Research Paper

Floxuridine Amino Acid Ester Prodrugs: Enhancing Caco-2 Permeability and Resistance to Glycosidic Bond Metabolism

Christopher P. Landowski,[1] Xueqin Song,[1] Philip L. Lorenzi,[1] John M. Hilfinger,[2] and Gordon L. Amidon[1,3]

Purpose. The aim of this study was to synthesize amino acid ester prodrugs of 5-fluoro-2′-deoxyuridine (floxuridine) to enhance intestinal absorption and resistance to glycosidic bond metabolism.

Methods. Amino acid ester prodrugs were synthesized and examined for their hydrolytic stability in human plasma, in Caco-2 cell homogenates, and in the presence of thymidine phosphorylase. Glycyl-L-sarcosine uptake inhibition and direct uptake studies with HeLa/PEPT1 cells [HeLa cells overexpressing oligopeptide transporter (PEPT1)] were conducted to determine PEPT1-mediated transport and compared with permeability of the prodrugs across Caco-2 monolayers.

Results. Isoleucyl prodrugs exhibited the highest chemical and enzymatic stability. The prodrugs enhanced the stability of the glycosidic bond of floxuridine. Thymidine phosphorylase rapidly cleaved floxuridine to 5-fluorouracil, whereas with the prodrugs no detectable glycosidic bond cleavage was observed. The 5′-L-isoleucyl and 5′-L-valyl monoester prodrugs exhibited 8- and 19-fold PEPT1-mediated uptake enhancement in HeLa/PEPT1 cells, respectively. Uptake enhancement in HeLa/PEPT1 cells correlated highly with Caco-2 permeability for all prodrugs tested. Caco-2 permeability of 5′-L-isoleucyl and 5′-L-valyl prodrugs was 8- to 11-fold greater compared with floxuridine.

Conclusions. Amino acid ester prodrugs such as isoleucyl floxuridine that exhibit enhanced Caco-2 transport and slower rate of enzymatic activation to parent, and that are highly resistant to metabolism by thymidine phosphorylase may improve oral delivery and therapeutic index of floxuridine.

KEY WORDS: Caco-2 permeability; floxuridine prodrugs; metabolism; PEPT1; thymidine phosphorylase.

INTRODUCTION

Effective cancer chemotherapy depends on control of drug levels at the tumor site. Many efforts have been made to improve the delivery and therapeutic index of anticancer drugs by chemically modifying the parent compound. Prodrugs of clinically effective cancer drugs such as 5-fluoro-2′-deoxyuridine (floxuridine) have been synthesized to improve its physiochemical properties and to reduce toxicity. Thus, a variety of alkyl ester prodrugs (1,2), phosphoramidate prodrugs (3), photoactivated prodrugs (4), and amino acid ester prodrugs (5,6) have been examined as potential improvements. The objective of such approaches is not only to enhance oral delivery but also to improve the metabolic disposition of floxuridine after systemic delivery. *In vitro* studies have demonstrated that the antiproliferative activity

of floxuridine was up to 5,000-fold greater than its metabolite, 5-fluorouracil (5-FU), in several cancer cell lines (7). Thus, the rapid phosphorolytic cleavage of floxuridine to 5-FU *in vitro* and *in vivo* (8,9) represents a major obstacle in delivering intact floxuridine to cells for enhanced cytotoxic action. In addition, the degradation of floxuridine in rat intestinal homogenates (1) and in humans after oral administration (10–12) clearly suggest that floxuridine administered orally would scarcely be absorbed as intact floxuridine.

A variety of chemical modifications have been shown to render floxuridine more resistant to cleavage by pyrimidine nucleoside phosphorylases (8). Aryl or long-chain alkyl diesters of floxuridine were found to exhibit 100-fold higher antitumor activity against L1210 leukemia cells after i.p. administration to mice compared with floxuridine (13). It was also clear from the studies in mice and rats that enhanced resistance to enzymatic ester hydrolysis and to metabolism of floxuridine was related to improved antitumor activity of the prodrug. Thus, the maintenance of floxuridine plasma levels after dosing of 3′,5′-dibenzoyl and 3′,5′-dipalmitoyl floxuridine suggested that protection from glycosidic bond destabilization coupled with the slow release of intact floxuridine from the prodrug leads to higher and more persistent plasma levels compared to direct administration of floxuridine (1,13). The release of active compound and its subsequent delivery

[1] Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48109-1065, USA.

[2] TSRL, Inc., 540 Avis Drive, Suite A, Ann Arbor, Michigan 48108, USA.

[3] To whom correspondence should be addressed. (e-mail: glamidon@umich.edu)

ABBREVIATIONS: HeLa/PEPT1, HeLa cells overexpressing PEPT1; PEPT1, oligopeptide transporter.

to the target site can therefore be controlled by controlling the rate of promoiety metabolism.

In addition to its conversion to a less active metabolite, floxuridine exhibits low and erratic oral bioavailability and is usually administered *via* intravenous infusion. Prodrug strategies have also been used to improve oral absorption of nucleoside analogs. Beauchamp *et al.* determined that some amino acid esters of acyclovir, an antiviral drug with a low oral bioavailability approximately 15% in humans (14), were more effective for oral use (15). Thus, the oral bioavailability of the L-valyl and L-isoleucyl esters of acyclovir in rats were 63 and 43%, respectively. Valacyclovir and valganciclovir, two commercially marketed prodrugs of acyclovir and ganciclovir, respectively, have been shown to be substrates of oligopeptide transporters such as PEPT1 (16,17). PEPT1 has a wide substrate specificity and transports di- and tripeptides as well as many peptidomemetic drugs (18) and thus is an attractive transporter target for prodrug design.

