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Enhanced Cancer Cell Growth Inhibition by Dipeptide Prodrugs of Floxuridine: Increased Transporter Affinity and Metabolic Stability

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Abstract: Dipeptide monoester prodrugs of floxuridine were synthesized, and their chemical stability in buffers, resistance to glycosidic bond metabolism, affinity for PEPT1, enzymatic activation and permeability in cancer cells were determined and compared to those of mono amino acid monoester floxuridine prodrugs. Prodrugs containing glycyl moieties were the least stable in pH 7.4 buffer ($t_{1/2}$ < 100 min). The activation of all floxuridine prodrugs was 2- to 30-fold faster in cell homogenates than their hydrolysis in buffer, suggesting enzymatic action. The enzymatic activation of dipeptide monoester prodrugs containing aromatic promoieties in cell homogenates was 5- to 20-fold slower than that of other dipeptide and most mono amino acid monoester prodrugs ($t_{1/2} \sim$ 40 to 100 min). All prodrugs exhibited enhanced resistance to glycosidic bond metabolism by thymidine phosphorylase compared to parent floxuridine. In general, the 5′-O-dipeptide monoester floxuridine prodrugs exhibited higher affinity for PEPT1 than the corresponding 5′-O-mono amino acid ester prodrugs. The permeability of dipeptide monoester prodrugs across Caco-2 and Capan-2 monolayers was 2- to 4-fold higher than the corresponding mono amino acid ester prodrug. Cell proliferation assays in AsPC-1 and Capan-2 pancreatic ductal cell lines indicated that the dipeptide monoester prodrugs were equally as potent as mono amino acid prodrugs. The transport and enzymatic profiles of 5′-L-phenylalanyl-L-tyrosyl-floxuridine, 5′-L-phenylalanyl-L-glycyl-floxuridine, and 5′-L-isoleucyl-L-glycyl-floxuridine suggest their potential for increased oral uptake, delayed enzymatic bioconversion and enhanced resistance to metabolism to 5-fluorouracil, as well as enhanced uptake and cytotoxic activity in cancer cells, attributes that would facilitate prolonged systemic circulation for enhanced therapeutic action.

Keywords: Dipeptide monoester floxuridine prodrugs; PEPT1; Caco-2 and Capan-2 permeability; metabolism; thymidine phosphorylase

Introduction

The anticancer agent 5-fluoro-2′-deoxyuridine (floxuridine) has been shown to be clinically effective in the treatment of colon carcinoma and colorectal cancer that has metastasized to the liver. However, the adverse effects associated with chemotherapeutics are still unresolved, and many efforts have been made to minimize side-effects and maximize therapeutic efficacy. Prodrug strategies have been increasingly utilized over the past two decades in order to overcome undesirable physicochemical properties of drugs, to improve oral bioavailability and to minimize toxic side-effects. A majority of the efforts have focused on antiviral and anticancer drugs and reflect the need for improved targeting, more selective action and further development of orally available alternatives. Amino acid ester prodrugs of poorly permeant anticancer and antiviral drugs have been designed for targeted delivery *via* specific transporters in order to improve their oral bioavailability and metabolic disposition.[1−3]

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Amino acid ester prodrugs have been shown to be substrates for PEPT1, PEPT2, and $ATB^{0,+}$ transporters, and their improved oral bioavailability has been attributed to enhanced transport *via* carrier-mediated mechanisms.[4−9] Alkyl ester prodrugs and amino acid ester prodrugs of floxuridine, for example, have been synthesized and tested for potential improvement of oral drug delivery.[10−14] Amino acid ester prodrugs of floxuridine and an antiviral agent, acyclovir, have been shown to be substrates of the PEPT1 transporter.[14,15] PEPT1 is stereoselective and exhibits greater affinity for L-enantiomers of amino acids than D-enantiomers.[16] PEPT1 is predominantly expressed in the small intestine, has broad substrate specificity and can transport dipeptides, tripeptides, and β-lactam antibiotics.[6,17−23] The valyl ester prodrug of acyclovir significantly enhances oral bioavailability of acyclovir, and several reports suggest that oligopeptide transporters are responsible for absorption enhancement.[15,24] Amino acid ester prodrugs may also facilitate enhanced delivery to pancreatic ductal cancer cells such as AsPC-1 and Capan-2, since these cells express oligopeptide transporters at relatively high levels.[25]

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The activation of prodrug to the parent drug following transport is an essential step and cannot be ignored. It has been shown that a specific enzyme, valacyclovirase, is primarily responsible for the conversion of valacyclovir to acyclovir. It has also been suggested that this enzyme might be involved in the activation of other amino acid prodrugs.[26] Kim and colleagues reported that the substrate specificity of valacyclovirase is largely determined by the amino acid acyl-linked promoiety of the prodrug.[27]

The metabolic conversion of floxuridine to 5-fluorouracil following systemic delivery has been shown to be detrimental to therapeutic efficacy of floxuridine.[28,29] The mechanism of action of 5-fluorouracil (5-FU) and floxuridine (FUdR) is well understood.[30] 5-FU toxicity is predominantly caused by 5-FU incorporation into RNA. However, unlike 5-FU, FUdR is specifically incorporated into DNA and not into RNA, which leads to the minimization of adverse effects.[31−33] Several groups have reported that floxuridine is more potent than 5-FU and that the inhibition of cell proliferation is 10- to 100-fold higher than that of 5-FU.[28,29] However, floxuridine is rapidly converted to 5-FU in many tissues, including the liver, by the enzyme thymidine phosphorylase.[34] As a consequence, higher doses of floxuridine are required for maintenance of clinical efficacy, leading to greater toxicity. Therefore, protection of the glycosidic bond of floxuridine is expected to maintain the high potency of the drug and facilitate administration of low doses that can selectively kill only proliferating cells by robust inhibition of DNA synthesis. Improving the chemical stability of floxuridine to thymidine phosphorylase may enhance its therapeutic efficacy at low doses and obviate toxicity concerns.

Although amino acid monoester prodrugs of floxuridine have been shown to provide enhanced PEPT1-mediated transport as well as enzymatic activation in intestinal and liver surrogate cell systems, dipeptide analogues may exhibit even higher affinity and transport *via* the oligopeptide transporter.[6,18,22,35] Therefore, dipeptide prodrugs may be delivered more to a target site by carrier mediated transporters, and extra amino acid attached prodrugs could be more suitable for specific enzymatic activation at a target site than mono amino acid ester prodrugs. Several studies on amino acid modifications to increase specific transporter affinity have been conducted. However, direct comparisons of mono amino acid and dipeptide prodrugs in terms of transporter affinity, prodrug stability and activation have not been reported.

