

Article

## Enhanced Absorption and Growth Inhibition with Amino Acid Monoester Prodrugs of Floxuridine by Targeting hPEPT1 Transporters

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**Abstract:** A series of amino acid monoester prodrugs of floxuridine was synthesized and evaluated for the improvement of oral bioavailability and the feasibility of target drug delivery via oligopeptide transporters. All floxuridine 5'-amino acid monoester prodrugs exhibited PEPT1 affinity, with inhibition coefficients of Gly-Sar uptake ( $IC_{50}$ ) ranging from 0.7 – 2.3 mM in Caco-2 and 2.0 – 4.8 mM in AsPC-1 cells, while that of floxuridine was 7.3 mM and 6.3 mM, respectively. Caco-2 membrane permeabilities of floxuridine prodrugs ( $1.01 - 5.31 \times 10^{-6}$  cm/sec) and floxuridine ( $0.48 \times 10^{-6}$  cm/sec) were much higher than that of 5-FU ( $0.038 \times 10^{-6}$  cm/sec). MDCK cells stably transfected with the human oligopeptide transporter PEPT1 (MDCK/hPEPT1) exhibited enhanced cell growth inhibition in the presence of the prodrugs. This prodrug strategy offers great potential, not

only for increased drug absorption but also for improved tumor selectivity and drug efficacy.

**Keywords:** 5-FU; floxuridine; prodrugs; Caco-2 permeability; cell proliferation assays; oligopeptide transporter 1 (PEPT1)

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## Introduction

The anti-metabolites 5-fluorouracil (5-FU) and floxuridine (5-fluoro-2'-deoxyuridine) continue to be mainstay drugs for colorectal cancer treatment after 50 years [1]. However, the improvement of 5-FU and floxuridine therapeutic efficacy is critical because of the poor response rate of 5-FU, erratic oral absorption of floxuridine, and the adverse effects associated with those chemotherapeutics [2]. The anabolic mechanism of 5-FU and floxuridine is well studied [3]. Floxuridine activity is quite specific for DNA related pathways and results in DNA-directed cytotoxicity with little or no RNA directed cytotoxicity, unlike 5-FU [4-6]. The utilization of the more potent floxuridine is appealing because it inhibits *in vitro* cell proliferation at 10- to 100-fold lower concentrations compared to 5-FU [7-9]. However, the abundant presence of thymidine phosphorylase (TP) in many tissues, including the liver and intestine, rapidly converts floxuridine to 5-FU [10]. Thus, improving the resistance of floxuridine to enzymatic degradation may also increase its therapeutic efficacy. With the consideration of improved chemical stability, the prodrugs must be converted to active compounds for the desired therapeutic effect. In prodrug development, the activation of the prodrug is an essential step. It has been suggested that the biphenyl hydrolase-like protein recently identified as being responsible for hydrolysis of the prodrug valacyclovir (VACVase), might be involved in the activation of other amino acid prodrugs [11]. Kim *et al.* suggest that the substrate specificity of this enzyme is largely determined by the amino acid acyl moiety of prodrug [12]. Another enzyme, carboxylesterase I, has been shown to preferentially hydrolyze phenylalanine containing nucleoside ester prodrugs, while also displaying 100-fold less activity toward aliphatic esters [13].