Thus, prodrug approaches that incorporate strategies to facilitate enhanced intestinal absorption and that minimize floxuridine metabolism by thymidine phosphorylase to 5-FU may allow enhanced floxuridine bioavailability and therapeutic action. In this report, we describe the synthesis and characterization of isoleucyl and leucyl ester prodrugs of floxuridine designed for targeted delivery to the PEPT1 transporter. In addition to uptake and permeability studies, 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 their activation to the parent drug and on parent drug metabolism.

MATERIALS AND METHODS

Materials

Floxuridine (FUdR) was obtained from Lancaster (Windham, NH, USA). The *tert*-butyloxycarbonyl (Boc) protected amino acids, Boc-L-Ile and Boc-L-Leu, were obtained from Calbiochem-Novabiochem (San Diego, CA, USA). High-performance liquid chromatography (HPLC)-grade acetonitrile was obtained from Fisher Scientific (St. Louis, MO. USA). N,N-Dicyclohexylcarbodiimide, N,N-dimethylformamide (DMF), N,N-dimethylaminopyridine, trifluoroacetic acid (TFA), dichloromethane, and all other reagents and solvents were purchased form Aldrich Chemical Company (Milwaukee, WI, USA). Valacyclovir was a gift from GlaxoSmithKline, Inc. (Research Triangle Park, NC, USA). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA) and cell culture supplies were obtained from Corning (Corning, NY, USA) and Falcon (Lincoln Park, NJ, USA). All chemicals were either analytical or HPLC grade. Recombinant human thymidine phosphorylase (TP) was purchased from Sigma (St. Louis, MO, USA). Human plasma was obtained from the University of Michigan Hospital Blood Bank (Ann Arbor, MI, USA) and stored at −80°C until used.

FUdR Prodrug Synthesis

Isoleucyl and leucyl prodrugs of floxuridine were synthesized in a manner similar to that reported for the valyl

prodrugs (5) (Fig. 1). Briefly, the Boc-protected amino acids (Boc-L-Ile, Boc-L-Leu; 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 h. The reaction progress was monitored by thin-layer chromatography (ethyl acetate). The reaction mixture was filtered and DMF removed under vacuum at 50–55°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, 1:1–1:0). Fractions from each spot were concentrated under vacuum separately. The Boc group was cleaved by treating the residues with 4 mL of TFA/dichloromethane/H_2O (6:3:1). After 4 h 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.

HPLC was used to evaluate the purity of the prodrugs. Prodrugs were between 96–98% pure. These prodrugs were easily separated from parent drug by HPLC. Electrospray ionization mass spectra (ESI-MS) were obtained on a Thermoquest LCQ electrospray ionization mass spectrometer. The observed molecular weights of all prodrugs were consistent with that required by their structures. The structural identity of the prodrugs was then confirmed using proton nuclear magnetic resonance (^{1}H NMR) spectra. ^{1}H NMR spectra were obtained on a 300-MHz Bruker DPX300 NMR spectrometer.

3'-L-Isoleucyl-FUdR: percent purity, 97%; ^{1}H NMR (DMSO-d_6) δ 0.85–0.94 (6H, m, δCH$_3$, β'CH$_3$), 1.24–1.28 (2H, m, γCH$_2$), 2.33–2.51 (3H, m, C$_2'$, βCH), 3.66–4.63 (4H, m, C$_4'$, C$_5'$, αCH), 5.10–5.36 (1H, m, C$_3'$), 6.22 (1H, t, C$_1'$, J = 6.1 Hz), 8.22 (1H, d, CHF, J = 7.2 Hz); ESI-MS: 360.4 (M + H)$^+$.

5'-L-Isoleucyl-FUdR: percent purity, 98%; ^{1}H NMR (DMSO-d_6) δ 0.82–0.91 (6H, m, δCH$_3$, β'CH$_3$), 1.25–1.28 (2H,m,γCH$_2$), 2.30–2.50 (3H, m, C$_2'$, βCH), 3.93–4.43 (5H, m, C$_3'$, C$_4'$, C$_5'$, αCH), 6.15–6.19 (1H, m, C$_1'$), 6.17 (1H, t, C$_1'$, J = 6.0 Hz), 7.92 (1H, d, CHF, J = 7.2 Hz); ESI-MS: 360.4 (M + H)$^+$.

3',5'-L-Isoleucyl-FUdR: percent purity, 96%; ^{1}H NMR (DMSO-d_6) δ 0.82–0.95 (12H, m, [δCH$_3$]$_2$, [β'CH$_3$]$_2$), 1.24–1.45 (4H, m, [γCH$_2$]$_2$), 2.30–2.71 (4H, m, C$_2'$, [βCH]$_2$), 4.04–4.56 (5H, m, C$_4'$, C$_5'$, [αCH]$_2$), 5.42–5.57 (1H, m, C$_3'$), 6.22 (1H, t, C$_1'$, J = 6 Hz), 8.10 (1H, d, CHF, J = 6.8 Hz); ESI-MS: 473.5 (M + H)$^+$.

3'-L-Leucyl-FUdR: percent purity, 98%; ^{1}H NMR (DMSO-d_6) δ 0.91–0.93 (6H, m, [δCH$_3$]$_2$), 1.59–1.71 (3H, m, βCH$_2$, γCH), 2.33–2.35 (2H, m, C$_2'$), 3.66–4.38 (4H, m, C$_4'$, C$_5'$, αCH), 5.34–5.43 (1H, m, C$_3'$), 6.24 (1H, t, C$_1'$, J = 6.2 Hz), 8.26 (1H, d, CHF, J = 6.5 Hz); ESI-MS: 360.4 (M + H)$^+$.

