Amino Acid Ester Prodrugs of Floxuridine: Synthesis and Effects of Structure, Stereochemistry, and Site of Esterification on the Rate of Hydrolysis

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Purpose. To synthesize amino acid ester prodrugs of floxuridine (FUdR) and to investigate the effects of structure, stereochemistry, and site of esterification of promoiety on the rates of hydrolysis of these prodrugs in Caco-2 cell homogenates.

Methods. Amino acid ester prodrugs of FUdR were synthesized using established procedures. The kinetics of hydrolysis of prodrugs was evaluated in human adenocarcinoma cell line (Caco-2) homogenates and pH 7.4 phosphate buffer.

Results. 3′-Monoester, 5′-monoester, and 3′,5′-diester prodrugs of FUdR utilizing proline, L-valine, D-valine, L-phenylalanine, and D-phenylalanine as promoieties were synthesized and characterized. In Caco-2 cell homogenates, the L-amino acid ester prodrugs hydrolyzed 10 to 75 times faster than the corresponding D-amino acid ester prodrugs. Pro and Phe ester prodrugs hydrolyzed much faster (3- to 30-fold) than the corresponding Val ester prodrugs. Further, the 5′-monoester prodrugs hydrolyzed significantly faster (3-fold) than the 3′,5′-diester prodrugs.

Conclusions. Novel amino acid ester prodrugs of FUdR were successfully synthesized. The results presented here clearly demonstrate that the rate of FUdR prodrug activation in Caco-2 cell homogenates is affected by the structure, stereochemistry, and site of esterification of the promoiety. Finally, the 5′-Val and 5′-Phe monoesters exhibited desirable characteristics such as good solution stability and relatively fast enzymatic conversion rates.

KEY WORDS: floxuridine; prodrug; hydrolysis; stability; amino acid; Caco-2.

INTRODUCTION

Floxuridine (FUdR, 5-fluoro-2-deoxyuridine) is a fluorinated pyrimidine that is primarily used for the treatment of metastatic carcinoma of the colon or following resection of hepatic metastases (1). FUdR and other pyrimidine analogs have been extensively used in the treatment of a variety of cancers for the past 40 years, and their mechanisms of action are well understood (2). Although clinically effective, FUdR exhibits various side effects as a result of its actions on highly mitotic tissues such as the gastrointestinal (GI) tract and bone marrow (2). Further, FUdR, like most other nucleoside anticancer agents, also suffers from low and erratic oral absorption (3). Hence, FUdR is administered as an intravenous infusion (4). Strategies that can improve the oral absorption of FUdR and reduce its toxicity can be of great benefit. In this regard, prodrug strategies offer maximum flexibility (5,6). In the past, a variety of prodrugs of FUdR were synthesized to improve its physicochemical properties and reduce toxicity. In this regard, a variety of alkyl ester prodrugs (7–10), phosphoramidate prodrugs (11), and photoactivated prodrugs (12) of FUdR have been investigated. Recently Xia *et al.* synthesized dual prodrugs of FUdR by attaching well-known cell-differentiating agents such as retinoic acid to FUdR (13).

The oral bioavailability of poorly absorbed drugs can also be enhanced by targeting them to oligopeptide transporters (14,15). Oligopeptide transporters are highly expressed in the intestine and are involved in transport of di/tri-peptides, β-lactam antibiotics, ACE inhibitors, and a variety of other clinically relevant drugs across the intestine (15,16). The enhanced oral bioavailability of valacyclovir and valganciclovir, amino acid ester prodrugs of acyclovir and ganciclovir, respectively, has been attributed to their enhanced intestinal transport via oligopeptide transporters (14,17). Further, Nakanishi *et al.* (18) and Gonzalez *et al.* (19) recently demonstrated that oligopeptide transporters are enriched in some cancer epithelial cells and can be used for delivery of peptidomimetic anticancer agents (20,21). We anticipate that amino acid ester prodrugs of anticancer agents such as floxuridine may also be transported by oligopeptide transporters and could provide enhanced oral absorption or improved targeting to cancer cells overexpressing these transporters. Although enhanced prodrug uptake via transporters is of primary importance, activation of the prodrug following its transport is also an essential step for drug activity; however, this process has not been well studied. It has generally been suggested that ubiquitous esterases and proteases are involved in such activation (22–24), but the enzymes involved have rarely been characterized. Activation of prodrugs can be influenced by both the promoiety and the drug itself. However, there are very limited structure–activity studies evaluating the influence of promoiety on the rate of hydrolysis/activation of prodrugs. An understanding of the influence of promoiety structural parameters on the rate of prodrug activation would therefore facilitate the design of prodrugs with optimum stability and activation profiles.

In an effort to determine the effects of structure, stereochemistry, and site of esterification of the promoiety on the rate and extent of prodrug activation, a variety of amino acid ester prodrugs of floxuridine (Fig. 1) were synthesized, and their hydrolysis profiles were determined in Caco-2 cell homogenates. To evaluate the effect of structure of the promoiety on the rate of hydrolysis of prodrugs, aromatic amino acid phenylalanine (Phe), aliphatic amino acid valine (Val), and secondary amino acid proline (Pro) were selected as promoieties. To evaluate the effect of stereochemistry on the rate of hydrolysis, L- and D-forms of Phe and Val were investigated. Further, to evaluate the effect of site of esterification on the rate of hydrolysis, 3′-monoester, 5′-monoester, and 3′,5′-diester prodrugs of FUdR were investigated. The hydrolysis of commercially available valine ester prodrugs of acyclovir

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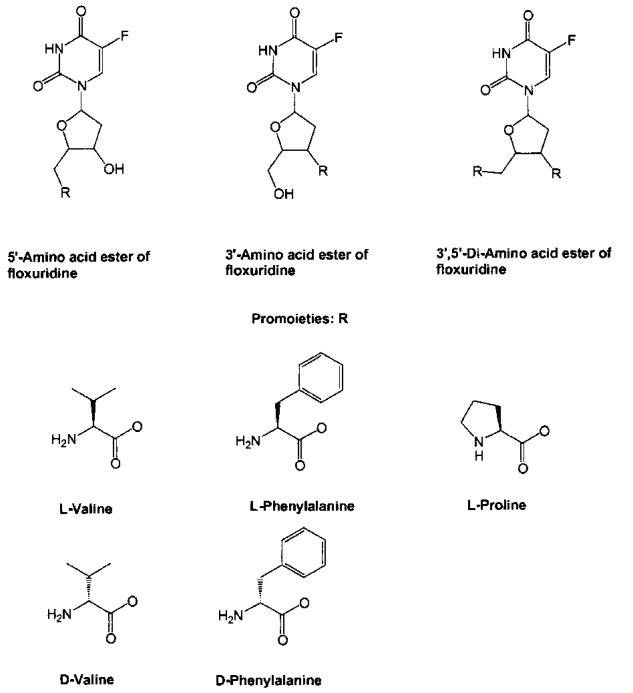

Fig. 1. Structures of amino acid ester prodrugs of floxuridine and promoieties.

and ganciclovir, valacyclovir and valganciclovir, respectively, were also evaluated for comparison.