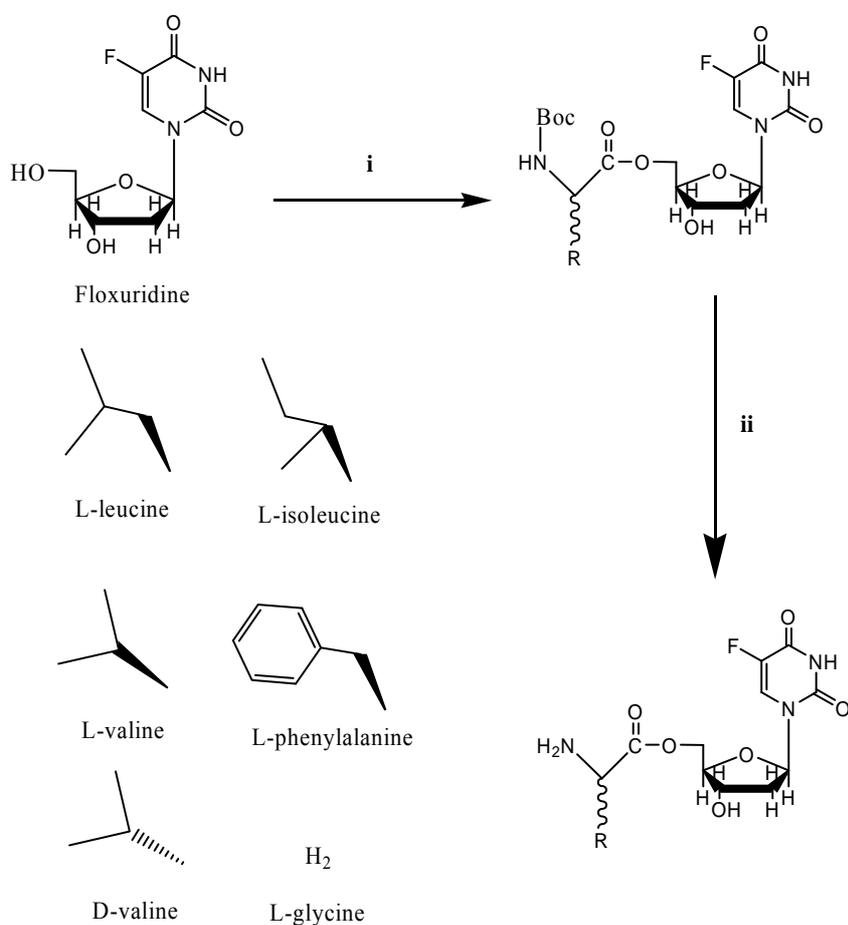
Amino acid ester prodrugs of floxuridine and the antiviral agent acyclovir have been shown to be substrates of the PEPT1 transporter [14, 15]. PEPT1 has broad substrate specificity for dipeptides, tripeptides, and  $\beta$ -lactam antibiotics [16-23]. Improved oral bioavailability of the valyl ester prodrug of acyclovir has been attributed to the presence of oligopeptide transporters [24, 25]. Two pancreatic cancer cell lines, AsPC-1 and Capan-2, have been reported to have significant expression of oligopeptide transporters, where they might represent possible targets for cancer therapy [26]. Selective growth inhibition studies in *in vitro* cell systems exogenously expressing PEPT1 have demonstrated more accumulation of cancer drug in tumor cells for an enhanced therapeutic effect [15, 27]. Those results support the notion that the moieties that incorporate amino acids, dipeptides, and tripeptides are well recognized by PEPT1, PEPT2, and ATB<sup>0+</sup> transporters [17, 28-32]. Thus, amino acid modification of cancer drugs represents a potential drug delivery strategy to target cells via transporters.

In this report, we briefly describe the synthesis and characterization of mono amino acid ester prodrugs of floxuridine. We evaluate the prodrug stability, Caco-2 membrane permeability and the feasibility of selective tumor growth inhibitory effect in MDCK and MDCK/hPEPT1 cells by cell proliferation assays.

## Results and Discussion

Prodrug approaches with amino acid modification have been widely employed to improve intestinal absorption of poorly permeant drugs [33]. The antiviral drug valacyclovir is an example of a successful amino acid ester prodrug strategy [34]. The improved oral bioavailability of valacyclovir has been attributed to the enhanced transport by intestinal oligopeptide transporters [14, 24, 35]. Dipeptide and tripeptide compounds, along with mono amino acid derivatives, have been investigated for their suitability as substrates for the oligopeptide transporter [16-18, 21, 28, 36-39]. Mono amino acid ester prodrugs of antiviral and anticancer drugs such as gemcitabine, acyclovir, and 2-bromo-5,6-dichloro-1-( $\beta$ -D-ribofuranosyl)benzimidazole (BDCRB) have been synthesized and evaluated for their suitability as transporter substrates in our previous reports [15, 24, 40-43]. Mono amino acid floxuridine prodrugs reported herein were synthesized as shown in Scheme 1.