5'-L-Leucyl-FUdR: percent purity, 98%; ^{1}H NMR (DMSO-d_6) δ 0.85–0.93 (6H, m, [δCH$_3$]$_2$), 1.59–1.70 (3H, m, βCH$_2$, γCH), 2.14–2.19 (2H, m, C$_2'$), 3.93–4.63 (5H, m, C$_3'$, C$_4'$, C$_5'$, αCH), 6.15 (1H, t, C$_1'$, J = 6.0 Hz), 7.93 (1H, d, CHF, J = 6.6 Hz); ESI-MS: 360.4 (M + H)$^+$.

3',5'-L-Leucyl-FUdR: percent purity, 96%; ^{1}H NMR (DMSO-d_6) δ 0.82–0.95 (12H, m, [δCH$_3$]$_4$), 1.24–1.88 (6H, m,
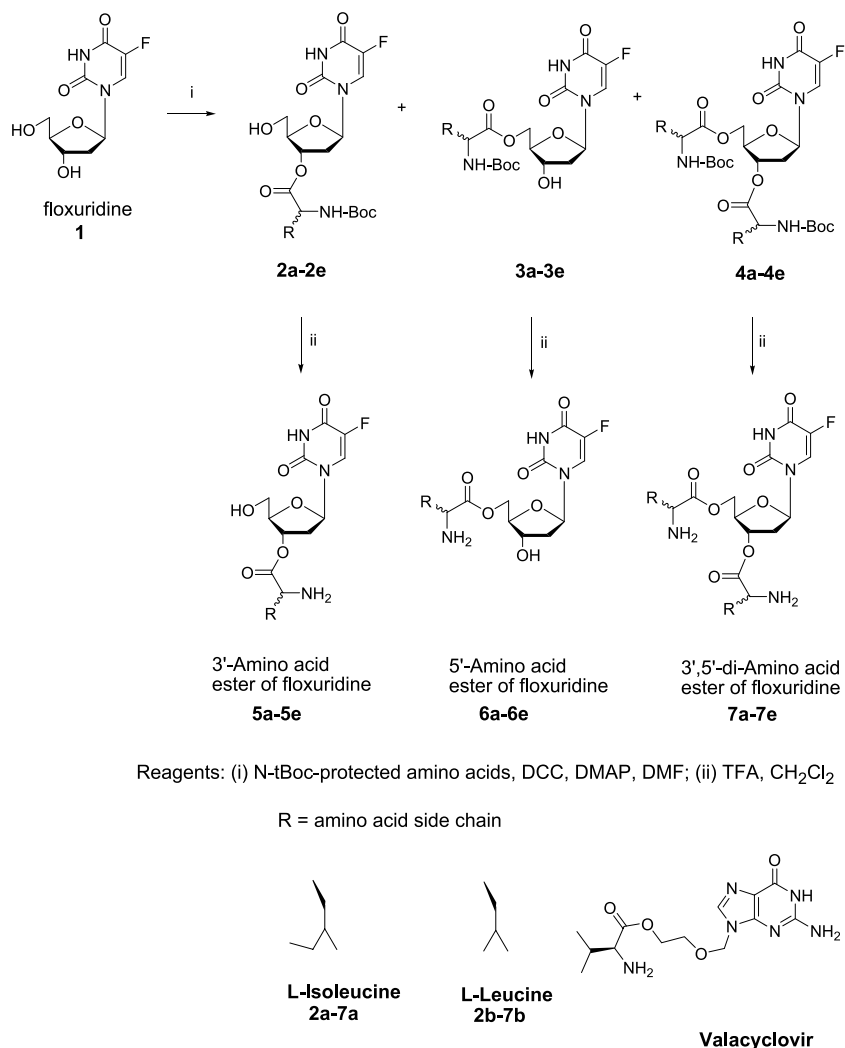
Fig. 1. Synthesis of floxuridine ester prodrugs.

$[\beta CH_2]_2$, $[\gamma CH]_2$), 2.35 (2H, m, C_2'), 4.27–4.56 (5H, m, C_4', C_5', $[\alpha CH]_2$), 5.42–5.57 (1H, m, C_3'), 6.22 (1H, t, C_1', $J = 6.0$ Hz), 8.06 (1H, d, CHF, $J = 6.6$ Hz); ESI-MS: 473.5 $(M + H)^+$.

Cell Culture

HeLa cells (passage 50–54), and Caco-2 cells (passage 30–35) from American Type Culture Collection (Rockville, MD, USA) were routinely maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% nonessential amino acids, 1 mM sodium pyruvate, and 1% L-glutamine. Cells were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C.

Enzymatic Stability

Confluent Caco-2 cells were washed with phosphate-buffered saline (pH 7.4) and then harvested with 0.05% trypsin–EDTA at 37°C for 5–10 min. Trypsin was neutralized by adding Dulbecco's modified Eagle's medium. The cells were washed off the plate and spun down by centrifugation. The pelleted cells were washed twice with phosphate buffer (10 mM, pH 7.4), and resuspended in phosphate buffer

(10 mM, pH 7.4) to obtain a final concentration of 4.7×10^6 cells/mL. The cells were then lysed with one volume 0.5% Triton X-100 solution. The cells were then homogenized by vigorous pipetting and total protein was quantified with the BioRad DC Protein Assay using bovine serum albumin as a standard. The hydrolysis reactions were carried out in 96-well plates (Corning). Caco-2 cell suspension (230 µL) was placed in triplicate wells; the reactions were started with the addition of substrate (40 µL) and incubated at 37°C for 30–60 min. The final prodrug concentration in the mixture was 400 µM. At each time point, 40-µL aliquot sample was removed and added to two volumes of 5% ice-cold TFA. The mixtures were centrifuged for 10 min 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 40 µL of substrate was added to initiate the reactions, which were conducted at 37°C for up to 3 h. At various time points,

40 μL aliquots were removed and added to two volumes of 5% TFA. The mixtures were centrifuged for 10 min 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 phosphate buffer (10 mM, pH 7.4) and in 4-morpholineethanesulfonic acid (MES) buffer (10 mM, pH 6.0) 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.