In this report, we describe the synthesis, characterization, and stability of dipeptide monoester prodrugs of floxuridine. Various dipeptides and peptidomimetics have been tested to characterize the hPEPT1 transporter and improve its affinity,[6,17−19,22,35−37] and mono amino acid ester prodrugs have been evaluated as hPEPT1 substrates.[3,13,14] Based on those reports, six amino acids were chosen to be N-terminal amino acids of the dipeptide. The smallest amino acid, glycine, and bulky amino acids like phenylalanine and tyrosine were paired with those six amino acids to form dipeptides to test the hypothesis that molecular sizes may structurally affect its ester bond stability. The six dipeptide promoieties, L-phenylalanalyl-L-glycine, L-leucyl-L-glycine, L-glycyl-L-leucine, L-isoleucyl-L-glycine, L-valyl-L-phenylalanine, and L-phenylalanyl-L-tyrosine, were designed for targeted delivery to an oligopeptide transporter and tested.

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Since 5′-ester prodrugs were found to exhibit higher affinity for transporters than 3′-ester prodrugs,[14] only 5′-dipeptide monoester floxuridine prodrugs were examined in this study. Uptake inhibition and permeability studies were conducted with Caco-2 cells as well as with AsPC-1 and Capan-2 cells. The chemical stability at physiological pH and the enzymatic activation of the prodrugs in Caco-2, AsPC-1, and Capan-2 cell homogenates were also evaluated to determine the effects of the amino acid/dipeptide promoiety structure on enzyme-mediated activation. The feasibility of selective antiproliferative action of amino acid/dipeptide floxuridine prodrugs was also explored using cancer cells that overexpress PEPT1. Finally, the stability and transport characteristics of the dipeptide monoester prodrugs of floxuridine were compared with those of the corresponding amino acid monoester prodrugs.

Materials and Methods

Materials. Floxuridine (FUdR) was obtained from Lancaster (Windham, NH). The *tert*-butyloxycarbonyl (Boc) protected amino acids Boc-L-isoleucine, Boc-L-glycine, Boc-L-valine, Boc-L-phenylalanine, Boc-L-leucine, Boc-L-glycyl-L-leucine, Boc-L-phenylalanalyl-L-glycine, Boc-L-leucyl-L-glycine, Boc-L-isoleucyl-L-glycine, Boc-L-valyl-L-phenylalanine, and Boc-L-phenylalanyl-L-tyrosine were obtained from Chem-Impex (Wood Dale, IL). High-performance liquid chromatography (HPLC) grade acetonitrile was obtained from Fisher Scientific (St. Louis, MO). *N*,*N*-Dicyclohexylcarbodiimide (DCC), *N*,*N*-dimethylaminopyridine
(DMAP), trifluoroacetic acid (TFA), and all other reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI). 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 analytical or HPLC grade.

Floxuridine Prodrug Synthesis. The synthesis and characterization of 5′-mono amino acid ester prodrugs of floxuridine have been reported previously.[11,13] Dipeptide prodrugs of floxuridine were synthesized in a similar manner. Briefly, Boc-protected dipeptides Boc-L-glycyl-L-leucine, Boc-L-phenylalanalyl-L-glycine, Boc-L-leucyl-L-glycine, Boc-L-isoleucyl-L-glycine, Boc-L-valyl-L-phenylalanine, and Boc-L-phenylalanyl-L-tyrosine, (1.1 mmol), DCC (1.1 mmol), and DMAP (0.1 mmol) were allowed to react with floxuridine (1 mmol) in 7 mL of dry DMF for 24 h. The reaction progress was monitored by TLC (ethyl acetate). The reaction mixture was filtered and DMF was removed under vacuum at 40 °C. The residue was extracted with ethyl acetate (30 mL) and washed with water (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 floxuridine prodrugs. The three spots observed on TLC were separated and purified using column chromatography (dichloromethane (DCM)/methanol, 20:1). Fractions from each spot were concentrated under vacuum separately. The Boc group was cleaved by treating the residues with 5 mL TFA:DCM (1:1). After 4 h, the solvent was removed and the residues were reconstituted with water and lyophilized. The TFA salts of amino acid prodrugs of floxuridine were obtained as white fluffy solids. The combined yield, consisting of 3′-monoester, 5′-monoester, and 3′,5′-diesters, of each floxuridine prodrug was ~60%. The prodrugs were determined to be 90%−99% pure by reverse-phase HPLC, and were easily separated from their parent compounds by reverse-phase HPLC. The observed molecular weights of all prodrugs, determined by electrospray ionization mass spectra (ESI-MS) obtained on a Micromass LCT ESI-MS, were found to be consistent with those predicted by their structures. The structural identities of the prodrugs were then confirmed using proton nuclear magnetic resonance spectra (^{1}H NMR), obtained on a 300 MHz Bruker DPX-300 NMR spectrometer.

5′-L-Leucyl-L-glycyl-floxuridine: yield, 15%; percent purity, 93%; ^{1}H NMR (DMSO-d_6) δ, 0.84−0.92 (6H, m, $^{\delta}(CH_3)_2$), 1.50−1.77 (3H, m, $^{\beta,\gamma}CH_2$), 2.12−2.28 (2H, m, C2′), 3.78−4.35 (7H, m, $^{\alpha}$CH of Leu and $^{\alpha}CH_2$ of Gly, C3′, C4′, C5′), 6.16 (1H, t, C1′, J = 6.0 Hz), 7.94 (1H, d, CHF, J = 6.9 Hz); ESI-MS, 417.1 $(M + H)^+$.

5′-L-Glycyl-L-leucyl-floxuridine: yield, 15%; percent purity, 96%; ^{1}H NMR (DMSO-d_6) δ, 0.85 (3H, d, $^{\delta}CH_3$, J = 6.5 Hz), 0.90 (3H, d, $^{\delta}CH_3$, J = 6.5 Hz), 1.51−1.68 (3H, m, $^{\beta,\gamma}CH_2$), 2.12−2.26 (2H, m, C2′), 3.54−4.40 (7H, m, $^{\alpha}$CH of Leu and $^{\alpha}CH_2$ of Gly, C3′, C4′, C5′), 6.16 (1H, t, C1′, J = 6.0 Hz), 7.90 (1H, d, CHF, J = 6.8 Hz); ESI-MS, 417.1 $(M + H)^+$.

5′-L-Valyl-L-phenylalanyl-floxuridine: yield, 30%; percent purity, 95%; ^{1}H NMR (DMSO-d_6) δ, 0.90 (3H, d, $^{\gamma}CH_3$, J = 6.9 Hz), 0.95 (3H, d, $^{\gamma}CH_3$, J = 6.9 Hz), 2.08−2.20 (3H, m, C2′, $^{\beta}$CH of Val), 3.02 (2H, m, $^{\beta}CH_2$ of Phe), 3.62−4.60 (6H, m, $^{\alpha}$CH of Val and Phe, C3′, C4′, C5′), 6.14 (1H, t, C1′, J = 6.1 Hz), 7.22−7.31 (5H, m, aromatic protons), 7.91 (1H, d, CHF, J = 6.9 Hz); ESI-MS, 494.1 $(M + H)^+$.