MATERIALS AND METHODS

Materials

Floxuridine was obtained from Lancaster (Windham, NH). The *tert*-butyloxycarbonyl (Boc) protected amino acids, Boc-L-Phe, Boc-D-Phe, Boc-L-Pro, Boc-L-Val, and Boc-D-Val were obtained from Calbiochem-Novabiochem (San Diego, CA). HPLC-grade acetonitrile was obtained from Fisher Scientific Company (St. Louis, MO). *N,N*-Dicyclohexylcarbodiimide (DCC), *N,N*-dimethylaminopyridine (DMAP), trifluoroacetic acid (TFA), and all other reagents and solvents were purchased form Aldrich Chemical Company (Milwaukee, WI). Valacyclovir and valganciclovir were obtained as gifts from GlaxoSmithKline, Inc. (Research Triangle Park, NC) and Hoffmann-La-Roche, Inc. (Nutley, NJ), respectively. Cell culture reagents were obtained from Invitrogen (Carlsbad, CA), and cell culture supplies were obtained from Corning (Corning, NY) and Falcon (Lincoln Park, NJ). All chemicals were either analytic or HPLC grade.

Prodrug Synthesis

Amino acid ester prodrugs of FUdR were synthesized as described in Fig. 2. Boc-protected amino acids (Boc-Val-OH, Boc-D-Val-OH, Boc-Phe-OH, Boc-D-Phe-OH, or Boc-Pro-OH) (1.5 mmole), DCC (1.5 mmole) and DMAP (0.15 mmole) were allowed to react with FUdR (1.5 mmole) in 10 ml of dry *N,N*-dimethylformamide (DMF). The reaction mixture was stirred at room temperature for 24 h. The progress of the reaction was monitored by TLC [dichloromethane (DCM):methanol, 9:1]. Each reaction yielded three products as determined by TLC. After 24 h, the reaction mixture was filtered, and DMF removed *in vacuo* at 55–60°C. The residue was dissolved in ethyl acetate (30 ml) and washed with water (2 × 20 ml), 0.1 N HCl (1 × 20 ml), saturated $NaHCO_3$ (2 × 20 ml), and brine (1 × 20 ml). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The three intermediates observed with TLC were separated and purified using column chromatography (ethyl acetate:hexane, 8:5). The fractions belonging to each intermediate were collected and analyzed for their purity by TLC. Pure fractions were pooled and concentrated *in vacuo*. Pure intermediates were then treated with 4 ml of TFA:DCM:water (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. The combined yields of pure Val, D-Val, Phe, D-Phe, and Pro prodrugs were 32%, 33%, 37%, 34%, and 41%, respectively. The actual amounts of each prodrug obtained and the percentage yields of the individual prodrugs are reported below.

The purity of prodrugs was determined by HPLC. All prodrugs were more than 93% pure. Prodrugs were easily separated from the 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 found to be similar to those predicted from their expected structures. For prodrugs such as the 3′ and 5′ monoesters with similar molecular weights but different HPLC retention times, structural identity was confirmed using proton nuclear magnetic resonance spectra (^{1}H NMR), which were obtained on a 300-MHz Bruker DPX-300 NMR spectrometer.

3′-L-Valyl-FUdR: amount obtained (% yield), 17 mg (10%); percent purity, 93%; ^{1}H NMR (DMSO-d_6) δ, 0.95–1.01 [6H, m, $(CH_3)_2CH$]; 2.14–2.18 (1H, m, $^{\beta}$CH); 2.33–2.37 (2H, m, C2′); 3.66–3.67 (2H, m, C5′); 3.98–3.99 (1H, d, $^{\alpha}$CH); 4.08–4.09 (1H, m, C4′); 5.37–5.41 (1H, m, C3′); 6.17–6.24 (1H, t, C1′); 8.20–8.27 (1H, d, CHCF); ESI-MS; 345.9 $(M+H)^+$.

5′-L-Valyl-FUdR: amount obtained (% yield), 67 mg (2%); percent purity, 98%; ^{1}H NMR (DMSO-d_6) δ, 0.92–0.98 [6H, m, $(CH_3)_2CH$]; 2.13–2.14 (2H, m, C2′); 2.25–2.34 (1H, m, $^{\beta}$CH); 3.94–3.95 (2H, m, C3′ and $^{\alpha}$CH); 4.23–4.24 (1H, m, C4′); 4.35–4.46 (2H, m, C5′); 6.14–6.19 (1H, t, C1′); 7.92–7.95 (1H, d, CHCF); ESI-MS; 346 $(M+H)^+$.

3′,5′-L-Divalyl-FUdR: amount obtained (% yield), 185 mg (19%); percent purity, 98%; ^{1}H NMR (DMSO-d_6) δ: 0.91–1.05 {12H, m, $[(CH_3)_2CH]_2$}; 1.99–2.37 [4H, m, C2′, $(^{\beta}CH)_2$], 3.96 [2H, m, $(^{\alpha}CH)_2$]; 4.27 (1H, m, C4′); 4.39–4.58 (2H, m, C5′); 5.35–5.36 (1H, m, C3′); 6.20–6.24 (1H, t, C1′); 8.01–8.03 (1H, d, CHCF); ESI-MS; 445.1 $(M+H)^+$.

3′-D-Valyl-FUdR: amount obtained (% yield), 69 mg (10%); percent purity, 96%; ^{1}H NMR (DMSO-d_6) δ: 0.96–1.01 [6H, m, $(CH_3)_2CH$]; 2.14–2.20 (1H, m, $^{\beta}$CH); 2.33–2.34 (2H, m, C2′); 3.66 (2H, m, C5′); 3.98 (1H, d, $^{\alpha}$CH); 4.07 (1H, m, C4′); 5.36–5.37 (1H, m, C3′); 6.21–6.25 (1H, t, C1′); 8.20–8.22 (1H, d, CHCF); ESI-MS; 345.9 $(M+H)^+$.

