 0.1	1.0

in Caco-2 cell homogenates (Table 2). The 5′ ester (**6c**) was significantly less stable compared with the 3′-prolyl ester (***5c***). The 5′ hydroxyl group is a primary alcohol group, whereas the 3′ hydroxyl is a secondary alcohol group that is in close proximity to the floxuridine sugar moiety. Serine hydrolase enzymes catalyze reactions through a classic serine hydrolase mechanism. Therefore, the interaction between the active site His and the substrate O_{ester} may be hindered by a bulky group close to leaving group O_{ester} (33). This hindrance of serine hydrolase enzymes, such as biphenyl hydrolase-like protein, may explain the increased selectivity for the 5′-prolyl ester over the 3′ ester. This selectivity preference for the 5′ ester was not observed with the lysyl and aspartyl prodrugs in Caco-2 cell homogenates, suggesting that a different class of enzymes may be acting on them.

Although all prodrugs exhibited affinity for the PEPT1 transporter comparable with that of valacyclovir, PEPT1-mediated transport was evident only with lysyl and prolyl prodrugs. Further, the lysyl monoesters and the 5′-prolyl prodrugs also exhibit transport similar to that of valacyclovir, suggesting that these prodrugs may exhibit enhanced cellular permeability *in vivo*. Aspartyl prodrugs did not exhibit PEPT1-mediated transport that is consistent with previous reports (31, 34). The lack of a correlation between direct PEPT1 uptake data with the corresponding Gly-Sar uptake inhibition data for the prodrugs suggests that competition studies need to be validated with other supportive evidence. Potential PEPT1 substrates can be evaluated with direct uptake studies or proton current detection methods, such as oocyte patch clamping or dye-based methods (34).

The feasibility of tumor-selective delivery of these amino acid ester prodrugs of floxuridine to cancer cells that overexpress PEPT1 was examined in an *in vitro* model developed in our laboratory. The results of cell proliferation studies suggest that prolyl and lysyl floxuridine prodrugs exhibited enhanced antiproliferative activity in MDCK/PEPT1 cells compared with MDCK control cells. The enhancement in antiproliferative action of these prodrugs in MDCK/PEPT1 cells is consistent with their enhanced uptake via PEPT1-mediated transport. Such selective antiproliferative activity was not observed with either the parent floxuridine or the aspartyl prodrug, a nonsubstrate of the PEPT1 transporter.

The success of this selective delivery approach would depend critically on the PEPT1 expression in tumor cells and tissues. The PEPT1 mRNA expression in the MDCK/PEPT1 cells was 3- to 6-fold lower compared with that in colon adenocarcinoma Caco-2 cells and the pancreatic cancer lines Capan-2 and Aspc-1. Functionally, these cancer cell lines, on average, accumulated 5-fold more dipeptide (Gly-Sar) compared with the MDCK/PEPT1 cells (data not shown). This indicates that PEPT1 uptake activity in our model system may underestimate the potential *in vivo* activity obtainable with pancreatic duct and colon carcinoma tumor cell lines. Previous studies with *in vivo* mouse xenograft models showed the accumulation of the dipeptide anticancer drug, bestatin, in tumor cells overexpressing PEPT1 that resulted in suppressed tumor growth (13). Therefore, our prodrug approach may potentially provide *in vivo* tumor accumulation of floxuridine that far exceeds that of the surrounding normal tissue. However, currently, there is limited information regarding expression of PEPT1 in tumor tissues. Identification of tumor tissues that express PEPT1 will be necessary to reveal its full potential in tumor targeting.

In conclusion, the structure and/or esterification site of the amino acid promoiety affect prodrug stability and transport in a marked manner. The wide range of chemical and enzymatic stability characteristics observed with the prodrugs suggests that the hydrolysis rate could be tailored to produce a prodrug with the desired half-life. A carefully selected amino acid can also make a nucleoside analogue, such as floxuridine, into a carrier-mediated substrate. Peptide transporter-mediated uptake of these prodrugs could potentially increase intestinal absorption if delivered orally or be used to target specific cancer cell types expressing these transporters. Further studies with more structurally diverse amino acids and

nucleoside analogues could eventually lead to the development of a structure-activity and structure-transport relationship database that could facilitate optimal prodrug design.

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