5′-L-Isoleucyl-L-glycyl-floxuridine: yield, 15%; percent purity, 93%; ^{1}H NMR (DMSO-d_6) δ, 0.87 (3H, t, $^{\delta}CH_3$, J=7.3 Hz), 0.94 (3H, d, $^{\gamma}CH_3$, J = 6.8 Hz), 1.17 (1H, m, $^{\gamma}CH_2$), 1.52 (1H, m, $^{\gamma}CH_2$), 1.81 (1H, m, $^{\beta}$CH), 2.11−2.28 (2H, m, C2′), 3.90−4.31 (7H, m, $^{\alpha}$CH of Ile and $^{\alpha}CH_2$ of Gly, C3′, C4′, C5′), 6.15 (1H, t, C1′, J = 6.5 Hz), 7.94 (1H, d, CHF, J = 6.9 Hz); ESI-MS, 416.9 $(M + H)^+$.

5′-L-Phenylalanyl-L-glycyl-floxuridine: yield, 16%; percent purity, 98%; ^{1}H NMR (DMSO-d_6) δ, 2.12−2.28 (2H, m, C2′), 2.92−3.16 (2H, m, $^{\beta}CH_2$ of Phe), 3.90−4.34 (7H, m, $^{\alpha}$CH of Phe and $^{\alpha}CH_2$ of Gly, C3′, C4′, C5′), 6.15 (1H, t, C1′, J = 6.5 Hz), 7.27−7.36 (5H, m, aromatic protons), 7.94 (1H, d, CHF, J = 6.5 Hz); ESI-MS, 451.1 $(M + H)^+$.

5′-L-Phenylalanyl-L-tyrosyl-floxuridine: yield, 3.2%; percent purity, 99%; ^{1}H NMR (DMSO-d_6) δ, 2.10−2.32 (2H, m, C2′), 2.72−2.92 (4H, m, $^{\beta}CH_2$ of Phe and Tyr), 3.90−4.29 (6H, m, $^{\alpha}$CH of Phe and Tyr, C3′, C4′, C5′), 6.15 (1H, m, C1′), 6.65−7.32 (9H, m, aromatic protons), 7.93 (1H, m, CHF); ESI-MS, 557.2 $(M + H)^+$.

Cell Culture. Capan-2 cells (passages 50−54), and AsPC-1 cells (passages 63−65) from American type Culture Collection (Rockville, MD) were routinely maintained in RPMI-1640 containing 10% fetal bovine serum. 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 at 5% CO_2 and 90% relative humidity at 37 °C. Cells were grown in antibiotic-free media to avoid the possible transport interference by antibiotics.

Hydrolysis Studies. (a) Enzymatic Stability. Confluent Caco-2, Capan-2, and AsPC-1 cells were rinsed twice with saline. The cells were washed with 5 mL of pH 7.4 phosphate buffer (10 mmol/L), lysed by ultrasonication (Micro ultrasonic cell disrupter model KT40, Kontes, Vineland, NJ), and pelleted by centrifugation for 5 min at 1000*g*. Protein amount was quantified with Bio-Rad (Hercules, CA) DC Protein Assay using bovine serum albumin as a standard. The protein amount was adjusted to 500 μg/mL, and the hydrolysis reactions were carried out in 96-well plates (Corning). Caco-2, AsPC-1, and Capan-2 cell suspensions (250 μL) were placed in triplicate wells, the reactions started with the addition of substrate, and cells were incubated at 37 °C for 120 min. At the desired time point, sample aliquots (35 μL) were removed and added to 150 μL of acetonitrile (ACN) containing 0.1% TFA. The mixtures were filtered with a 0.45 μm filter at 1000*g* for 10 min at 4 °C. The filtrate was then analyzed *via* reverse-phase HPLC.

(b) Stability in Human Plasma. The stability of the prodrugs in human plasma was determined using the procedure below. Undiluted plasma (250 μL) was added to each well in triplicate, and substrate was added to initiate the reactions that were conducted at 37 °C for 2 h. At various time points, aliquots (35 μL) were removed and added to 150 μL of ACN containing 0.1% TFA. The mixtures were filtered with a 0.45 μm filter at 1000*g* for 10 min at 4 °C. The filtrate was then analyzed *via* reverse-phase HPLC.

(c) Chemical Stability. The nonenzymatic hydrolysis of the prodrugs was determined as described above, except that each well contained pH 7.4 phosphate buffer (10 mmol/L) instead of cell homogenate or human plasma.

(d) 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 (200 μM) with TP (2.0 ng/μL) in phosphate buffer (pH 7.0) at 37 °C. Aliquots of the incubation mixture were sampled at 0, 1, 3, 5, 10, 30, 60, and 120 min, and quenched with cold acetonitrile (ACN) with 0.1% TFA, filtered through a 0.45 μm membrane, and analyzed for the concentrations of prodrug, floxuridine, and 5-FU by HPLC.

[^{3}H]Gly-Sar Uptake Inhibition. Caco-2 cells at nine days postseeding, and AsPC-1 and Capan-2 cells, both at four days postseeding, 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 (5−0.05 mmol/L) for 30 min. The cells were washed three times with ice-cold PBS and solubilized with 10 mL of scintillation cocktail (ScintiVerse, Fisher Scientific, St.Louis, MO), and the amount of cell-associated radioactivity was determined by scintillation counting (Beckman LS-9000, Beckman Instruments, Fullerton, CA). IC_{50} values were determined using nonlinear data fitting (GraphPad Prism version 3.0).

Transport Studies. Caco-2 cell monolayers were grown on collagen-coated polytetrafluoroethylene membranes for 21 to 24 days, and Capan-2 cell monolayers were grown on the same type of membrane for 14 days. Transepithelial electrical resistance (TEER) was monitored, and values of 240−280 Ω/cm^2 in Caco-2 and 380−420 Ω/cm^2 in Capan-2 (total area for both cells was 4.67 cm^2) were used in the study. Apical side and basolateral sides of transwell inserts were washed with MES (pH 6.0) and HEPES (pH 7.4), respectively. Fresh MES and HEPES buffers were reapplied to transwell inserts and incubated at 37 °C for 15 min. Freshly prepared 0.1 mM drug solution in MES buffer (total 1.5 mL) was placed in the donor chamber, and the receiver chamber was filled with HEPES buffer (total 2.5 mL) Sampling from the receiver chamber (200 μL) was conducted up to a period of 2 h at time intervals of 15, 30, 45, 60, 75, 90, and 120 min, at 37 °C and replaced with an equal volume of fresh HEPES buffer to maintain sink conditions in the receiver chamber. All samples were immediately acidified with 0.1% TFA and analyzed by reverse-phase 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 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

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

The degradation half-lives were then calculated by the equation

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

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

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

$$\text{Flux} = J_{ss} = \mathrm{d}M/\mathrm{d}t \quad (3)$$

where J_{ss} is the steady state flux, M is the cumulative amount of prodrug, and regenerated mono amino acid prodrug, drug and 5-FU in the receiver compartment. The apparent permeability was calculated from steady state flux as follows:

$$P_{app} = \frac{J_{ss}}{A \times C_0} \quad (4)$$

where A is the surface area of monolayer exposed to the permeant and C_0 is the concentration of the prodrug in the donor solution. The concentrations of floxuridine and its