5′-D-Valyl-FUdR: amount obtained (% yield), 70 mg (10%); percent purity, 98%, ^{1}H NMR (DMSO-d_6) δ: 0.93–0.98 [6H, m, $(CH_3)_2CH$]; 2.13–2.14 (2H, m, C2′); 2.26–2.28

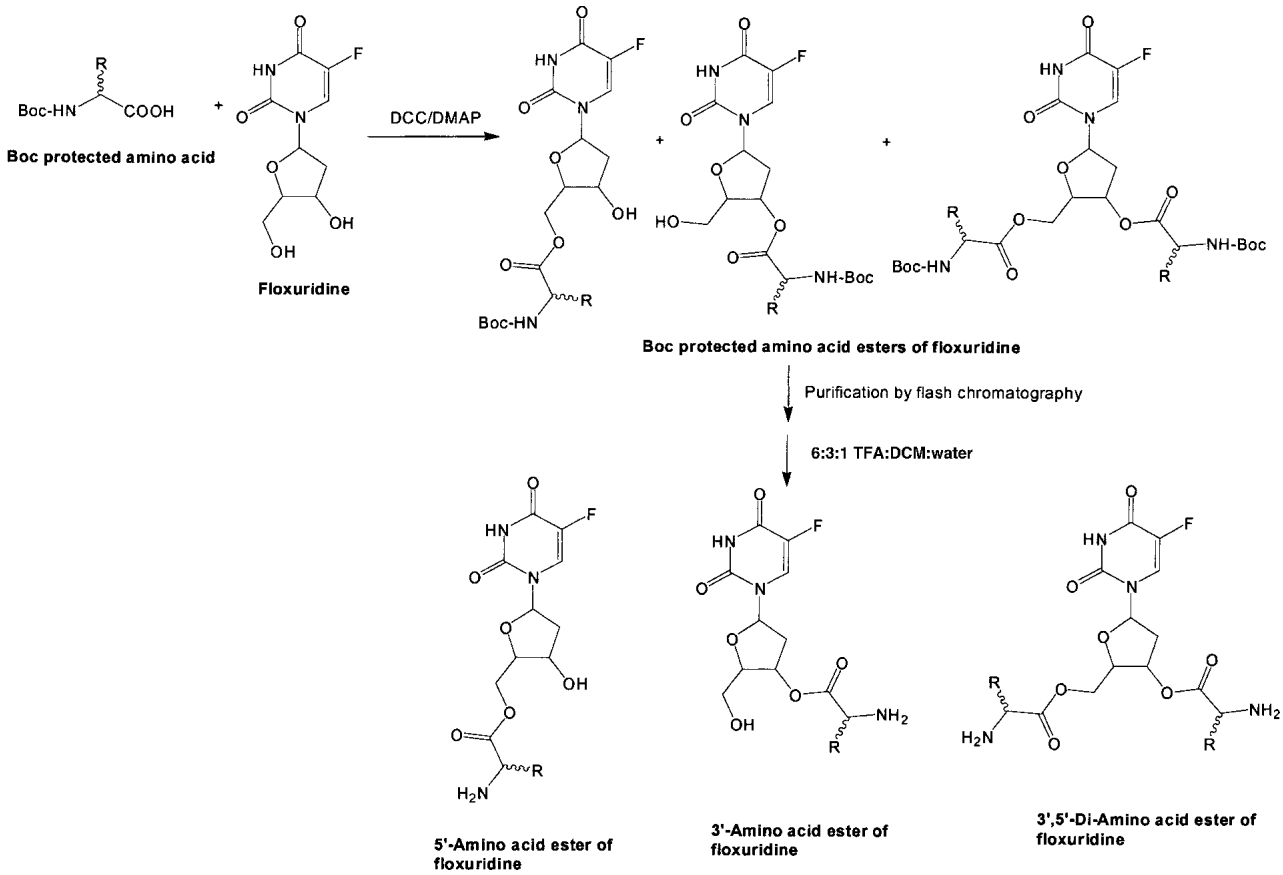

Fig. 2. Schematic of synthesis of amino acid ester prodrugs of floxuridine.

(1H, m, $^{\beta}$CH); 3.93–4.00 (2H, m, C3 and $^{\alpha}$CH), 4.23 (1H, m, C4′); 4.39 (2H, m, C5′); 6.1–6.2 (1H, t, C1′); 7.9–7.96 (1H, d, CHCF); ESI-MS; 346 $(M+H)^+$.

3′,5′-D-Divalyl-FUdR: amount obtained (% yield), 123 mg (13%); percent purity, 98%; ^{1}H NMR (DMSO-d_6) δ: 0.91–1.05 [12H, m, $(CH_3)_2CH)_2$]; 1.98–2.39 [4H, m, C2′, $(^{\beta}CH)_2$]; 3.96–4.03 [2H, m, $(^{\alpha}CH)_2$]; 4.27 (1H, m, C4′); 4.41–4.54 (2H, m, C5′); 5.34 (1H, m, C3′); 6.20–6.24 (1H, t, C1′); 8.01–8.03 (1H, d, CHCF); ESI-MS; 445.1 $(M+H)^+$.

3′-L-Phenylalanyl-FUdR: amount obtained (% yield), 66 mg (9%); percent purity, 96%; ^{1}H NMR (DMSO-d_6) δ: 2.21–2.26 (2H, m, C2′); 3.04–3.21 (2H, m, $^{\beta}CH_2$); 3.56 (2H, m, C5′); 3.71 (1H, m, $^{\alpha}$CH); 4.32–4.37 (1H, m, C4′); 5.19–5.20 (1H, m, C3′); 6.04–6.09 (1H, t, C1′); 7.25–7.37 (5H, m, aromatic protons); 8.15–8.18 (1H, d, CHCF); ESI-MS; 394 $(M+H)^+$.

5′-L-Phenylalanyl-FUdR: amount obtained (% yield), 131 mg (18%); percent purity, 93%; ^{1}H NMR (DMSO-d_6) δ: 2.07–2.15 (2H, m, C2′); 3.07–3.12 (2H, m, $^{\beta}CH_2$); 3.83–3.88 (1H, m, C3′); 4.05–4.09 (1H, m, $^{\alpha}$CH); 4.24–4.55 (3H, m, C4′,C5′); 6.11–6.16 (1H, t, C1′); 7.21–7.34 (5H, m, aromatic protons); 7.88–7.95 (1H, d, CHCF); ESI-MS; 394 $(M+H)^+$.