[³H]Glycyl-L-Sarcosine Uptake Inhibition

HeLa cells were infected with adenovirus containing PEPT1 as described previously (19). Two days after viral infection the HeLa/PEPT1 cells (HeLa cells overexpressing PEPT1) were incubated with 10 μM glycyl-L-sarcosine (9.98 μM Gly-Sar and 0.02 μM [³H]Gly-Sar) along with various prodrug concentrations (5.0–0.01 mM) for 30 min. The cells were washed three times with ice-cold phosphate-buffered saline 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, USA). IC_{50} values were determined using nonlinear data fitting (Graph Pad Prism v3.0).

Uptake Studies

Carrier-mediated transport of the prodrugs was screened in HeLa/PEPT1 cells as described previously (20). Briefly, 2 days postinfection, the cells were incubated with 0.5 mL of freshly prepared drug solution (1 mM) in uptake buffer. After 45 min, cells were washed with ice-cold uptake buffer and 0.3 mL of 0.1% sodium dodecyl sulfate in water was added to each well. The protein was precipitated from the cell lysates by 5% TFA (final concentration) and pelleted by centrifugation. Filtered supernatant was analyzed by HPLC to determine prodrug/drug concentrations. Control experiments were performed in normal HeLa cells.

Caco-2 Permeability

Transcellular transport studies were performed in triplicate with minor modifications as described previously (21). Uptake buffers contained 1 mM $CaCl_2$, 1 mM $MgCl_2 \cdot 6H_2O$, 150 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 5 mM D-glucose, and 5 mM MES or 5 mM Tris. Briefly, 3.75×10^5 cells were seeded onto collagen-coated membranes (0.4-μm pore size, 12 mm diameter, Costar, Cambridge, MA, USA) and cells were allowed to grow for 21–26 days. Mannitol permeability was assayed for each batch of Caco-2 monolayers ($n = 3$) and TEER measurements were performed on all monolayers. Monolayers with apparent [¹⁴C]mannitol permeability $< 3 \times 10^{-7}$ cm/s and TEER values > 250 Ω/cm^2 were used for the study. 1.5 mL of prodrug solution (0.8 mM) in MES buffer (pH 6.0) was added to the apical side of the monolayer and 2.6 mL of Tris buffer (pH 7.4) to the receiver compartment

on the basolateral side of the monolayer. Samples were taken from both sides (200 μL from basolateral side, 20 μL from apical side) at various time points up to 120 min. To minimize enzymatic degradation, samples were acidified with 0.1% TFA and stored at −80°C until HPLC analysis.

Resistance to Metabolism of Floxuridine and Its Prodrugs by Thymidine Phosphorylase

The stability of floxuridine and its prodrugs in the presence of thymidine phosphorylase (TP) was assessed by incubating the desired substrates (400 μM) with TP (2 ng/μL) in phosphate buffer (pH 6.4) at 37°C. The pyrimidine nucleoside gemcitabine was also tested as a positive control. Aliquots of the incubation mixture were periodically sampled over 30 min, quenched with cold TFA (2% final), and analyzed for prodrug, parent, and metabolite concentrations *via* HPLC.

Data Analysis

The initial rates of hydrolysis were used to obtain the apparent first-order rate constants and to calculate the half-lives. The apparent first-order degradation rate constants of various floxuridine prodrugs (and valacyclovir) 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

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

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

$$t_{1/2} = 0.693/k \tag{2}$$

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

The apparent permeability (P_{app}) for the prodrugs was calculated using the following equation:

$$P_{app} = \frac{V_r}{A \times C_0} \times \frac{dC_r}{d_t} \tag{3}$$

where V_r is the receiver volume, A is the surface area of the exposed monolayer, C_0 is the concentration of the prodrug in the donor solution, and dC_r/dt is the rate of change of concentration in the receiver solution. The concentrations of floxuridine and its prodrugs in the receiver and donor compartments were analyzed using HPLC.

HPLC Analysis

The concentrations of floxuridine and its prodrugs 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} reversed phase column (5 μm, 4.6 × 250 mm) equipped

Table I. Stability of Floxuridine Prodrugs in Buffers and Biological Media (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)
3′,5′-L-Leucyl floxuridine	144.6 ± 2.5	40.6 ± 0.3	36.8 ± 1.0	8.4 ± 0.6
3′-L-Leucyl floxuridine	227.9 ± 12.6	73.6 ± 2.9	87.4 ± 2.1	8.0 ± 0.2
5′-L-Leucyl floxuridine	225.1 ± 3.8	77.3 ± 1.2	57.6 ± 1.4	4.8 ± 0.2
3′,5′-L-Isoleucyl floxuridine	943.2 ± 49.2	126.9 ± 5.9	162.1 ± 4.2	19.8 ± 2.5
3′-L-Isoleucyl floxuridine	1,486.3 ± 25.9	333.4 ± 10.3	294.1 ± 9.5	28.9 ± 1.9
5′-L-Isoleucyl floxuridine	1,453.5 ± 60.2	323.52 ± 1.5	271.4 ± 3.2	24.8 ± 1.7
Valacyclovir	5,700[a]	1,029.1 ± 11.2	312.0 ± 2.6	10.3 ± 0.8

[a] From Ref. (15).

with a guard column. The HPLC and analysis methods for floxuridine and its prodrugs and for valacyclovir were described previously (5). Standard curves generated for each prodrug and their parent drugs were used for quantitation of integrated area under peaks.