Table 1. Analytical Data for Amino Acid Ester Prodrugs of Floxuridine

prodrug	% purity (HPLC)	ESI-MS (M + H)$^+$ required	ESI-MS (M + H)$^+$ obsd	MW (TFA salt)	CLogP[a]
5′-L-valyl-L-phenylalanyl-floxuridine	95.3	493.2	494.1	606.4	0.04
5′-L-leucyl-L-glycyl-floxuridine	93.2	417.2	417.1	544.4	−0.80
5′-L-glycyl-L-leucyl-floxuridine	96.0	417.2	417.1	544.4	−0.80
5′-L-phenylalanyl-L-tyrosyl-floxuridine	99.0	557.2	557.2	670.4	−0.14
5′-L-phenylalanyl-L-glycyl-floxuridine	98.1	451.2	451.1	578.4	−1.20
5′-L-isoleucyl-L-glycyl-floxuridine	92.7	417.2	416.9	544.4	−0.89

[a] Calculated using BioLoom.

prodrugs in the receiver and donor compartments were analyzed using HPLC.

HPLC Analysis. The concentrations of prodrugs and their metabolites 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) controlled by Waters Millennium 32 software (version 3.0.1). Samples were resolved in a Waters Xterra C_{18} reverse-phase column (5 μm, 4.6 × 250 mm) equipped with a guard column. The mobile phase consisted of 1% HFBA/water (solvent A) and 1% HFBA/acetonitrile (solvent B) with the solvent B gradient changing from 0−56% at a rate of 2%/min during a 28 min run. Standard curves generated for each prodrug, and their parent drugs were utilized for quantitation of integrated area under peaks. The detection wavelength was 254 nm, and spectra were acquired in the 220−380 nm range.

Cell Proliferation Assays. Cell proliferation studies were conducted with AsPC-1 and Capan-2 cell lines. The cells were seeded onto 96-well plates at 125,000 cells per well and allowed to attach/grow for 24 h before drug solutions were added. The culture medium (RPMI-1640 + 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 2-fold serially diluted in pH 6.0 uptake buffer from 4 to 0.25 mmol/L. Buffer alone was used as 100% viability control. The wash buffer was removed, and 25 μL of drug solution per well was added and incubated at 37 °C for 2 h with AsPC-1 cells and 4 h with Capan-2 cells in the cell incubator. After this time period, the drug solutions were removed and the cells were gently washed twice with sterile uptake buffer. Fresh culture medium was then added to each well after washing, and the cells were allowed to recover for 24 h before evaluating cell viability *via* 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt (XTT) assays. A mixture (30 μL) containing XTT (1 mg/mL) in sterile RPMI-1640 without phenol red and phenazine methosulfate (*N*-methyl dibenzopyrazine methyl sulfate in sterile PBS, 0.383 mg/mL) reagents was added to the cells and incubated at 37 °C for 1 h, after which the absorbance at 450 nm was read. GI_{50} values were calculated using GraphPad Prism version 3.0 by nonlinear data fitting.

Results

Floxuridine Prodrug Synthesis. Dipeptide floxuridine prodrugs were synthesized using a method similar to that described earlier.[11,13] The total prodrug yield for each dipeptide was >15%, and the purity for all prodrugs was >90% as determined by HPLC, where the impurity was the parent drug, floxuridine. All prodrug structures and their identities were confirmed by ESI-MS and NMR. The data for prodrug purity and mass are shown in Table 1.

Stability Studies. The experiments concerning prodrug stability were performed at 37 °C in pH 7.4 phosphate buffer. The estimated half-lives ($t_{1/2}$) obtained from linear regression of pseudo-first-order plots of prodrug concentration vs time for floxuridine prodrugs in pH 7.4 phosphate buffers alone and in Caco-2, AsPC-1, and Capan-2 cell homogenates are listed in Table 2. Prodrug metabolites such as floxuridine and 5-FU were monitored along with prodrug disappearance in this experiment. However, mass balance was not achieved because 5-FU was metabolized even further and those metabolites beyond 5-FU were not quantified (Figure 1). 5′-L-Isoleucyl-floxuridine exhibited the highest stability in all media tested. Dipeptide prodrugs with at least one aromatic amino acid moiety were next-best with regard to stability in enzyme-containing solutions such as plasma and cell homogenates, and those with two aromatic amino acids also exhibited chemical stability that was similar to that of 5′-L-isoleucyl-floxuridine. All prodrugs exhibited 3- to 30-fold shorter half-lives in cell homogenates than in pH 7.4 phosphate buffer suggesting enzyme-catalyzed hydrolysis. With a few exceptions, the half-lives of all monoester and dipeptide prodrugs tested exhibited similar trends in the three cell homogenates and showed good linear correlations ($r^2 = 0.87-0.93$). The composition of the amino acids in the dipeptide moiety exerted a profound effect on the stability of the ester bond regardless of the mode of attachment. Thus, glycyl-containing dipeptide prodrugs were less stable in buffer alone compared to dipeptide prodrugs containing one or two aromatic amino acids. Glycyl-containing dipeptide prodrugs were also less stable than the 5′-L-glycyl ester prodrug. A comparison of the stability of 5′-L-isoleucyl-floxuridine in various media with 5′-L-isoleucyl-L-glycyl-floxuridine dramatically illustrates this effect (Table 2).

Table 2. Stability of Floxuridine Prodrugs in pH 7.4 Buffer and Biological Media[a]

	$t_{1/2}$ (min)				
			cell homogenates		
prodrug	buffer pH 7.4	human plasma	Caco-2	AsPC-1	Capan-2
5′-L-valyl-floxuridine	304.0 ± 33.3	131.5 ± 54.1	9.4 ± 0.5	18.7 ± 6.7	5.2 ± 2.4
5′-L-phenylalanyl-floxuridine	187.0 ± 19.0	62.8 ± 0.8	11.1 ± 9.9	11.8 ± 1.7	3.0 ± 0.1
5′-L-leucyl-floxuridine	83.2 ± 1.7	82.4 ± 8.7	3.2 ± 0.2	2.0 ± 0.1	4.7 ± 2.1
5′-L-isoleucyl-floxuridine	1194.5 ± 660.6	271.4 ± 3.2	192.3 ± 31.8	198.0 ± 70.2	139.9 ± 15.3
5′-L-glycyl-floxuridine	85.5 ± 3.2	72.1 ± 15.0	24.1 ± 2.0	27.6 ± 5.8	49.7 ± 5.6
5′-L-valyl-L-phenylalanyl-floxuridine	104.7 ± 7.0	76.0 ± 14.1	57.6 ± 9.3	51.6 ± 4.2	56.2 ± 12.8
5′-L-leucyl-L-glycyl-floxuridine	23.2 ± 4.1	6.7 ± 0.3	4.1 ± 0.1	3.6 ± 0.8	3.9 ± 1.1
5′-L-glycyl-L-leucyl-floxuridine	35.7 ± 0.9	24.6 ± 0.3	25.4 ± 2.7	13.0 ± 1.4	29.2 ± 0.7
5′-L-phenylalanyl-L-tyrosyl-floxuridine	233.9 ± 6.6	80.6 ± 3.7	103.8 ± 55.5	59.7 ± 1.4	42.8 ± 0.0
5′-L-phenylalanyl-L-glycyl-floxuridine	132.1 ± 10.2	9.7 ± 0.8	6.3 ± 0.6	10.2 ± 0.3	4.3 ± 0.9
5′-L-isoleucyl-L-glycyl-floxuridine	33.5 ± 2.4	9.5 ± 0.5	20.5 ± 1.1	25.1 ± 5.8	18.7 ± 1.4

[a] Mean ± SD, $n = 3$.