**Scheme 1.** Synthesis of amino acid ester prodrugs of floxuridine.



Reagents: (i) *N*-*t*-BOC-protected amino acids, DCC, DMAP, DMF; (ii) TFA, CH<sub>2</sub>Cl<sub>2</sub>

The total prodrug yields for each amino acid ranged between 2 and 18 % and the purity for all prodrugs was > 95 %, as determined by HPLC. The impurities were easily separated from their parent compounds by reverse-phase HPLC. All prodrug structures and identities were confirmed by ESI-MS and NMR. The prodrug purity and mass spectral data are shown in Table 1.

**Table 1.** Analytical data for amino acid ester prodrugs of floxuridine.

	% Purity (HPLC)	ESI-MS (M + H) <sup>+</sup>		Molecular Weight (TFA salt)	log P <sup>*</sup>
		Required	Observed		
5'-O-L-leucyl-floxuridine	96.77	360.2	360.4	473.4	-0.95
5'-O-L-phenylalanyl-floxuridine	96.10	394.2	394.0	507.4	-0.51
5'-O-L-valyl-floxuridine	98.51	346.2	346.0	459.4	-1.30
5'-O-D-valyl-floxuridine	99.52	346.2	346.0	459.4	-1.30
5'-O-L-isoleucyl-floxuridine	95.96	360.2	360.4	473.4	-0.78
5'-O-L-glycyl-floxuridine	95.23	304.2	303.9	417.4	-2.68

\* Calculated using ChemDraw 7.0.

The experiments concerning prodrug stability were performed at 37°C in pH 7.4 phosphate buffers and Caco-2, AsPC-1, and MDCK cell homogenates. Table 2 displays the estimated half-lives ( $t_{1/2}$ ) obtained from linear regression of pseudo-first-order plots of prodrug concentration vs. time for the floxuridine prodrugs in pH 7.4 phosphate buffers alone and in Caco-2, AsPC-1, and MDCK cell homogenates.

**Table 2.** Half-lives of the hydrolytic degradation of floxuridine prodrugs in pH 7.4 buffer, and in homogenates from Caco-2 cells, AsPC-1 cells, and MDCK cells.

Prodrug	Buffer pH 7.4	Half Life (min)		
		Homogenates from Caco-2 cells	Homogenates from AsPC-1 cells	Homogenates from MDCK cells
Floxuridine	nd	5.7 ± 0.3	6.4 ± 3.2	68.9 ± 12.8
5'-O-L-valyl-floxuridine	303.9 ± 17.8	9.4 ± 0.6	18.7 ± 6.7	74.7 ± 5.3
5'-O-D-valyl-floxuridine	344.9 ± 10.2	342.6 ± 120.2	290.9 ± 48.9	311.6 ± 45.4
5'-O-L-phenylalanyl-floxuridine	221.7 ± 56.7	11.1 ± 9.9	11.8 ± 1.7	6.0 ± 0.6
5'-O-L-leucyl-floxuridine	77.3 ± 1.2	4.8 ± 0.2	2.0 ± 0.1	9.2 ± 1.1
5'-O-L-isoleucyl-floxuridine	323.5 ± 1.5 <sup>a</sup>	192.3 ± 31.8	198.0 ± 34.1	244.9 ± 18.3
5'-O-L-glycyl-floxuridine	85.5 ± 3.2	24.1 ± 2.0	27.6 ± 5.8	11.2 ± 2.0

Values are presented as mean ± S.D.; nd = not determined; <sup>a</sup>from Ref. [40].