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 more than 60% and the purity for all prodrugs was ≥96% 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.

Stability Studies

The half-lives ($t_{1/2}$), obtained from linear regression of pseudo-first-order plots of prodrug concentration *vs*. time in MES buffer (pH 6.0) and in phosphate buffer (pH 7.4) at 37°C are shown in Table I. The mass balance for prodrug disappearance and parent drug appearance was excellent under our HPLC analysis conditions (100 ± 2%). All prodrugs evaluated were more stable at pH 6.0 than at pH 7.4. At both acidic and neutral pH, the L-isoleucyl ester prodrugs of floxuridine were approximately 4- to 7-fold more stable than the L-leucyl esters, but not as stable as valacyclovir. The chemical stability of the 3′ and 5′ monoester prodrugs was similar; however, diester prodrugs exhibited a 2-fold lower stability compared with the monoesters.

The half-lives of the floxuridine prodrugs determined in Caco-2 cell homogenates and in human plasma at 37°C are also summarized in Table I. Again, isoleucyl ester prodrugs were about 4- to 7-fold more resistant to enzymatic hydrolysis compared with the corresponding leucyl ester prodrug. The enzymatic activity in Caco-2 cell homogenates was about 10-fold greater than that in human plasma and in pH 7.4 buffer. Thus, the hydrolysis rates of the isoleucyl and leucyl floxuridine prodrugs in plasma and in phosphate buffer (pH 7.4) were similar and highly correlated ($r^2 = 0.95$). With the exception of leucyl prodrugs in Caco-2 cell homogenates, the stability of the esters in buffers and in biological media were in the order: 3′ monoester ≥ 5′ monoester 3′,5′ diester. Lastly, the enzymatic stability of the isoleucyl monoester prodrugs of floxuridine in Caco-2 cell homogenates was about 2- to 4-fold greater than that of the leucyl prodrugs and the commercially marketed valacyclovir prodrug.

Uptake Inhibition Studies

The affinity of the floxuridine prodrugs for PEPT1 was measured by the extent of Gly-Sar uptake in HeLa/PEPT1 cells. IC_{50} values determined in HeLa/PEPT1 cells are presented in Table II. All prodrugs displayed increased affinity for PEPT1 compared with floxuridine, which exhibited no apparent affinity over the concentration range tested. With the exception of the 5′-L-leucyl prodrug, all floxuridine prodrugs examined in this study exhibited IC_{50} values that were similar to those obtained with valacyclovir, a well-established PEPT1 substrate.

Direct Uptake Studies

With the exception of the diester prodrugs, the uptake of all floxuridine prodrugs examined in this study was enhanced

Table II. Uptake and [³H]Gly-Sar Uptake Inhibition in HeLa and HeLa/PEPT1 Cells (Mean ± SD, $n = 3$)

| Prodrugs	Uptake inhibition, IC_{50} (mM)	Uptake (HeLa/PEPT1) (nmol/mg per 45 min)	Uptake (HeLa control) (nmol/mg per 45 min)	PEPT1/Control
3′,5′-L-Leucyl floxuridine	1.16 ± 0.18	0.45 ± 0.06	0.41 ± 0.02	1.1
3′-L-Leucyl floxuridine	0.74 ± 0.12	1.19 ± 0.11	0.54 ± 0.01	2.2
5′-L-Leucyl floxuridine	2.02 ± 0.32	1.28 ± 0.13	0.42 ± 0.04	3.1
3′,5′-L-Isoleucyl floxuridine	0.60 ± 0.10	0.42 ± 0.07	0.31 ± 0.06	1.3
3′-L-Isoleucyl floxuridine	1.19 ± 0.24	4.97 ± 0.35	0.62 ± 0.07	8.0
5′-L-Isoleucyl floxuridine	0.86 ± 0.08	5.30 ± 0.26	0.50 ± 0.05	10.7
5′-L-Valyl floxuridine	0.97 ± 0.09	3.42 ± 0.09	0.18 ± 0.01	19.2
Valacyclovir	1.46 ± 0.36	2.83 ± 0.30	0.60 ± 0.05	4.7

in HeLa/PEPT1 cells compared with uptake in HeLa cells (Table II). The prodrug uptake enhancement ranged between 2.2- to 19.2-fold. In comparison, the transport of valacyclovir, the reference prodrug, was 4.7-fold higher in HeLa/PEPT1 cells compared with that in control HeLa cells. PEPT1-mediated enhancement in uptake with isoleucyl monoester prodrugs was roughly 4-fold higher than that observed with leucyl monoester prodrugs. For these monoester prodrugs, the site of esterification affected PEPT1-mediated transport in a modest manner. 5′-L-Valyl floxuridine exhibited the highest uptake enhancement. The uptake of 5′-L-valyl, 3′-L-isoleucyl, and 5′-L-isoleucyl floxuridine prodrugs exceeded that observed with the reference prodrug valacyclovir.
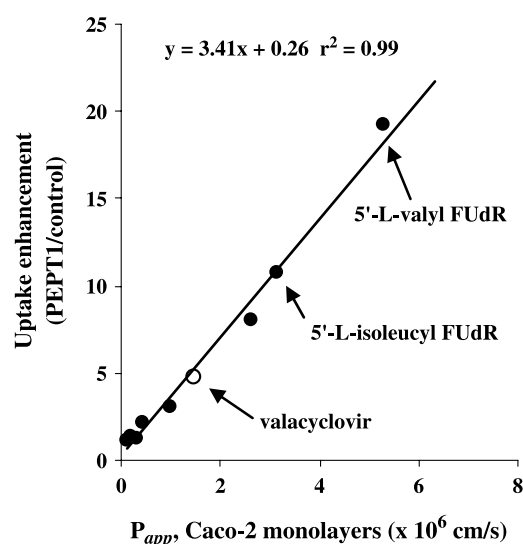
Caco-2 Permeability