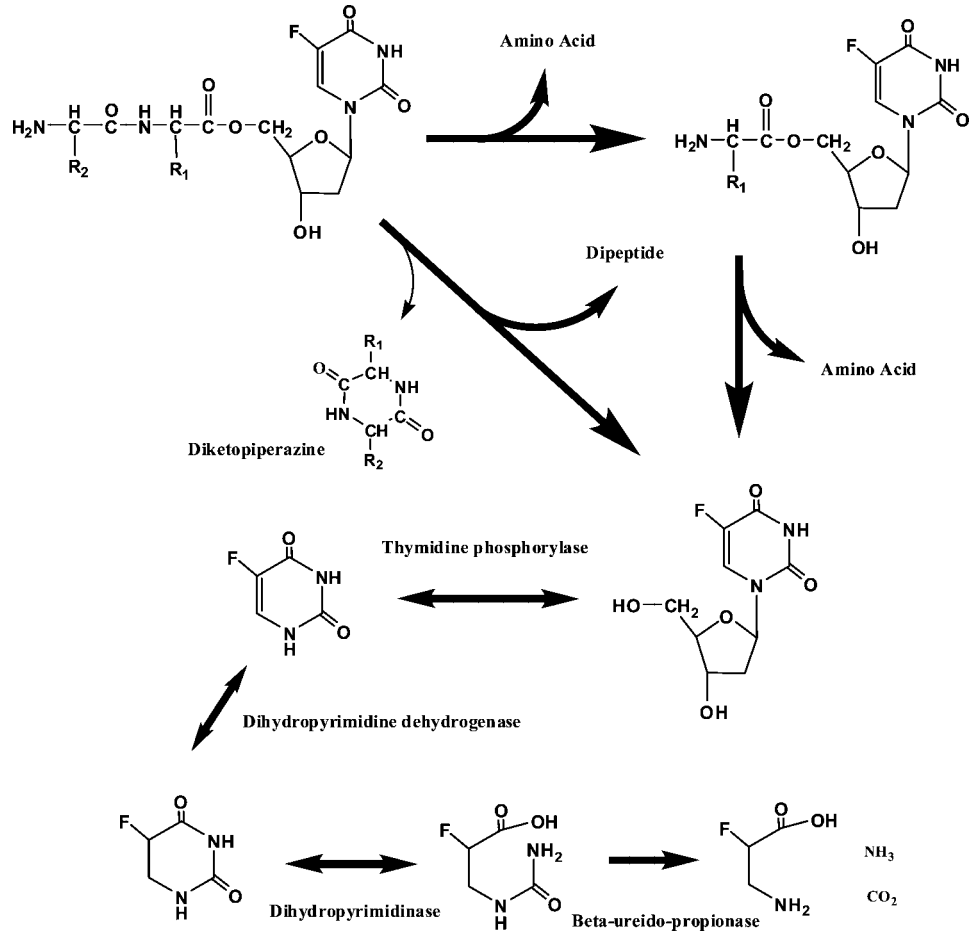

Figure 1. The metabolic pathway of floxuridine and floxuridine prodrugs with enzymes.

Thymidine Phosphorylase Activity against Floxuridine and Prodrugs. The metabolic stability of floxuridine and its amino ester prodrugs was assessed using thymidine phosphorylase. The results shown in Table 3 indicate that floxuridine was rapidly degraded to the less active metabolite, 5-fluorouracil, by thymidine phosphorylase. The amino acid ester prodrugs of floxuridine were found to be quite resistant to degradation by thymidine phosphorylase. Prodrugs containing the glycyl moiety, 5′-L-glycyl, 5′-L-leucyl-L-glycyl, 5′-L-glycyl-L-leucyl, 5′-L-phenylalanyl-L-glycyl, and 5′-L-isoleucyl-L-glycyl floxuridine were 20- to 40-fold more stable than floxuridine to degradation by thymidine phosphorylase. The half-lives of 5′-L-phenylalanyl-floxuridine, 5′-L-isoleucyl-floxuridine, 5′-L-valyl-L-phenylalanyl-floxuridine, and 5′-L-phenylalanyl-L-tyrosyl-floxuridine were in excess of 500 min reflecting their superior resistance to metabolic degradation by thymidine phosphorylase. The results are consistent with the stability of the prodrugs in buffer systems.

Table 3. Stability of Floxuridine and Floxuridine Prodrugs in the Presence of Thymidine Phosphorylase[a]

prodrug/drug	$t_{1/2}$ (min)
floxuridine	6 ± 3
5′-L-phenylalanyl-floxuridine	>500
5′-L-isoleucyl-floxuridine	>500
5′-L-glycyl-floxuridine	250 ± 54
5′-L-valyl-L-phenylalanyl-floxuridine	>500
5′-L-leucyl-L-glycyl-floxuridine	138 ± 11
5′-L-glycyl-L-leucyl-floxuridine	142 ± 10
5′-L-phenylalanyl-L-tyrosyl-floxuridine	>500
5′-L-phenylalanyl-L-glycyl-floxuridine	119 ± 30
5′-L-isoleucyl-L-glycyl-floxuridine	223 ± 54

[a] Mean ± SD, $n = 3$.

Uptake Inhibition Studies. IC_{50} values of the amino acid/dipeptide monoester prodrugs of floxuridine for PEPT1 determined using inhibition of Gly-Sar uptake in Caco-2, AsPC-1, and Capan-2 cells are summarized in Table 4. All ester prodrugs of floxuridine exhibited greater affinity for PEPT1 than the parent drug. Floxuridine showed minimal inhibition of Gly-Sar uptake, and IC_{50} values were 8.43 ± 2.66 mM, 6.63 ± 1.52 mM, and 16.06 ± 6.71 mM in Caco-2, AsPC-1, and Capan-2 cells, respectively. 5′-L-Isoleucyl-L-glycyl-floxuridine displayed the highest affinity for oligopeptide transporters in Caco-2 cells ($IC_{50} = 0.39 \pm 0.01$ mM) and in AsPC-1 cells ($IC_{50} = 0.29 \pm 0.00$ mM) and was second-best in Capan-2 cells ($IC_{50} = 0.44 \pm 0.02$ mM). With the exception of 5′-L-leucyl and 5′-L-glycyl dipeptide analogs with Caco-2 cells, all dipeptide prodrugs of floxuridine exhibited 2- to 14-fold higher affinity for oligopeptide transporters than the corresponding 5′-L-monoester prodrug. In particular, dipeptide prodrugs with at least one aromatic amino acid component consistently exhibited 3- to 5-fold higher affinity for the oligopeptide transporters than the corresponding monoester prodrug in all three cell lines tested.