The 5'-O-D-valyl-floxuridine prodrug exhibited the highest stability in all conditions and thus confirmed previous reported results [44, 45]. These results indicate that there does not appear to be any significant enzymatic component in the breakdown of the 5'-O-D-valyl ester prodrug in cell homogenates. All prodrugs except 5'-O-L-glycyl-floxuridine and 5'-O-L-leucyl-floxuridine exhibited the same degree of stability in phosphate buffers. The phenylalanine monoester prodrug exhibited 20-fold less stability in cell homogenates compared to pH 7.4 phosphate buffers. This result suggests that the phenylalanine moiety was recognized by enzymes more readily than other amino acid residues and its ester prodrugs were enzymatically degraded. This observation agrees well with our previous enzymatic hydrolysis results [13]. The stability of the 5'-O-D-valyl, 5'-O-L-phenylalanyl, 5'-O-L-isoleucyl, and 5'-O-L-glycyl prodrugs were comparable in both Caco-2 and AsPC-1 cell homogenates. On the other hand, 5'-O-L-valyl and 5'-O-L-leucyl prodrugs exhibited significantly different half-lives in each cell homogenate and were stable in MDCK cell homogenates. In Caco-2 cell homogenates, the 5'-O-L-valyl ester prodrug displayed a 2-fold shorter half-life than in AsPC-1 cell homogenates, while 5'-O-L-leucyl ester prodrug was hydrolyzed 2.4- and 4.7-fold more quickly in AsPC-1 cells homogenates than in Caco-2 and MDCK cell homogenates, respectively. The IC<sub>50</sub> values of amino acid monoester prodrugs of floxuridine for an oligopeptide transporter determined using inhibition of Gly-Sar uptake in Caco-2 and AsPC-1 cells are summarized in Table 3.

**Table 3.** Concentrations of floxuridine and floxuridine prodrugs required to inhibit Gly-Sar uptake by 50 % (IC<sub>50</sub>) in Caco-2 and AsPC-1 cells.

Prodrug	IC <sub>50</sub> Value (mM)	
	Caco-2 cell	AsPC-1 cell
Floxuridine	7.3 ± 1.6	6.3 ± 2.3
5'-O-L-valyl-floxuridine	1.0 ± 0.1	2.9 ± 0.4
5'-O-D-valyl-floxuridine	2.6 ± 0.1	4.8 ± 1.3
5'-O-L-phenylalanyl-floxuridine	2.1 ± 0.1	2.0 ± 0.1
5'-O-L-leucyl-floxuridine	2.0 ± 0.3	2.6 ± 0.2
5'-O-L-isoleucyl-floxuridine	0.7 ± 0.0	4.1 ± 1.8
5'-O-L-glycyl-floxuridine	2.3 ± 0.6	2.7 ± 0.5

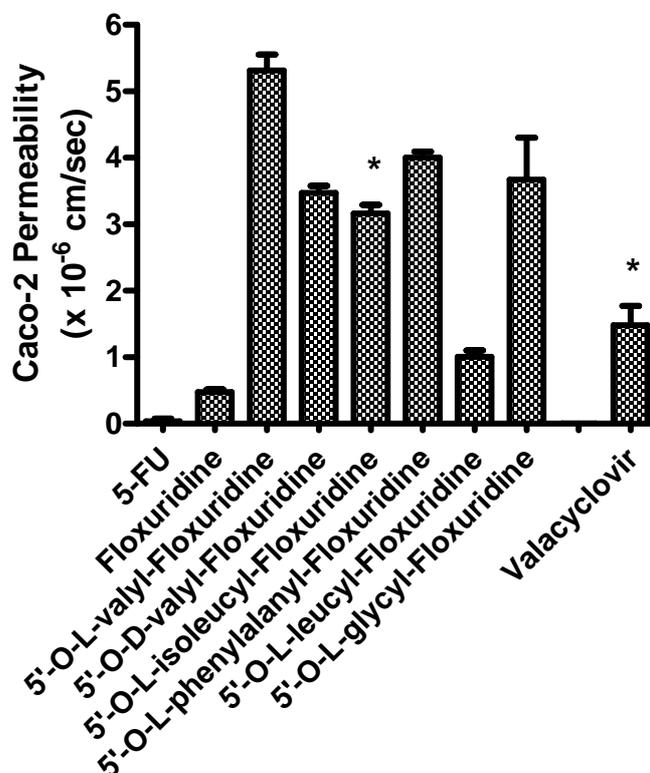
Values are presented as mean ± S.D.