Apical to basolateral permeability was evaluated for eight floxuridine prodrugs and several controls (Fig. 2). The apparent permeability of the two pyrimidine nucleoside drugs, gemcitabine and floxuridine, across Caco-2 monolayers was lower than 1×10^{-6} cm/s. Of the floxuridine prodrugs tested, only the 5′-L-valyl, 5′-L-leucyl, 5′-L-isoleucyl, and 3′-L-isoleucyl monoester prodrugs exhibited significant enhancement compared with floxuridine. 3′-L-Leucyl and 5′-L-aspartyl floxuridine, the two other monoester prodrugs examined, exhibited permeability that was poor and similar to that of floxuridine itself. The isoleucyl and leucyl diesters were even less permeable than the floxuridine parent drug. The uptake enhancement observed with HeLa/PEPT1 cells compared with control HeLa cells for the compounds tested (Table II) correlated highly with their permeability across Caco-2 monolayers ($r^2 = 0.99$) (Fig. 3).

The extent of hydrolysis of select prodrugs in Caco-2 monolayer transport experiments is summarized in Table III. The results indicate that ester hydrolysis of the prodrugs is not extensive when in contact with the apical side of the monolayers (<5% at 120 min). The fate of the prodrug after transport across the monolayers was, however, dramatically dependent on the amino acid promoiety and its stereochemistry. Thus, 5′-L-valyl-FUdR was extensively hydrolyzed
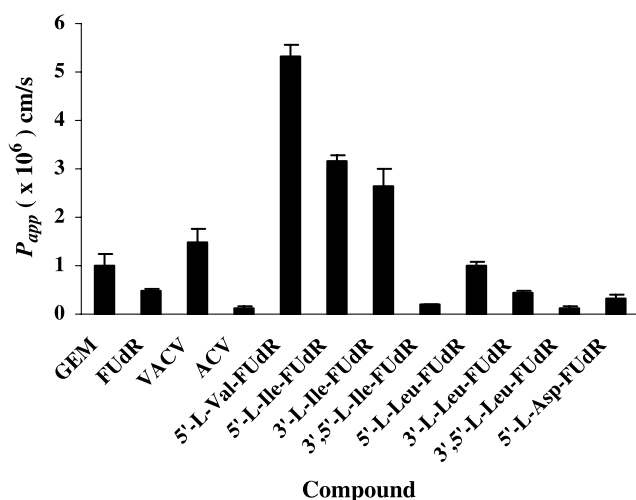
Fig. 2. Apparent permeability coefficients (P_{app}) of floxuridine and its amino ester prodrugs in the apical-to-basolateral direction across Caco-2 monolayers (mean ± SD, $n = 3$).

Fig. 3. Correlation of PEPT1-mediated uptake enhancement in HeLa/PEPT1 cells with apparent apical-to-basolateral permeability across Caco-2 monolayers.

(~35%), whereas its D analog exhibited much greater stability after transport (~15% hydrolysis). In comparison, the hydrolysis of 5′-L-isoleucyl-FUdR after transport seems to be quite moderate (~20%). The reference prodrug valacylovir was extensively hydrolyzed (~85%) after transport across Caco-2 monolayers.

Thymidine Phosphorylase Activity Against Floxuridine and Prodrugs

The metabolic stability of floxuridine and its amino ester prodrugs was assessed using pure thymidine phosphorylase. The results shown in Fig. 4a indicate that floxuridine was rapidly degraded to the less active metabolite, 5-FU, by thymidine phosphorylase. It is also evident from Fig. 4a that an excellent mass balance between the disappearance of floxuridine and the appearance of 5-FU is maintained at all time points examined. The other pyrimidine nucleoside drug gemcitabine, however, exhibited high resistance to degradation by thymidine phosphorylase to cytidine (Fig. 4b). The amino acid ester prodrugs of floxuridine were also quite

Table III. Stability of Select Prodrugs in Caco-2 Cell Permeability Studies (Mean ± SD, $n = 3$)

	Stability[a]	
Prodrugs	Apical donor compartment	Basolateral receiver compartment
5′-L-Leucyl floxuridine	92.0 ± 3.0	77.0 ± 1.2
5′-L-Isoleucyl floxuridine	97.0 ± 6.0	77.1 ± 5.8
5′-D-Isoleucyl floxuridine	100.0 ± 1.0	99.7 ± 2.5
5′-L-Valyl floxuridine	75.9 ± 8.1	34.7 ± 3.3
5′-D-Valyl floxuridine	95.3 ± 2.2	83.9 ± 5.7
Valacyclovir	91.3 ± 2.4	15.5 ± 6.0

[a] Percent prodrug remaining intact in donor and receiver solutions at 120 min.