Caco-2 and Capan-2 Cell Permeability of Prodrugs and Floxuridine. The apical-to-basolateral permeability of mono amino acid/dipeptide monoester prodrugs of floxuridine and parent floxuridine were determined at 37 °C in Caco-2 and Capan-2 cell monolayers. Table 5 shows the permeability values in the two cell systems. All ester prodrugs of floxuridine exhibited 4- to 20-fold higher permeability than floxuridine in Caco-2 cells. With the exception of 5′-L-valyl-L-phenylalanyl-floxuridine, the permeability of all other dipeptide prodrugs of floxuridine across Caco-2 monolayers was 2- to 4-fold higher than the corresponding 5′-L-mono amino acid ester floxuridine prodrug. The permeability trends in Caco-2 monolayers are consistent with the affinity data observed in uptake inhibition studies; however, no simple correlations between permeability and affinity were evident. Floxuridine was impermeant with Capan-2 cell monolayers; neither floxuridine nor its metabolite 5-FU could be detected suggesting the possibility of upregulation of metabolic enzymes such as TP and dihydropyrimidine dehydrogenase (DPD) in Capan-2 cells. DPD is the rate-limiting enzyme involved in the catabolism of pyrimidines and is also the main enzyme involved in the degradation of structurally related compounds like 5-FU (Figure 1). Floxuridine permeability was indeed observed in the presence of DPD inhibitors, dipyridamole and cisplatin; however, it was 1,000- or 10,000-fold lower than that obtained with Caco-2 cells (data not shown). The permeability of 5′-L-valyl-L-phenylalanyl-floxuridine across Caco-2 monolayers was quite low. 5′-L-Phenylalanyl-L-tyrosyl-floxuridine, however, exhibited dramatically lower permeability in Capan-2 cells compared to its permeability in Caco-2 cells. With the exception of 5′-L-phenylalanyl-L-tyrosyl-floxuridine, the permeability of the floxuridine ester prodrugs in Caco-2 cells was about 2-fold higher than the corresponding value in Capan-2 cells ($r^2 = 0.83$). Unlike Caco-2 monolayers, general permeability enhancement effects with dipeptide prodrugs compared to the corresponding 5′-L-mono amino ester prodrug were not apparent with Capan-2 cell monolayers.

Cell Proliferation Assays. GI_{50} values for floxuridine and its 5′-mono amino acid/dipeptide monoester prodrugs determined in cell proliferation studies with the pancreatic cancer cell lines, AsPC-1 and Capan-2, are shown in Table 6. All prodrugs exhibit 4- to 8-fold enhanced antiproliferative activity in the two cell lines compared to parent floxuridine. Thus, the GI_{50} values of all floxuridine prodrugs were in the range of 1.65−3.36 mM in AsPC-1 and Capan-2 cells as opposed to GI_{50} values of 22.85 mM and 17.63 mM for floxuridine in AsPC-1 and Capan-2 cells, respectively. These results are consistent with trends observed in Gly-Sar uptake inhibition studies. Gly-Sar and Gly-Pro did not inhibit the growth rate of Capan-2 cells. Additionally, 5′-D-valyl-floxuridine exhibited antiproliferative activity similar to that of floxuridine and Gly-Sar, despite an affinity for PEPT1 that was similar to that of other mono amino acid/dipeptide monoester prodrugs of floxuridine.

Discussion

Amino acid ester prodrugs have been widely employed to improve intestinal absorption of poorly permeant drugs. The antivirals valacyclovir and valganciclovir [8,38] are early examples of the clinical and commercial success of amino acid ester prodrug strategies. The enhanced oral bioavailability of these prodrugs [24,39] has been attributed to their enhanced transport by intestinal oligopeptide transporters,[1,15,40] and to their efficient bioconversion to the parent drug by

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Table 4. [^{3}H]Gly-Sar Uptake Inhibition of Floxuridine and Floxuridine Prodrugs in Caco-2, AsPC-1, and Capan-2 Cells[a]

	IC_{50}		
prodrug/drug	Caco-2 (mM)	AsPC-1 (mM)	Capan-2 (mM)
floxuridine	8.43 ± 2.66	6.63 ± 1.52	16.06 ± 6.71
5′-L-valyl-floxuridine	1.88 ± 0.18	2.91 ± 0.38	2.41 ± 0.28
5′-L-phenylalanyl-floxuridine	1.99 ± 0.82	1.97 ± 0.09	3.71 ± 2.13
5′-L-leucyl-floxuridine	3.51 ± 0.11	2.60 ± 0.16	1.45 ± 0.07
5′-L-isoleucyl-floxuridine	0.72 ± 0.03	4.12 ± 1.75	2.38 ± 0.12
5′-L-glycyl-floxuridine	2.28 ± 0.59	2.71 ± 0.53	2.79 ± 0.08
5′-L-valyl-L-phenylalanyl-floxuridine	2.88 ± 0.01	1.03 ± 0.08	0.46 ± 0.02
5′-L-leucyl-L-glycyl-floxuridine	3.80 ± 0.60	0.51 ± 0.03	0.34 ± 0.01
5′-L-glycyl-L-leucyl-floxuridine	5.49 ± 1.48	1.89 ± 0.11	1.67 ± 0.05
5′-L-phenylalanyl-L-tyrosyl-floxuridine	0.66 ± 0.25	0.61 ± 0.18	1.20 ± 0.16
5′-L-phenylalanyl-L-glycyl-floxuridine	0.78 ± 0.56	0.88 ± 0.22	0.79 ± 0.02
5′-L-isoleucyl-L-glycyl-floxuridine	0.39 ± 0.01	0.29 ± 0.00	0.44 ± 0.02

[a] Mean ± SD, $n = 3$.