All floxuridine ester prodrugs exhibited greater inhibition of Gly-Sar uptake by for hPEPT1 than did the parent drug, floxuridine. 5'-O-L-isoleucyl-floxuridine displayed the highest inhibition of oligopeptide transporters in Caco-2 cells (IC<sub>50</sub>, 0.7 ± 0.0 mM), whereas 5'-O-L-phenylalanyl-floxuridine had the highest affinity in AsPC-1 cells (IC<sub>50</sub>, 2.0 ± 0.1 mM). Not surprisingly, the D-stereoisomer ester prodrug 5'-O-D-valyl-floxuridine showed the least inhibition of Gly-Sar uptake in both cells (IC<sub>50</sub>, 2.6 ± 0.1 mM in Caco-2 cells, IC<sub>50</sub>, 4.8 ± 1.3 mM in AsPC-1 cells). 5'-O-D-valyl-floxuridine inhibited the transporters 1.6-fold to 2.5-fold less than did 5'-O-L-valyl-floxuridine in AsPC-1 cells and Caco-2 cells, respectively, but the monoester prodrug with an unnatural form of amino acid still inhibited to an extent similar to that of the parent drug. With the exception of 5'-O-L-

phenylalanyl-floxuridine, all monoester prodrugs of floxuridine exhibited better  $IC_{50}$  values in Caco-2 cells than in AsPC-1 cells, likely due to over-expression of PEPT1 transporters in AsPC-1 cells [26].

The apical-to-basolateral permeabilities of amino acid monoester prodrugs of floxuridine and their parent drugs, floxuridine and 5-FU, were determined at 37°C in Caco-2 cell monolayers. Figure 1 shows the membrane permeability values in Caco-2 cell system. Floxuridine, a prodrug form of 5-FU, showed a 12-fold higher Caco-2 membrane permeability than 5-FU. All floxuridine prodrugs exhibited 2- to 11-fold higher and 26- and 140-fold higher membrane permeability than floxuridine and 5-FU, respectively. Surprisingly, 5'-O-D-valyl-floxuridine exhibited permeability that was as high as that of other amino acid ester prodrugs. No simple correlations between membrane permeability and transporter affinity in Caco-2 cells were evident in the prodrugs, even though our group previously reported excellent linear correlations between them in HeLa/PEPT1 cells [40]. The floxuridine amino acid monoester prodrugs, with the exception of 5'-O-L-leucyl-floxuridine, demonstrated excellent membrane permeability in Caco-2 cells, better than that of a reference compound, valacyclovir (Figure 1). The detection of only 5-FU in the basolateral receiver compartment following transport of floxuridine across Caco-2 cell monolayers suggests the instability of glycosidic bond. Drug/prodrug stability in the mucosal cell would affect its drug membrane permeability and, therefore, the observation of low permeability of 5'-O-L-leucyl-floxuridine among those prodrugs may be partly due to its rapid hydrolysis to floxuridine, 5-FU and beyond (Table 2).

**Figure 1.** Caco-2 Permeability of 5-fluorouracil, floxuridine, and floxuridine prodrugs (Mean  $\pm$  S.D.).



The permeability values were calculated using the total amount of permeant drug (prodrug + floxuridine + 5-FU). \* from Ref. [40].