3′,5′-L-Diphenylalanyl-FUdR: amount obtained (% yield), 115 mg (10%); percent purity, 94%; ^{1}H NMR (DMSO-d_6) δ: 2.08–2.33 (2H, m, C2′); 3.05–3.19 [4H, m, $(^{\beta}CH_2)_2$]; 3.81–3.85 (1H, m, C4′); 4.28–4.43 [4H, m, C5′, $(^{\alpha}CH)_2$]; 4.94–4.96 (1H, m, C3′); 6.01–6.05 (1H, t, C1′); 7.21–7.37 (10H, m, aromatic protons); 7.89–7.97 (1H, d, CHCF); ESI-MS; 541.1 $(M+H)^+$.

3′-D-Phenylalanyl-FUdR: amount obtained (% yield), 67 mg (9%); percent purity, 94%; ^{1}H NMR (DMSO-d_6) δ: 1.99–2.24 (2H, m, C2'), 3.01–3.20 (2H, m, $^{\beta}CH_2$), 3.61 (2H, m, C5′), 3.94 (1H, m, $^{\alpha}$CH); 4.35 (1H, m, C4′); 5.28 (1H, m, C3′); 6.07 (1H, t, C1′); 7.26–7.35 (5H, m, aromatic protons); 8.15–8.24 (1H, d, CHCF); ESI-MS; 394.1 $(M+H)^+$.

5′-D-Phenylalanyl-FUdR: amount obtained (% yield), 104 mg (14%); percent purity, 96%; ^{1}H NMR (DMSO-d_6) δ: 2.12–2.28 (2H, m, C2′); 3.02–3.16 (2H, m, $^{\beta}CH_2$); 3.79 (1H, m, C3′); 4.16–4.37 (4H, m, C4′, C5′, $^{\alpha}$CH); 6.14 (1H, t, C1′); 7.21–7.32 (5H, m, aromatic protons); 7.93–7.95 (1H, d, CHCF); ESI-MS; 394 $(M+H)^+$.

3′,5′-D-Diphenylalanyl-FUdR: amount obtained (% yield); 118 mg (11%), percent purity; 95%, ^{1}H NMR (DMSO-d_6) δ: 1.88–2.04 (2H, m, C2'), 3.03–3.19 (4H, m, $(^{\beta}CH_2)_2$), 4.01 (1H, m, C4'), 4.23–4.44 (4H, m, C5′, $(^{\alpha}CH)_2$), 5.14 (1H, m, C3′), 6.04–6.08 (1H, t, C1'), 7.20–7.36 (10H, m, aromatic protons), 7.91–7.98 (1H, d, CHCF), ESI-MS; 541.1 $(M+H)^+$.

3′-L-Prolyl-FUdR: amount obtained (% yield), 66 mg (10%); percent purity, 99%; ^{1}H NMR (DMSO-d_6) δ: 1.86–2.43 (6H, m, C2′, $^{\beta}CH_2$, $^{\gamma}CH_2$); 3.23–3.25 (2H, m, $^{\delta}CH_2$); 3.66–3.67 (2H, m, C5′); 4.09–4.10 (1H, m, $^{\alpha}$CH); 5.35–5.39 (1H, m, C3′); 6.20–6.25 (1H, t, C1′); 8.22–8.24 (1H, d, CHCF); ESI-MS; 344 $(M+H)^+$.

5′-L-Prolyl-FUdR: amount obtained (% yield), 131 mg (20%); percent purity, 98%; ^{1}H NMR (DMSO-d_6) δ: 1.87–2.33

(6H, m, C2′, $^{\beta}CH_2$, $^{\gamma}CH_2$); 3.23–3.25 (2H, m, $^{\delta}CH_2$); 3.93–4.43 (5H, m, C3′, C4′,C5′, $^{\alpha}CH$); 6.12–6.17 (1H, t, C1′); 7.92–7.99 (1H, d, CHCF); ESI-MS; 344.1 $(M+H)^+$.

3′,5′-L-Diprolyl-FUdR: amount obtained (% yield), 115 mg (12%); percent purity, 94%; ^1H NMR (DMSO-d_6) δ: 1.53–2.33 [10H, m, C2′, $(^{\beta}CH_2)_2$, $(^{\gamma}CH_2)_2$]; 3.23–3.25 [2H, m, $(^{\delta}CH_2)_2$]; 4.29–4.53 [5H, m, C4′,C5′, $(^{\alpha}CH)_2$], 5.34 (1H, m, C3′); 6.17–6.21 (1H, t, C1′); 7.99–8.02 (1H, d, CHCF); ESI-MS; 441.1 $(M+H)^+$.

Cell Culture

Caco-2 cells (passage 44–52) from American Type Culture Collection (Rockville, MD) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 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.

Caco-2 Cell Homogenates

Confluent Caco-2 cells were washed with phosphate-buffered saline (PBS, pH 7.4) and then incubated with 0.05% trypsin-EDTA at 37°C for 5–10 min. Trypsin was neutralized by adding DMEM. The cells were washed off the plate and spun down by centrifugation. The pelleted cells were washed twice with pH 7.4 phosphate buffer (100 mM) and resuspended in pH 7.4 phosphate buffer (100 mM) to obtain a final concentration of approximately 5×10^6 cells/ml. The hydrolysis reactions were carried out in 96-well plates (Corning #3960). The Caco-2 cell suspension (600 μl) was placed in a well, and 600 μl 0.5% Triton X-100 was added. The cells were then homogenized by vigorous pipeting, and total protein was quantified with the Bio-Rad DC Protein Assay using bovine serum albumin as a standard.

Hydrolysis of Prodrugs in Caco-2 Cell Homogenates

Hydrolysis of prodrugs was determined in Caco-2 cell homogenates at room temperature. The hydrolysis reaction was initiated by adding 200 μl of 1.4 mM prodrug solution (prepared fresh in pH 7.4, 100 mM phosphate buffer) to a reaction well containing Caco-2 cell homogenates. At various time points (0, 5, 10, 15, 20, and 30 min), 200 μl of the reaction mixture was removed and added to a quenching plate containing 200 μl of ice-cold TFA in each well. Following collection of all samples, the quenching plate was centrifuged at 1800 *g* for 1 h at 4°C to pellet the protein precipitate. The supernatant was removed and assayed for prodrug and the parent drug by HPLC. Triplicate determinations of the extent of hydrolysis of each prodrug at a given time point were conducted.