Table 5. Apparent Permeability Coefficients (P_{app}) of Floxuridine and Its Amino Acid Ester Prodrugs in the Apical-to-Basolateral Direction Across Caco-2 and Capan-2 Monolayers[a]

	P_{app} ($\times 10^6$ cm/s)	
prodrug/drug	Caco-2	Capan-2
floxuridine	0.69 ± 0.10	0.00 ± 0.00
5′-L-phenylalanyl-floxuridine	4.09 ± 0.52	2.20 ± 1.33
5′-L-glycyl-floxuridine	3.76 ± 0.46	1.67 ± 0.42
5′-L-isoleucyl-floxuridine	2.56 ± 0.13	0.76 ± 0.10
5′-L-valyl-L-phenylalanyl-floxuridine	0.83 ± 0.09	1.16 ± 0.42
5′-L-leucyl-L-glycyl-floxuridine	3.79 ± 0.56	1.50 ± 0.15
5′-L-glycyl-L-leucyl-floxuridine	6.29 ± 2.18	1.42 ± 0.08
5′-L-phenylalanyl-L-tyrosyl-floxuridine	7.50 ± 0.78	0.24 ± 0.10
5′-L-phenylalanyl-L-glycyl-floxuridine	12.60 ± 1.61	6.46 ± 2.09
5′-L-isoleucyl-L-glycyl-floxuridine	11.40 ± 1.10	4.09 ± 2.90

[a] Mean ± SD, $n = 3$.

valacyclovirase.[26,27] A variety of dipeptide and tripeptide compounds and prodrugs have also been investigated for their suitability as substrates for the PEPT1 transporter.[4,6,17,18,22,35,41−43] We had previously reported the synthesis and evaluation of mono amino acid ester prodrugs of antiviral and anticancer drugs such as floxuridine,[11,13,14] gemcitabine,[2] acyclovir,[15] and 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (BDCRB).[2,44] These studies revealed that mono amino acid ester prodrugs in general provide enhanced PEPT1-mediated transport, a range of bioactivation rates, and

Table 6. Cell Growth Inhibition in AsPC-1 and Capan-2 Cells[a]

	GI_{50} (mM)	
prodrug	AsPC-1	Capan-2
floxuridine	22.9 ± 5.7	17.6 ± 2.2
Mono Amino Acid Prodrugs		
5′-L-phenylalanyl-floxuridine	1.8 ± 0.1	2.4 ± 0.2
5′-L-glycyl-floxuridine	2.6 ± 0.5	3.4 ± 0.3
5′-L-isoleucyl-floxuridine	3.9 ± 0.8	2.8 ± 0.4
5′-L-leucyl-floxuridine	2.9 ± 0.4	6.8 ± 4.1
5′-L-valyl-floxuridine	3.9 ± 0.1	3.0 ± 0.1
Dipeptide Prodrugs		
5′-L-valyl-L-phenylalanyl-floxuridine	1.8 ± 0.5	1.8 ± 0.2
5′-L-leucyl-L-glycyl-floxuridine	1.7 ± 0.1	3.0 ± 0.3
5′-L-glycyl-L-leucyl-floxuridine	1.8 ± 0.3	2.0 ± 0.3
5′-L-phenylalanyl-L-tyrosyl-floxuridine	4.0 ± 0.7	2.2 ± 0.3
5′-L-phenylalanyl-L-glycyl-floxuridine	2.8 ± 0.3	2.6 ± 0.5
5′-L-isoleucyl-L-glycyl-floxuridine	7.0 ± 3.1	2.4 ± 0.4
Controls		
5′-D-valyl-floxuridine	nd[b]	19.6 ± 2.9
glycylsarcosine (Gly-Sar)	nd	25.7 ± 8.1
glycylproline (Gly-Pro)	nd	29.5 ± 6.5

[a] Mean ± SD, $n = 3$. [b] Not determined.

enhanced glycosidic bond resistance to metabolic enzymes such as thymidine phosphorylase and cytidine deaminase. In this report, we describe the synthesis of dipeptide monoester prodrugs of floxuridine and their chemical stability, bioactivation and transport in Caco-2 cells, a surrogate for intestinal transport, and in two pancreatic duct cell lines, AsPC-1 and Capan-2, that overexpress the PEPT1 transporter. We also conducted similar studies with the mono amino acid analogues for comparison.

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The dipeptide prodrugs appeared to be less stable in pH 7.4 buffers than the corresponding mono amino acid ester prodrugs. Since no mono amino ester prodrug degradation products were detected, it is quite likely that the dipeptide monoester prodrugs degrade *via* parallel pathways similar to those suggested for Gly-Phe dipeptide alkyl ester prodrugs by Larsen and colleagues.[45] Thus, in addition to hydrolysis of the ester bond producing the dipeptide, a diketopiperazine cyclization product is also possible due to intramolecular condensation of the ester group with the free amino group of the dipeptide monoester prodrug. It has been reported that the rate of intramolecular aminolysis is comparable to that of ester hydrolysis and that cyclization is negligible at pH values below 6.[45−47] Indeed, the formation of diketopiperizine was observed in a chemical stability study at pH 10 but not at lower pH values (data not shown). The stability of the prodrugs in buffer was clearly influenced by the prodrug moiety of amino acids; dipeptide prodrugs containing glycyl and leucyl moieties were less stable than those containing phenylalanyl dipeptide prodrugs.

Dipeptide prodrugs with two aromatic residues were the most stable in buffer as well as in cell homogenates. The enzymatic stabilities of 5′-L-phenylalanyl-L-tyrosyl-floxuridine and 5′-L-valyl-L-phenylalanyl-floxuridine were significantly enhanced compared to the other prodrugs, suggesting that bulky amino acids such as tyrosine and phenylalanine protect against enzyme-catalyzed hydrolysis of the ester linkage. The stability profiles of 5′-L-phenylalanyl-L-tyrosyl-floxuridine and 5′-L-valyl-L-phenylalanyl-floxuridine in cell homogenates, particularly Caco-2, suggest that activation to the parent drug following transport would be much slower than monoester prodrugs such as 5′-L-valyl-floxuridine and reference prodrugs such as valacyclovir. The improved stability in biological surrogate media would facilitate prolonged systemic circulation of intact prodrugs for enhanced therapeutic action.

The results of the affinity studies of the mono amino acid ester prodrugs for the oligopeptide transporter in Caco-2 cells were generally consistent with previous findings in our laboratory,[14] as well as with amino acid prodrugs of acyclovir reported by Beuchamp and colleagues.[48] No significant trends were noticeable regarding the affinity of the mono amino acid ester prodrugs in AsPC-1 and Capan-2 cells. With the exception of leucyl floxuridine, the affinities of the mono amino ester prodrugs were lower in AsPC-1 and Capan-2 compared to those observed with Caco-2 cells. Dipeptide monoester prodrugs exhibited enhanced affinity in all cell lines depending on the nature of the N-terminal amino acid moiety. Thus, attachment of isoleucyl, phenylalanyl, or leucyl groups to glycyl floxuridine yielded 3- to 9-fold enhancement in affinity for the transporter. However, attachment of a glycyl promoiety to the N-terminus of leucyl floxuridine did not result in affinity enhancement. These findings are consistent with previous observations on the importance of the amino acid composition at the N-terminus in improving affinity for the PEPT1 oligopeptide transporter.[22]

The results of apparent permeability of the floxuridine prodrugs across Caco-2 monolayers are consistent with the affinity trends observed in Gly-Sar uptake inhibition studies. In light of previous studies with mono amino acid prodrugs of floxuridine that revealed excellent linear correlations between Caco-2 permeability and PEPT1-mediated transport in HeLa/PEPT1 cells,[13] the enhanced permeability of the dipeptide monoester prodrugs across Caco-2 monolayers may indicate enhanced PEPT1-mediated transport of the dipeptide prodrugs. The extremely low permeability of 5′-L-valyl-L-phenylalanyl-floxuridine in Caco-2 and Capan-2 cells and that of 5′-L-phenylalanyl-L-tyrosyl-floxuridine in Capan-2 cells are not consistent with permeability profiles of 5′-L-valyl-floxuridine in Caco-2 cells reported earlier[13] or of 5′-L-phenylalanyl-floxuridine in this study. The low permeability of these prodrugs is similar to the low permeability across Caco-2 monolayers observed for monoester prodrugs containing L-valyl-L-tyrosyl dipeptide promoieties.[17] The estimated CLogP values (Table 1) of these two were indicative of their being the most lipophilic prodrugs examined, and the contribution of MDR and MRP efflux transporters in permeability studies, therefore, was tested. However, the permeability of 5′-L-phenylalanyl-L-tyrosyl-floxuridine in Capan-2 cells was not affected by 1 mM verapamil, a known efflux pump inhibitor (data not shown).