The concentrations of floxuridine 5' amino acid monoester required for 50% growth inhibition ( $GI_{50}$ ) of MDCK cells with and without exogenous hPEPT1 (MDCK/hPEPT1) are shown in Table 4. The prodrug  $GI_{50}$  values are significantly lower in MDCK/hPEPT1 cells, compared to those in MDCK, indicating increased growth inhibition due to enhanced drug delivery by carrier-mediated transporters. The cell growth inhibition effect was improved 18-fold with the presence of hPEPT1 transporters when cells were treated with 5'-O-L-isoleucyl-floxuridine. 5'-O-L-leucyl-floxuridine exhibited the lowest  $GI_{50}$  values (2.3  $\mu$ M in MDCK/hPEPT1 cells) and 10-fold greater growth inhibition with the expression of exogenous oligopeptide transporters. 5'-O-L-valyl-floxuridine displayed about 6-fold enhancement in cell growth inhibition, while 5'-O-D-valyl-floxuridine showed only 25% improvement of cell growth inhibition in MDCK/hPEPT1 cells. Floxuridine exhibited no enhancement (Enhancement Factor = 1.00) between MDCK and MDCK/hPEPT1 cells (data not shown). The lower observed effectiveness of 5'-O-D-valyl-floxuridine in cell growth inhibition might be due to the prodrug stability in MDCK cells (Table 2). This observation indicates the necessity of prodrug activation for cancer cell growth inhibition and for the reduction in side-effects of the prodrug in non-tumor cells.

**Table 4.** Effect of hPEPT1 in MDCK Cells for Cell Proliferation Assay.

Prodrug	$GI_{50}$ ( $\mu$ M)		Enhancement Factor
	MDCK	MDCK/hPEPT1	
5'-O-L-valyl-floxuridine	126.6 $\pm$ 7.7	21.1 $\pm$ 4.2	5.91
5'-O-D-valyl-floxuridine	88.5 $\pm$ 2.6	71.0 $\pm$ 2.7	1.25
5'-O-L-phenylalanyl-floxuridine	41.3 $\pm$ 3.9	4.7 $\pm$ 2.4	8.80
5'-O-L-leucyl-floxuridine	23.5 $\pm$ 3.0	2.3 $\pm$ 1.0	10.09
5'-O-L-isoleucyl-floxuridine	186.5 $\pm$ 4.2	10.3 $\pm$ 2.9	18.04

Values are presented as mean  $\pm$  S.D.; The prodrug concentration required to inhibit growth by 50 % ( $GI_{50}$ ) was determined in MDCK cells and MDCK cells that overexpressed hPEPT1 (MDCK/hPEPT1). The ratios of  $GI_{50}$  in MDCK and MDCK/hPEPT1 cells are presented as Enhancement Factor.

In all cases, the prodrug forms of floxuridine improved permeability in Caco-2 cells by as much as 11-fold over that of floxuridine and 140-fold over that of 5-FU. It was also demonstrated by MDCK cell growth inhibition assays that transporters could potentially serve as excellent delivery targets to improve chemotherapeutic efficacy.

## Conclusions

In summary, amino acid monoester prodrugs of floxuridine were synthesized and were demonstrated to be suitable substrates for a human PEPT1 transporter to improve oral absorption and the possibility of target drug delivery via transporters on target sites. All prodrugs exhibited better

transport values than floxuridine and 5-FU. 5'-O-D-valyl-floxuridine displayed good uptake inhibition of Gly-Sar and much less enzymatic breakdown in cell homogenates compared with 5'-O-L-valyl-floxuridine. The prodrug stability in GI tract, blood, and organs such as the liver is a critical factor for successful oral drug delivery. From this point of view, 5'-O-D-valyl-floxuridine exhibited 11 to 213-fold more enzymatic stability than the other prodrugs without compromising transporter affinity (IC<sub>50</sub>) and Caco-2 membrane permeability. Thus, 5'-O-D-valyl-floxuridine may be the best candidate for *in vivo* oral bioavailability study.