Hydrolysis of Prodrugs in pH 7.4 Phosphate Buffer

The degradation profiles of the prodrugs were determined in pH 7.4 phosphate buffer (100 mM) alone in order to obtain the contribution of nonenzymatic hydrolysis. The experiments were carried out in triplicate as described above except that each well contained pH 7.4 phosphate buffer (100 mM) without Caco-2 cell homogenates.

HPLC Analysis

The concentrations of prodrugs and their parent drugs were determined on a Waters HPLC system (Waters Inc., Milford, MA). The HPLC system consisted of two Waters pumps (Model 515), a Waters auto-sampler (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 with a guard column. The compounds were eluted using a gradient method. Table I lists the solvent compositions, retention times, and detection wavelengths for all prodrugs and their parent drugs. The aqueous mobile phase (solvent A) was 0.1% (v/v) TFA in distilled water, and organic mobile phase (solvent B) was 0.1% (v/v) TFA in acetonitrile. Standard curves generated for each prodrug, and their parent drugs were used for quantification of integrated area under peaks.

Data Analysis

The initial rates of hydrolysis were used to obtain the apparent first-order rate constants and estimate the half-lives. The apparent first-order degradation rate constants of various

Table I. HPLC Methods and Retention Times for Floxuridine Prodrugs

Prodrug	Gradient description (% solvent B)[a]	λ (nm)	Retention time (min)			
			Prodrug			Parent
			$Rt_{3',5'}$	$Rt_{3'}$	$Rt_{5'}$	Rt_{Flox}
Val-FUdR	2 to 26% in 12 min	267	11.7	11.0	9.7	6.7
D-Val-FUdR	2 to 26% in 12 min	267	11.8	10.5	10.2	6.7
Phe-FUdR	2 to 34% in 16 min	267	16.3	14.7	13.1	6.7
D-Phe-FUdR	2 to 34% in 16 min	267	16.4	14.4	13.4	6.7
Pro-FUdR	0% for 6 min, followed by 0 to 20% in 10 min	267	14.9	15.3	10.7	9.2
Valacyclovir	0% for 6 min, followed by 0 to 14% in 7 min	254		15.2		7.1
Valganciclovir	0% for 6 min, followed by 0 to 14% in 7 min	254		14.1		6.0

[a] Solvent A, 0.1% (v/v) TFA in distilled water; solvent B, 0.1% (v/v) TFA in acetonitrile; flow rate, 1 ml/min.

prodrugs and their parent drugs at 25°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 estimated 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.

RESULTS

Synthesis of FUdR Prodrugs

The aromatic amino acids L-Phe and D-Phe, the aliphatic amino acids L-Val and D-Val, and the secondary amino acid Pro were selected as promoieties for synthesis of FUdR prodrugs (Fig. 1). Amino acid ester prodrugs of FUdR were synthesized as described in Fig. 2. The reaction of Boc-protected amino acids with FUdR resulted in production of three new intermediates as observed by TLC. The expected intermediates were Boc-protected 3′-amino acid ester, 5′-amino acid ester, and 3′,5′-amino acid diester prodrugs of FUdR. Following purification by column chromatography, the Boc group was removed by treating the pure intermediates with TFA. After removal of excess TFA, the resulting residue was reconstituted with water and lyophilized. The TFA salts of 3′-amino acid ester, 5′-amino acid ester, and 3′,5′-diamino acid ester prodrugs of FUdR were obtained as white fluffy powders. The combined yield of pure prodrugs was more than 33%. The purity of all prodrugs was ≥93% as determined by HPLC. The impurities were generally the known amino acid ester prodrugs or the parent drug. The λ_{max} and molar absorptivities for parent drug, floxuridine, and its prodrugs were found to be similar (267 nm and 0.00147 ± 0.00015 μM^{-1}, respectively). In some cases, other unknown impurities (1–2%) were also present. However, it was found that both the λ_{max} and the proportionality of HPLC areas and peak heights for these unknown impurities were similar to those of prodrugs and floxuridine. This similarity suggests that these unknown contaminants are probably floxuridine-related entities. The identity of the prodrugs was confirmed by electrospray ionization-mass spectrometry (ESI-MS) and proton nuclear magnetic resonance (^{1}H NMR). All prodrugs resulted in correct molecular weight and expected ^{1}H NMR.

Hydrolysis of FUdR Prodrugs

Nonenzymatic Hydrolysis in Phosphate Buffer

The estimated half-lives ($t_{1/2}$), obtained from linear regression ($r^2 \geq 0.95$) of pseudo-first-order plots of prodrug concentration vs. time for FUdR prodrugs in 100 mM phosphate buffer, pH 7.4, are listed in Table II. The results indicate that the structure of the promoiety influenced the rate of hydrolysis of amino acid ester prodrugs of FUdR. Pro ester prodrugs ($t_{1/2}$ ~ 24 to 90 min) hydrolyzed significantly faster than Phe ($t_{1/2}$ ~ 190 to 820 min, $p < 0.001$) and Val ($t_{1/2}$ ~ 1030 to 3520 min, $p < 0.001$) ester prodrugs. Phe ester prodrugs hydrolyzed faster than Val ester prodrugs. The stereochemistry of the amino acid promoiety did not influence the rate of hydrolysis of the ester prodrugs of FUdR in phosphate buffers. Thus, hydrolysis rates (and estimated half-lives) of both the L and D forms of Phe and of Val were similar, irrespective of the site of esterification. With few exceptions, the site of esterification did not influence the rate of hydrolysis of amino acid ester prodrugs of FUdR in a significant manner. The control prodrugs, valacyclovir and valganciclovir, exhibited high stability ($t_{1/2}$ ~ 3790–5860 min) in pH 7.4 phosphate buffer.