The permeabilities of the floxuridine prodrugs were consistently lower in Capan-2 cells compared to their corresponding values in Caco-2 monolayers. Dipeptide monoester prodrugs did not exhibit any significant enhancement in permeability compared to the mono amino acid ester prodrugs in Capan-2 cells. Although the permeability across Capan-2 cells for all prodrugs was significantly higher than that of floxuridine alone, meaningful trends based on structure−activity correlations between transporter affinity and membrane permeability are not evident with the limited set of promoieties examined in this study.

The detection of only 5-FU in the basolateral receiver compartment following transport of floxuridine across Caco-2 monolayers suggests the instability of the glycosidic bond of floxuridine. The extent of conversion of prodrugs to 5-FU following transport was substantially lower in Caco-2 and Capan-2 cells. The average percent 5-FU observed in the basolateral compartment in Caco-2 monolayer studies (43%; range 0−92%) was higher than the corresponding average with Capan-2 monolayers (15%; range 0−35%). In general, conversion of dipeptide prodrugs to 5-FU following transport

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across the monolayers was about 2-fold lower than that observed with mono amino ester prodrugs. The results are consistent with stability profiles of floxuridine and its prodrugs in the presence of thymidine phosphorylase, an enzyme involved in the *in vivo* for phosphorytic cleavage of floxuridine.[49] Floxuridine was rapidly cleaved by thymidine phosphorylase, while all amino acid ester prodrugs examined in this study were at least 20-fold more stable to glycosidic bond cleavage by thymidine phosphorylase (Table 3). The role of esterification of the hydroxyl groups in protecting glycosidic bond cleavage by thymidine phosphorylase, the rate-determining step in deprotection and in metabolic conversion of floxuridine to 5-FU, has been discussed in a previous study.[13]

The cell proliferation studies in the pancreatic duct cancer cell lines confirmed the enhanced potency of the amino acid ester prodrugs compared to parent floxuridine. In many cases, dipeptide prodrugs exhibited better GI_{50} values even though the GI_{50} values for dipeptide monoester prodrugs in the two cell lines were not significantly different from those obtained with mono amino acid ester prodrugs. The GI_{50} values of prodrugs did not exhibit any discernible correlations with their permeability and/or bioactivation profiles in these cells. The lack of potency enhancement of 5′-D-valyl-floxuridine in Capan-2 cells compared to floxuridine suggests that activation of the prodrugs to the parent is essential for cytotoxic action and is enzyme-specific. The different amino acid promoieties of prodrugs may contribute to the different rates of prodrug activation inside cancer cells by particular activation enzymes. Therefore, it would be difficult to discern a meaningful correlation between GI_{50} values and prodrug permeabilities with a limited experimental time course. This characteristic could lead to enzyme targeted activation of prodrugs at target sites after their membrane permeation.

Intracellular anabolism of floxuridine prodrugs may illustrate that transported drugs are converted to floxuridine and 5-FU via a sequential enzymatic pathway with higher concentrations of TP present in tumor tissue (Figure 2).[14,49] Taken together, our results indicate that the dipeptide monoester prodrugs exhibit significantly higher affinity for the PEPT1 oligopeptide transporter and 2- to 4-fold higher permeability in Caco-2 and Capan-2 cells than the corresponding mono amino acid ester prodrugs, suggesting their potential for improved oral absorption and uptake in cancer cells. Therefore, dipeptide prodrugs might possess an advantage over amino acid monoester prodrugs for cancer target delivery. Three dipeptide prodrugs of floxuridine, 5′-L-phenylalanyl-L-tyrosyl-floxuridine, 5′-L-phenylalanyl-L-glycyl-floxuridine and 5′-L-isoleucyl-L-glycyl-floxuridine, displayed significantly higher affinity for the PEPT1 oligopeptide transporter and Caco-2 permeability. The delayed enzymatic activation, enhanced metabolic resistance and superior affinity to oligopeptide transporters of dipeptide prodrugs may facilitate their prolonged systemic circulation and enhanced therapeutic action. With its display of respectable stability in biological surrogate media and its approximately 2- to 3-fold shorter half-life in cancer cell homogenates than ones in human plasma and Caco-2 cell homogenates, 5′-L-phenylalanyl-L-tyrosyl-floxuridine could be an optimal candidate for cancer cell targeting with enzyme-specific activation.

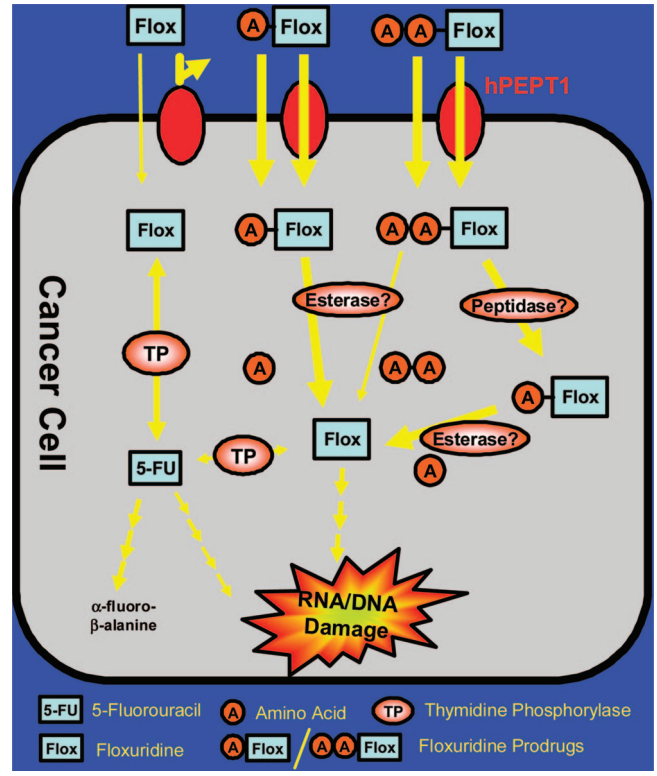

Figure 2. Intracellular anabolism of floxuridine prodrugs.

Abbreviations Used

XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate; PMS, *N*-methyl dibenzopyrazine methyl sulfate.

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