## Experimental

### Materials

Floxuridine (Floxuridine) was obtained from Lancaster (Windham, NH, USA). The *tert*-butyloxycarbonyl (Boc) protected amino acids Boc-L-Leucine, Boc-L-Phenylalanine, Boc-L-Valine, Boc-D-Valine, Boc-L-Isoleucine and Boc-L-Glycine 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). 5-Fluorouracil (5-FU), *N,N*-dicyclohexylcarbodiimide, *N,N*-dimethylaminopyridine, trifluoroacetic acid (TFA), and all other reagents and solvents were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI, 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.

### Floxuridine Prodrug Synthesis

The synthesis and characterization of 5'-mono amino acid ester prodrugs of floxuridine have been reported previously [40, 43]. The L-glycyl prodrug of floxuridine was synthesized in a similar manner. Briefly, Boc-protected amino acids (Boc-L-glycine; 1 mmol), *N,N*-dicyclohexylcarbodiimide (1 mmol), and *N,N*-dimethylaminopyridine (0.1 mmol) were allowed to react with floxuridine (1 mmol) in dry DMF (7 mL) for 24 hours. 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 x 20 mL), and saturated NaCl (20 mL). The organic layer was dried over MgSO<sub>4</sub> 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/methanol, 20:1). Fractions from each spot were concentrated under vacuum separately. The Boc group was cleaved by treating the residues with 1:1 TFA-dichloromethane (5 mL). After 4 hours, 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'-diester floxuridine prodrugs of floxuridine, was ~60%. Prodrugs were determined to be 95-99% 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 confirmed using proton nuclear magnetic resonance spectra ( $^1\text{H-NMR}$ ) were obtained in  $\text{DMSO-d}_6$  on a 300 MHz Bruker DPX-300 NMR spectrometer.

*5'-L-glycyl-floxuridine*: yield, 8.5%; percent purity, 95%;  $^1\text{H-NMR}$   $\delta$ : 2.10-2.33 (2H, m, C2'), 3.80-3.97 (3H, m,  $\alpha\text{-CH}_2$ , C3'), 4.26 (1H, m, C4'), 4.38 (2H, d,  $J = 5.0$  Hz, C5'), 6.17 (1H, t,  $J = 6.4$  Hz, C1'), 7.95 (1H, d,  $J = 7.0$  Hz, CHF); ESI-MS, 303.9 (M + H) $^+$ .

### Cell Culture

AsPC-1 cells (passages 44-49, American Type Culture Collection, Rockville, MD, USA) were routinely maintained in RPMI-1640 containing 10% fetal bovine serum and Caco-2 cells (passages 30-55) and MDCK cells (Passages 35-40) (both also from the American Type Culture Collection) 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%  $\text{CO}_2$  and 90% relative humidity at 37°C and in antibiotic-free media to avoid the possible transport interference by antibiotics.

### Hydrolysis Studies

*Enzymatic Stability.* Confluent Caco-2 cells, AsPC-1 cells, and MDCK cells were rinsed twice with saline. The cells were washed with pH 7.4 phosphate buffer (5 mL, 10 mmol/L), lysed by ultrasonication (Micro ultrasonic cell disrupter Model KT40, Kontes, Vineland, NJ, USA), and pelleted by centrifugation for 5 minutes at 1,000 x g. Protein amount was quantified with the Bio-Rad (Hercules, CA, USA) DC Protein Assay using bovine serum albumin as a standard. The amount of protein was adjusted to 500  $\mu\text{g/mL}$  and hydrolysis reactions were carried out in 96-well plates (Corning). Caco-2, AsPC-1, and MDCK cell suspensions (250  $\mu\text{L}$ ) were placed in triplicate wells, the reactions were started with the addition of substrate, and cells were incubated at 37°C for 120 minutes. At the desired time point, sample aliquots (35  $\mu\text{L}$ ) were removed and added to acetonitrile (ACN, 150  $\mu\text{L}$ ) with 0.1% TFA. The mixtures were filtered with a 0.45  $\mu\text{m}$  filters at 1,000 x g for 10 minutes at 4°C. The filtrate was then analyzed via reverse-phase HPLC.