a Floxuridine

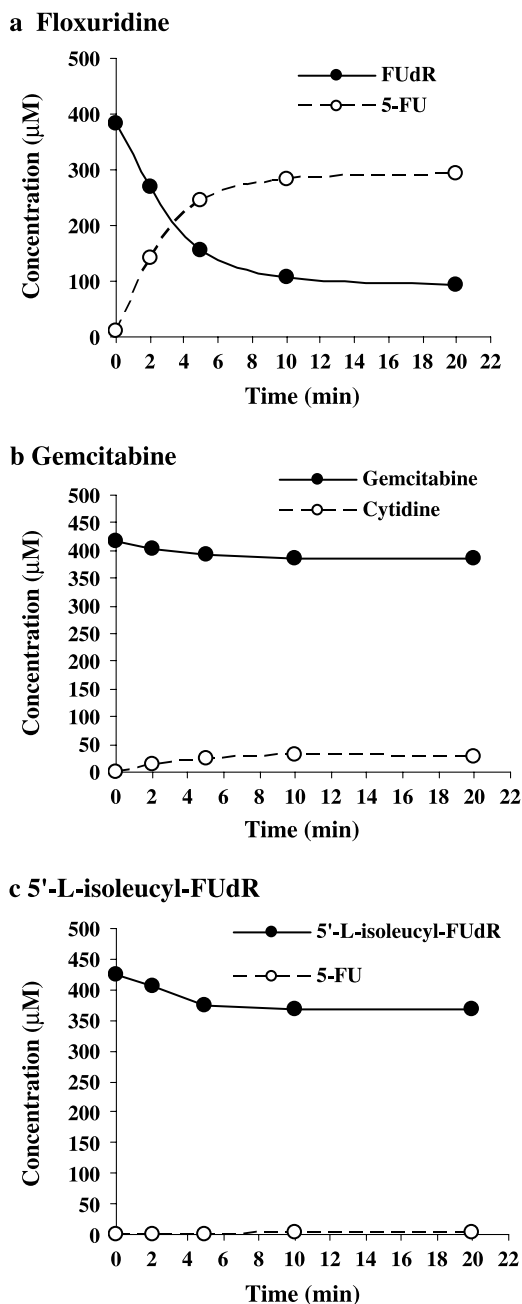### b Gemcitabine

c 5'-L-isoleucyl-FUdR

Fig. 4. Thymidine phosphorylase activity against nucleosides.

resistant to degradation by thymidine phosphorylase as depicted for the 5′-L-isoleucyl prodrug in Fig. 4c.

DISCUSSION

The development of oral alternatives to intravenous administration of fluoropyrimidines such as floxuridine would not only avert the high costs dictated by hospital treatments but also be more patient-friendly. In addition, it may also be possible to incorporate prodrug strategies that could reduce undesirable side effects such as toxicity and improve therapeutic action into such oral alternatives. As a first step toward such a realization, it would be necessary to address three key

issues that would determine overall floxuridine disposition. The first of these involves a predictable assurance of desirable intestinal transport characteristics. The second is the avoidance or slowing of the metabolic conversion of floxuridine to its less active metabolite, 5-FU, in the liver. Thirdly, and possibly *via* a combined effect of intestinal absorption and liver metabolism, it would be essential to maintain therapeutic profiles of floxuridine that can mimic an intravenous infusion.

In this report, we describe the synthesis of leucyl and isoleucyl amino acid ester prodrugs of floxuridine and their suitability as substrates of the PEPT1 intestinal transporter. The chemical stability in buffers and enzymatic activation in surrogate cell systems and the ability to evade extensive metabolism by thymidine phosphorylase were also determined to identify prodrugs that could be potentially useful for oral delivery of floxuridine.

Amino acid esters of nucleoside analogs such as acyclovir have been designed and evaluated for their transport across intestinal membranes (15). These studies indicated that optimal transport occurred with valyl esters and led to the development of valacyclovir. The enhanced oral bioavailability of valacyclovir in humans (22) has been suggested to be the result of enhanced intestinal transport by peptide transport systems (16,23,24). Consistent with previous findings, 5′-L-valyl floxuridine was the most efficiently transported floxuridine prodrug, exhibiting the highest PEPT1-mediated transport and permeability across Caco-2 monolayers (15,25). In comparison, the slightly more branched isoleucyl side chain reduced the transport almost by half. In addition, it seems that branching at the γ carbon in leucine chains as opposed to branching at the β carbon in isoleucine and valine decreases the transport efficiency even further. The lack of direct uptake despite the significant inhibition of Gly-Sar transport by the diesters suggests uptake inhibition studies alone may not be sufficient.

The transport of floxuridine, a hydrophilic drug, across Caco-2 monolayers was limited but not insignificant. Although floxuridine is not expected to be passively transported, it is quite likely that they are actively transported *via* pyrimidine nucleoside transporters such as CNT2 (5,26). The permeability of 5′ monoester prodrugs of floxuridine across Caco-2 monolayers was significantly higher than that of floxuridine itself and reflected a profound promoiety dependency. Thus, the permeability of the 5′-L-valyl prodrug was roughly 2- and 5-fold higher than the permeability of the 5′-L-isoleucyl and 5′-L-leucyl floxuridine prodrugs, respectively. This trend is consistent with their PEPT1-mediated uptake enhancement in HeLa cells and resembles the trends reported for amino acid ester prodrugs of acyclovir in rats (15). The poor permeability of 5′-L-aspartyl floxuridine across Caco-2 monolayers is consistent with previous reports that suggest that this prodrug is not a substrate of PEPT1 (6,24). The enhanced transport of the 3′-L-isoleucyl floxuridine prodrug across Caco-2 cells comparable to its 5′ analogue is in contrast to the behavior of 3′-L-leucyl floxuridine, which exhibited a 2-fold lower transport compared with 5′-L-leucyl floxuridine.