Table II. Estimated Half-Lives (Expressed as Mean ± SEM) of the Hydrolytic Degradation of Floxuridine Prodrugs in pH 7.4 Phosphate Buffer and in Caco-2 Cell Homogenates (n = 3)

Prodrug	Estimated $t_{1/2}$ (min) ± SEM		$t_{1/2}$ ratios buffer/ Caco-2
	Buffer pH 7.4	Caco-2 cell homogenates	
Valacyclovir	5860 ± 1500	16.9 ± 1.0	347
Valganciclovir	3790 ± 240	25.3 ± 0.3	150
3′,5′-di-*O*-valyl	1090 ± 440	30.3 ± 0.3	36
3′-*O*-valyl	1160 ± 230	31.8 ± 0.4	36
5′-*O*-valyl	3520 ± 650	9.8 ± 0.5	360
3′,5′-di-*O*-D-valyl	1030 ± 54	365.0 ± 16.0	3
3′-*O*-D-valyl	1300 ± 96	742.0 ± 7.0	2
5′-*O*-D-valyl	1630 ± 320	392.0 ± 23.0	4
3′,5′-di-*O*-phenylalanyl	190 ± 54	7.7 ± 0.6	24
3′-*O*-phenylalanyl	790 ± 24	2.2 ± 0.4	359
5′-*O*-phenylalanyl	820 ± 30	2.5 ± 0.3	328
3′,5′-di-*O*-D-phenylalanyl	306 ± 30	100.0 ± 4.0	3
3′-*O*-D-phenylalanyl	582 ± 18	196.0 ± 27.0	3
5′-*O*-D-phenylalanyl	612 ± 18	28.6 ± 0.3	21
3′,5′-di-*O*-prolyl	24 ± 6	0.9 ± 0.0	27
3′-*O*-prolyl	84 ± 6	4.9 ± 0.5	17
5′-*O*-prolyl	90 ± 6	ND[a]	ND[a]

[a] ND, Not determined.

Hydrolysis of Prodrugs in Caco-2 Cell Homogenates

The estimated half-lives ($t_{1/2}$) obtained from linear regression ($r^2 \geq 0.95$) of pseudo-first-order plots of prodrug concentration vs. time for FUdR prodrugs in Caco-2 cell homogenates are also listed in Table II. The structure, stereochemistry, and site of esterification of the amino acid promoiety affected the hydrolysis of the amino acid ester prodrugs of FUdR in a significant manner.

In general, Pro and Phe ester prodrugs hydrolyzed significantly faster ($p < 0.05$) than their corresponding Val ester prodrugs. L-Pro and L-Phe ester prodrugs exhibited short half-lives of 1 to 8 min, whereas L-Val ester prodrugs exhibited half-lives of 10 to 32 min. A similar influence of structure on hydrolysis rates was also evident with the D-stereoisomers. Thus, the D-Phe ester prodrugs hydrolyzed significantly faster ($p < 0.001$) than their corresponding D-Val ester prodrugs. The control prodrugs, valacyclovir and valganciclovir, exhibited similar enzymatic stability ($t_{1/2}$ ~ 17–25 min) to L-Val ester prodrugs of floxuridine ($t_{1/2}$ ~ 10–32 min).

The stereochemistry of the amino acid ester promoiety had a profound effect on the rate of hydrolysis of prodrugs. The L-amino acid ester prodrugs of FUdR hydrolyzed significantly faster ($p < 0.001$) than the D-amino acid ester prodrugs.

In case of Phe, the L-Phe ester prodrugs hydrolyzed 11- to 48-fold faster than their corresponding D-Phe ester prodrugs, whereas for Val the L-Val ester prodrugs hydrolyzed 12- to 75-fold faster than their corresponding D-Val ester prodrugs.

Although the site of esterification greatly influenced the hydrolysis of prodrugs by Caco-2 cell homogenates, the effects varied depending on the promoiety. Thus, in case of L-Val ester prodrugs, enzymatic stability was in the order 5′-monoester << 3′,5′-diester ≤ 3′-monoester prodrug. For D-Val ester prodrugs the order of stability was 3′,5′-diester ≤ 5′-monoester << 3′-monoester prodrug. In case of L-Phe ester prodrugs, the 3′,5′-diester exhibited the highest stability, and 3′-monoester and 5′-monoester prodrugs exhibited similar rates of hydrolysis. For D-Phe ester prodrugs, the 3-monoester prodrug was most stable, followed by 3′,5′-diester and 5′-monoester prodrugs. In case of Pro ester prodrugs, the 3′,5′-diester prodrug hydrolyzed significantly faster than the 3′-monoester prodrug.

DISCUSSION

Prodrug strategies are often used to improve the efficacy, safety, and biopharmaceutical properties of clinically important agents (5,6). The prodrug strategy offers a high degree of flexibility and has several advantages over other drug delivery strategies (5,6). Currently, a number of prodrugs are being used for treatment of a variety of disease states. Although alkyl groups have been extensively used as promoieties, the use of amino acids as promoieties provides several advantages: (a) a number of structurally diverse amino acids (aliphatic, aromatic, acidic, basic, neutral, etc.) are commercially available, (b) there are fewer safety concerns regarding the use of amino acids as promoieties, (c) amino acid and peptide chemistries are well established, and (d) amino acid prodrugs can potentially target carrier-mediated transporters for their transport across cell membranes.

Thus, a variety of amino acid ester prodrugs of FUdR, a well-established anticancer agent, were synthesized. FUdR has two free hydroxyl groups that are amenable to attachment of promoiety. Based on the site(s) of attachment of the promoiety, three types of FUdR prodrugs can be obtained: the 3′-monoester prodrug, the 5′-monoester prodrug, and 3′,5′-diester prodrug. In this study, all three types of FUdR prodrugs were synthesized. The promoieties selected for hydrolysis studies were the aromatic amino acids L-Phe and D-Phe, the aliphatic amino acids L-Val and D-Val, and the secondary amino acid Pro. All amino acid ester prodrugs of FUdR were synthesized in good yield and with high purity.

An ideal prodrug should exhibit good chemical stability but must be enzymatically converted to parent drug following transport across the biological membrane. The desired stability and activation characteristics of prodrug will depend on the purpose and the route of administration of the prodrug. However, a prodrug should exhibit as high a chemical stability as possible as long as it is activated before or at the site of action. Although detailed and rigorous stability studies are important in the characterization of promising prodrug candidates, rapid screening of stability is helpful for identification of such candidates. Such screening studies may reveal the effects of structure, stereochemistry, and site of esterification on the rate of hydrolysis of the amino acid ester prodrugs of FUdR. In this study, chemical and enzymatic hydrolysis of the FUdR prodrugs in pH 7.4 phosphate buffer and Caco-2 cell homogenates, respectively, were evaluated. The hydrolysis of commercially available valine ester prodrugs of acyclovir and ganciclovir, valacyclovir and valganciclovir, respectively, were also evaluated for comparison. Because both the prodrugs and the parent drug could be simultaneously analyzed by HPLC, the disappearance of the prodrugs and the appearance of the parent drug were calculated. Generally, an excellent mass balance (100 ± 2%) of loss of prodrug and generation of parent drug was obtained. However, here only the estimated half-lives for disappearance of the prodrugs are reported.