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

### 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 post-hoc Tukey's test to compare means.

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

$$\text{Flux} = J_{ss} = dM / dt \quad (3)$$

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,  $C_0$  is the concentration of the prodrug in the donor solution. The concentrations of floxuridine and its 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, USA). The HPLC system consisted of two Waters pumps (model 515), a Waters autosampler (WISP model 712) and a Waters 996 photodiode array UV detector controlled by Waters Millennium<sup>®</sup> 32 software (version 3.0.1). Samples were resolved on a Waters Xterra C<sub>18</sub> reverse-phase column (5  $\mu$ m, 4.6 x 250 mm) equipped with a guard column. The mobile phase consisted of 0.1% TFA/water (Solvent A) and 0.1% TFA/ACN (Solvent B) with the solvent B gradient changing from 0-56% at a rate of 2%/minute during a 28 minute run. Prodrugs, their metabolites, and their parent drugs were easily separated and identified. 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.

#### [<sup>3</sup>H]Gly-Sar Uptake Inhibition

Caco-2 cells at nine days post-seeding, and AsPC-1 cells at four days post-seeding were incubated with 10  $\mu$ mol/L Gly-Sar (9.98  $\mu$ mol/L Gly-Sar and 0.02  $\mu$ mol/L [<sup>3</sup>H]Gly-Sar) along with various prodrug concentrations (5–0.05 mmol/L) for 30 minutes. The cells were washed three times with ice-cold PBS and solubilized with 10 mL of scintillation cocktail (ScintiVerse<sup>®</sup>, Fisher Scientific, St.Louis, MO, USA). The amount of cell-associated radioactivity was determined by scintillation counting (Beckman LS-9000, Beckman Instruments, Fullerton, CA, USA). The half maximal inhibitory concentration (IC<sub>50</sub>) values were determined using nonlinear data fitting (GraphPad Prism version 3.0).

### *Caco-2 permeability study*

Caco-2 cell monolayers were grown on collagen-coated polytetrafluoroethylene membrane for 21 to 24 days. Transepithelial electrical resistance (TEER) was monitored and values of 240-280  $\Omega/\text{cm}^2$  (total growth area was 4.67  $\text{cm}^2$ ) was used in the study. Apical 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 minutes. Each drug was individually tested from freshly prepared solutions in MES buffer (0.1 mM, total 1.5 mL). The solution was placed in the donor chamber, while the receiver chamber was filled with HEPES buffer (total 2.5 mL). Sampling from the receiver chamber was conducted up to a period of 2 hours at time intervals of 15, 30, 45, 60, 75, 90, and 120 minutes, 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 HPLC.

### *Cell Proliferation Assays*

Cell proliferation studies were conducted with MDCK cells and MDCK cells that express hPEPT1 (MDCK/hPEPT1). The cells were seeded into 96-well plates at 125,000 cells per well and allowed to attach/grow for 24 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 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 30  $\mu\text{L}$  drug solution per well was added and incubated at 37°C for 2 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. The culture medium was then added to each well after washing. The cells were allowed to recover for 24 hours before evaluating cell viability via 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assays. A mixture (30  $\mu\text{L}$ ) containing XTT (1 mg/mL) in sterile DMEM without phenol red and phenazine methosulfate (*N*-methylidibenzopyrazine methyl sulfate in sterile PBS, 0.383 mg/mL) reagents were added to the cells and incubated at 37°C for 1 hour, after which the absorbance at 450 nm was read. The concentrations required to inhibit cell growth by 50% ( $\text{GI}_{50}$ ) were calculated using GraphPad Prism version 3.0 by nonlinear data fitting.

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### **References and Notes**

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