The excellent linear correlation between uptake enhancement of the amino acid ester prodrugs in HeLa cells mediated by PEPT1 and AP-BL permeability across Caco-2 monolayers ($r^2 = 0.99$) indicates the high likelihood of their

active transport in Caco-2 cells as well. The overlay of the reference prodrug valacyclovir on this correlation plot and the observation that the 5′-L-isoleucyl and 5′-L-valyl floxuridine prodrugs both exhibit substantially higher Caco-2 permeability than valacyclovir provide some assurance that these two prodrugs may indeed exhibit enhanced *in vivo* intestinal transport.

Although Caco-2 cells are used as a model for human intestine, they lack expression of many metabolizing enzymes found in the intestine. Caco-2 cells do not express thymidine or uridine phosphorylase; however, high levels of both are expressed in the intestine (27). Thymidine phosphorylase, an enzyme that has been suggested to be important *in vivo* for phosphorytic cleavage of floxuridine (28), was therefore used to examine metabolism by nucleoside phosphorylases that would normally be observed in the intestine and liver. As expected, floxuridine was rapidly cleaved by thymidine phosphorylase in this study, whereas the other pyrimidine nucleoside analog, gemcitabine, resisted breakdown. It has been proposed that the mechanism of thymidine phosphorylase-catalyzed cleavage of pyrimidine nucleosides involves the initial attack of a phosphate ion on the C1′ position of the sugar moiety that leads to glycosidic bond cleavage (29). The attachment of two fluorine groups at the adjacent C2′ position of the sugar moiety in gemcitabine may prevent the initial phosphate ion attack on the C1′ position and obviate thymidine phosphorylase catalytic action. All amino acid ester prodrugs examined in this study were stable to glycosidic bond cleavage by thymidine phosphorylase. Thus, the chemical modification of floxuridine by esterification of one or both of the free hydroxyl groups on the sugar moiety seems to provide protection from glycosidic bond cleavage. Structural studies with purine nucleoside phosphorylase revealed that these hydroxyl groups are critical for efficient substrate binding (30), which suggests that it may also be important for pyrimidine nucleoside phophorylases. Enzymatic activation of the prodrug *via* ester hydrolysis would therefore deprotect it from thymidine phosphorylase action. Thus, the rate of bioactivation of the amino acid ester prodrugs of floxuridine is likely the rate-limiting step to glycosidic bond cleavage of floxuridine to 5-FU. Thus, the rate of conversion of the prodrugs to the parent drug after transport would determine floxuridine disposition and therapeutic action.

The roughly 5- to 12-fold higher activity in Caco-2 cell homogenates compared with pH 7.4 buffer suggests the predominance of enzymatic bioconversion of the prodrugs. The stability profiles of leucyl ester prodrugs of floxuridine in Caco-2 cell homogenates (and in human plasma) indicate that they would not be suitable candidates. In comparison, isoleucyl ester prodrugs of floxuridine are enzymatically more stable than the valyl ester floxuridine prodrugs previously investigated (5) and also more stable than the reference prodrug valacyclovir. The preference for 5′ monoesters over the corresponding 3′ monoester is consistent with the trends reported for activation of amino acid ester prodrugs of floxuridine and gemcitabine by biphenyl hydrolase-like protein (BPHL) or valacyclovirase, an enzyme responsible for activation of valacyclovir and amino acid ester prodrugs (31) and by carboxylesterase (Landowski *et al.*, unpublished data). It has been suggested

that 3′ floxuridine esters exhibit higher antitumor effect than 5′ esters partly due to their enhanced enzymatic stability (32,33).

Furthermore, assessment of the basolateral compartment at 2 h (Table III) indicates that around 77% of 5′-L-isoleucyl-FUdR remains intact after passage across Caco-2 monolayers. In comparison, only 15% of the reference prodrug valacyclovir, and 35% of 5′-L-valyl-FUdR were found as intact prodrug in the basolateral compartment at 2 h. Thus, it seems that at least in the Caco-2 model, ester hydrolysis of 5′-L-isoleucyl-FUdR was not extensive. It is quite possible that the extent of ester hydrolysis *in vivo* after transport across the human intestine (and in liver) may be higher than that predicted with the Caco-2 model. In this regard, we have compared the hydrolysis kinetics of amino acid ester prodrugs by BPHL and by carboxylesterase, both of which are highly expressed in the human liver and intestine. The results (Landowski *et al.*, unpublished data) indicated that 5′-L-isoleucyl-containing prodrugs are poor substrates for both BPHL and carboxylesterase. Accordingly, the BPHL specific activity toward 5′-L-isoleucyl-FUdR was found to be 15- to 20-fold lower than that observed with two very good BPHL substrates, valacyclovir and 5′-L-valyl-FUdR. Carboxylesterase 1 catalytic activity toward 5′-L-valyl and 5′-L-isoleucyl-FUdR was even lower than that obtained with BPHL. These data indicate that ester bond hydrolysis, especially with 5′-L-isoleucyl floxuridine, may be limited *in vivo*. However, *in vivo* experiments with the floxuridine prodrugs would be necessary to determine the actual combined metabolism effects of the intestine and liver.

The combined results of the *in vitro* studies suggest that isoleucyl monoesters of floxuridine may be potentially promising candidates for improving oral bioavailability of floxuridine *in vivo*. The prodrugs given orally could improve the intestinal uptake of floxuridine as well as shield it from unwanted degradation. Although the 5′-L-valyl floxuridine prodrug may be transported to a much greater extent across the intestine, its more rapid activation *in vivo* may not be beneficial in evading metabolism by thymidine phosphorylase. Rapid intestinal activation is also expected to lead to increased intestinal toxicity. The disposition of floxuridine after oral administration of the candidate prodrugs in mice will be determined to corroborate some of these indications.

ACKNOWLEDGMENT

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