The results of the chemical stability studies in pH 7.4 phosphate buffer indicate that with the exception of Pro ester prodrugs, all other FUdR prodrugs exhibited good stability. Although detailed studies are required to evaluate the mechanisms involved in the hydrolysis of FUdR prodrugs, these prodrugs may hydrolyze by one or more mechanisms as suggested by Varia and co-workers (25). The low chemical stability of Pro ester prodrugs of FUdR may result from the higher pK_a of the secondary α-amino group of Pro (pK_a of the alpha amine of Pro, Val, and Phe are 10.61, 9.61, and 9.12, respectively) (25). At pH 7.4, the α-amine of Pro will be highly ionized, and substitution of an ionized amine in the acyl portion of the esters is known to increase the hydrolysis of ester bond (26). The high chemical instability of Pro ester prodrugs of FUdR renders them unsuitable for further development. The slightly higher chemical stability of Val ester prodrugs compared to Phe ester prodrugs may be caused by the electron-donating isopropyl side chain of Val, which renders the ester linkage less labile, or by the excess of protons on the γ carbon of the side chain, which can hinder the nucleophilic attack on carbonyl carbon as suggested by Wolfenden (27).

It appears that the chemical stability of the ester bond in amino acid ester prodrugs of FUdR is influenced by the characteristics of the amino acid promoieties. Further, as expected, the stereochemistry of the promoiety did not influence the chemical stability of amino acid ester prodrugs of FUdR. In addition, as expected, the stability profiles of the 3′-monoester prodrugs and the 5′-monoester prodrugs in phosphate buffer were similar. These results are consistent with previous studies on the hydrolysis of 3′- and 5′-monoalkyl esters of FUdR in 0.01 N NaOH (9), suggesting that the site of esterification had no effect on the alkali lability of the monoalkyl esters of FUdR (9). Although less stable than valacyclovir and valganciclovir, the Val ester prodrugs of FUdR exhibited the highest chemical stability of all the FUdR prodrugs synthesized. Based on the results of the chemical hydrolysis screening studies, Val ester prodrugs of FUdR appear to be the most promising candidates for further rigorous examination of stability characteristics.

Caco-2 cells are routinely used as *in vitro* models to evaluate the intestinal transport and disposition of drugs and drug candidates, and preliminary studies demonstrated significant prodrug hydrolytic activity (28). Caco-2 cell homogenates were therefore used to evaluate the hydrolysis of FUdR prodrugs. In Caco-2 cell homogenates, FUdR prodrugs exhibited a wide range of hydrolytic profiles, with D-Val ester prodrugs being the most stable and Pro ester prodrugs the least. In comparison, valacyclovir and valganciclovir exhibited intermediate stability. The rates of hydrolysis of the amino acid ester prodrugs of FUdR were much faster in Caco-2 cell

homogenates than in phosphate buffer, which were used as controls for nonenzymatic hydrolysis (Table II). These results clearly suggest that hydrolysis of prodrugs was predominantly due to the enzymes present in Caco-2 cell homogenates. In Caco-2 cell homogenates, the rates of hydrolysis were significantly affected by the structure, stereochemistry, and site of esterification of the promoiety. The Pro and Phe ester prodrugs hydrolyzed faster than Val ester prodrugs, suggesting that Pro and Phe ester prodrugs exhibit higher conformational compatibility with the enzyme(s) responsible for their hydrolysis. A similar conformational preference may also explain the influence of site of esterification on the rate of hydrolysis of FUdR prodrugs. The finding that L-amino acid ester prodrugs hydrolyzed faster than the D-amino acid ester prodrugs is consistent with previous studies with peptides and peptidomimetic drugs that demonstrated that D-amino acid–containing analogs were less susceptible to enzymatic hydrolysis (29). Finally, a comparison of the ratios of half-lives of chemical (phosphate buffer pH 7.4) to enzymatic (Caco-2 cell homogenates) hydrolysis indicated that the highest ratios were obtained with the 3′-Phe-, 5′-Phe-, and 5′-Val monoester prodrugs of FudR, which were similar to that for valacyclovir. Thus, the 3′-Phe, 5′-Phe, and 5′-Val ester prodrugs of FUdR possess desirable prodrug characteristics such as good solution stability and relatively fast enzymatic conversion rates. However, from a prodrug design perspective, only 5′-Phe- and 5′-Val-FUdR prodrugs block the hydroxyl group essential for the activity of FUdR. Therefore, these prodrugs will be further evaluated in detailed stability studies.

The results of this study clearly indicate that the structure, stereochemistry, and site of esterification of the amino acid promoiety profoundly affect the rates of activation of the FUdR prodrugs. The significant differences in activation rates of FUdR prodrugs in the presence of Caco-2 cell homogenates suggest that it may be possible to modulate the rate of activation of the prodrug following its transport across the cell membrane. Thus, depending on the structure of the promoiety, either a rapid or slow rate of hydrolysis can be achieved and may be beneficial in the design of a prodrug with a desired half-life. A controlled rate of hydrolysis can therefore be achieved based on the structure of promoiety.

In conclusion, the hydrolysis of amino acid ester prodrugs of FUdR is a function of the promoiety. A careful selection of promoiety can lead to a controlled activation of the prodrug. Similar studies involving structurally diverse amino acids and nucleosidic drugs will provide further insights into the role of the structure of promoiety and parent drug in the hydrolysis of the prodrug. Such studies will eventually lead to a database of structure–activity correlations that can be used to design prodrugs with optimum stability and activation profiles.

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REFERENCES

1. N. Kemeny, Y. Huang, A. M. Cohen, W. Shi, J. A. Conti, M. F. Brennan, J. R. Bertino, A. D. Turnbull, D. Sullivan, J. Stockman, L. H. Blumgart, and Y. Fong. Hepatic arterial infusion of chemotherapy after resection of hepatic metastases from colorectal cancer. *N. Engl. J. Med.* **341**:2039–2048 (1999).
2. J. L. Grem. 5-Fluorouracil: Forty-plus and still ticking. A review of its preclinical and clinical development. *Invest. New Drugs* **18**:299–313 (2000).
3. B. A. Chabner, D. P. Ryan, L. Paz-Area, R. Garcia-Carbonero, and P. Calabresi. Antineoplastic agents. In G. Hardman and L. E. Limbird (eds.), *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 10th ed., Mc-Graw Hill, New York, 2001, pp. 1389–1459.
4. J. A. Van Laar, Y. M. Rustum, S. P. Ackland, C. J. Van Groeningen, and G. J. Peters. Comparison of 5-fluoro-2′-deoxyuridine with 5-fluorouracil and their role in the treatment of colorectal cancer. *Eur. J. Cancer* **34**:296–306 (1998).
5. H. K. Han and G. L. Amidon. Targeted prodrug design to optimize drug delivery. *AAPS Pharmsci.* **2**:E6 (2000).
6. W. A. Denny. Prodrug strategies in cancer therapy. *Eur. J. Med. Chem.* **36**:577–595 (2001).
7. R. A. Schwendener, A. Supersaxo, W. Rubas, H. G. Weder, H. R. Hartmann, H. Schott, A. Ziegler, and H. Hengartner. 5′-O-Palmitoyl- and 3′,5′-O-dipalmitoyl-5-fluoro-2′-deoxyuridine—novel lipophilic analogues of 5′-fluoro-2′-deoxyuridine: Synthesis, incorporation into liposomes and preliminary biological results. *Biochem. Biophys. Res. Commun.* **126**:660–666 (1985).
8. S. Fukushima, T. Kawaguchi, M. Nishida, K. Juni, Y. Yamashita, M. Takahashi, and M. Nakano. Selective anticancer effects of 3′,5′-dioctanoyl-5-fluoro-2′-deoxyuridine, a lipophilic prodrug of 5-fluoro-2′-deoxyuridine, dissolved in an oily lymphographic agent on hepatic cancer of rabbits bearing vx-2 tumor. *Cancer Res.* **47**:1930–1934 (1987).
9. T. Kawaguchi, S. Fukushima, Y. Hayashi, and M. Nakano. Nonenzymatic and enzymatic hydrolysis of 5-fluoro-2′-deoxyuridine (FUdR) esters. *Pharm. Res.* **5**:741–744 (1988).
10. T. Halmos, P. Moroni, K. Antonakis, and J. Uriel. Fatty acid conjugates of 2′-deoxy-5-fluorouridine as prodrugs for the selective delivery of 5-fluorouracil to tumor cells. *Biochem. Pharmacol.* **44**:149–155 (1992).
11. S. C. Tobias and R. F. Borch. Synthesis and biological studies of novel nucleoside phosphoramidate prodrugs. *J. Med. Chem.* **44**: 4475–4480 (2001).
12. Y. Wei, Y. Yan, D. Pei, and B. Gong. A photoactivated prodrug. *Bioorg. Med. Chem. Lett.* **8**:2419–2422 (1998).
13. Z. Xia, L. I. Wiebe, G. G. Miller, and E. E. Knaus. Synthesis and biological evaluation of butanoate, retinoate, and bis(2,2,2-trichloroethyl)phosphate derivatives of 5-fluoro-2′-deoxyuridine and 2′,5-difluoro-2′-deoxyuridine as potential dual action anticancer prodrugs. *Arch. Pharm. (Weinheim)* **332**:286–294 (1999).
14. H. K. Han, D. M. Oh, and G. L. Amidon. Cellular uptake mechanism of amino acid ester prodrugs in caco-2/hpept1 cells overexpressing a human peptide transporter. *Pharm. Res.* **15**:1382–1386 (1998).
15. I. Rubio-Aliaga and H. Daniel. Mammalian peptide transporters as targets for drug delivery. *Trends Pharmacol. Sci.* **23**:434–440 (2002).
16. D. M. Oh, H. Han, and G. L. Amidon. Drug transport and targeting. Intestinal transport. *Pharm. Biotechnol.* **12**:59–88 (1999).
17. M. Sugawara, W. Huang, Y. L. Fei, F. H. Leibach, V. Ganapathy, and M. E. Ganapathy. Transport of valganciclovir, a ganciclovir prodrug, via peptide transporters pept1 and pept2. *J. Pharm. Sci.* **89**:781–789 (2000).
18. T. Nakanishi, I. Tamai, A. Takaki, and A. Tsuji. Cancer cell-targeted drug delivery utilizing oligopeptide transport activity. *Int. J. Cancer* **88**:274–280 (2000).
19. D. E. Gonzalez, K. M. Covitz, W. Sadee, and R. J. Mrsny. An oligopeptide transporter is expressed at high levels in the pancreatic carcinoma cell lines aspc-1 and capan-2. *Cancer Res.* **58**: 519–525 (1998).
20. R. J. Mrsny. Oligopeptide transporters as putative therapeutic targets for cancer cells. *Pharm. Res.* **15**:816–818 (1998).
21. V. H. Lee. Mucosal drug delivery. *J. Natl. Cancer Inst. Monogr.* **41**:4 (2001).
22. F. M. Williams. Clinical significance of esterases in man. *Clinical Pharmacokin.* **10**:392–403 (1985).
23. P. K. Banerjee and G. L. Amidon. Design of prodrugs based on

enzyme-substrate specificity. In H. Bundgaard (ed.), *Design of Prodrugs*, Elsevier, Sciences Publisher, New York, 1985, pp. 93–133.
24. T. Satoh, P. Taylor, W. F. Bosron, S. P. Sanghani, M. Hosokawa, and B. N. La Du. Current progress on esterases: From molecular structure to function. *Drug Metab. Dispos.* **30**:488–493 (2002).
25. S. A. Varia, S. Schuller, and V. J. Stella. Phenytoin prodrugs iv: Hydrolysis of various 3-(hydroxymethyl)phenytoin esters. *J. Pharm. Sci.* **73**:1074–1080 (1984).
26. T. C. Bruice and S. Benkovic. *Bioorganic mechanisms*, W. A. Benjamin, Inc., New York (1966).
27. R. Wolfenden. The mechanism of hydrolysis of amino acyl RNA. *Biochemistry* **2**:1090–1092 (1963).
28. I. J. Hidalgo, T. J. Raub, and R. T. Borchardt. Characterization of the human colon carcinoma cell line (caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **96**:736–749 (1989).
29. K. Tamura, C. P. Lee, P. L. Smith, and R. T. Borchardt. Metabolism, uptake, and transepithelial transport of the stereoisomers of val-val-val in the human intestinal cell line, caco-2. *Pharm. Res.* **13**:1663–1667 (1996).