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Amino Acid Ester Prodrugs of the Anticancer Agent Gemcitabine: Synthesis, Bioconversion, Metabolic Bioevasion, and hPEPT1-Mediated Transport

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Abstract: Gemcitabine, a clinically effective nucleoside anticancer agent, is a polar drug with low membrane permeability and is administered intravenously. Further, extensive degradation of gemcitabine by cytidine deaminase to an inactive metabolite in the liver affects its activity adversely. Thus, strategies that provide both enhanced transport and high metabolic bioevasion would potentially lead to oral alternatives that may be clinically useful. The objective of this study was to evaluate whether amino acid ester prodrugs of gemcitabine would (a) facilitate transport across intestinal membranes or across cells that express hPEPT1 and (b) provide resistance to deamination by cytidine deaminase. 3′-Monoester, 5′-monoester, and 3′,5′-diester prodrugs of gemcitabine utilizing aliphatic (L-valine, D-valine, and L-isoleucine) and aromatic (L-phenylalanine and D-phenylalanine) amino acids as promoieties were synthesized and evaluated for their affinity and direct hPEPT1-mediated transport in HeLa/hPEPT1 cells. All prodrugs exhibited enhanced affinity (IC_{50}: 0.14−0.16 mM) for the transporter. However, only the 5′-L-valyl and 5′-L-isoleucyl monoester prodrugs exhibited (a) increased uptake (11.25- and 5.64-fold, respectively) in HeLa/hPEPT1 cells compared to HeLa cells and (b) chemical stability in buffers, that were comparable to valacyclovir, a commercially marketed oral amino acid ester prodrug. The widely disparate enzymatic bioconversion profiles of the 5′-L-valyl and 5′-L-isoleucyl prodrugs in Caco-2 cell homogenates along with their significant resistance to deamination by cytidine deaminase suggest that the disposition of gemcitabine following oral administration would be controlled by the rate of bioconversion following transport across the intestinal epithelial membrane. The combined results also suggest that it may be possible to modulate these characteristics by the choice of the amino acid promoiety.

Keywords: Peptide transporter; gemcitabine; amino acid esters; prodrug; cytidine deaminase

Introduction

Gemcitabine (2′-deoxy-2′,2′-difluorocytidine, dFdC) is a pyrimidine nucleoside analogue with therapeutic activity against solid tumors and hematological malignancies.[1,2] Gemcitabine is clinically effective in the treatment of advanced or metastatic pancreatic cancer and non-small cell lung cancer.[1] Gemcitabine exerts its antiproliferative activity via multiple mechanisms of action. Gemcitabine is phosphorylated intracellularly by deoxycytidine kinase (dCK, EC 2.7.1.74) and subsequently by nucleotide kinases to its active metabolites, gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP). The triphosphate is incorporated into DNA and blocks DNA synthesis.[3,4] Gemcitabine is also known to undergo self-potentiated intracellular activation.[5−7] However, intracellular deamination of gem-

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citabine by cytidine deaminase (CDA; EC 3.5.4.5) to an inactive metabolite, 2′,2′-difluorodeoxyuridine, may affect gemcitabine activity adversely.[3,7−10] Although clinically effective, gemcitabine exhibits various side effects such as myelosuppression, hepatotoxicity, and renal toxicity, which are attributed to its inability to distinguish between normal cells and target cells.[11] Further, gemcitabine, like most other nucleoside antiviral and anticancer agents, is a polar drug with low membrane permeability and is administered intravenously.

Thus, oral alternatives to intravenous gemcitabine administration must incorporate strategies that facilitate (a) enhanced intestinal absorption and (b) circumvention of metabolic inactivation by cytidine deaminase in the liver. Prodrug strategies have often been used to overcome undesirable pharmaceutical properties of a variety of drugs.[12,13] Several prodrugs targeted to nutrient transporters expressed in the gastrointestinal (GI) tract have been developed in order to improve oral bioavailability.[14−17] Nutrient transporters play a vital role in the transport of nutrients and various therapeutically important drugs.[14] Oligopeptide transporters such as hPEPT1 are promising targets due to their presence in the GI tract and ability to transport a wide variety of di- and tripeptides as well as many peptidomimetic drugs.[18−22] The significantly enhanced bioavailability of nucleoside analogues such as acylovir and ganciclovir following oral administration of their valyl ester prodrugs has been attributed to their transport by oligopeptide transporters.[16,17,23−25]

Further, recent reports on the functional expression of oligopeptide transporters in a fibrosarcoma cell line HT-1080,[26] and in two pancreatic adenosarcoma ductal cell lines Aspc-1 and Capan-2,[27] suggest the possibility of targeting the oligopeptide transporters for anticancer therapy. On the

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basis of these findings, it is hypothesized that amino acid ester prodrugs of the anticancer drug gemcitabine would also be potential hPEPT1 substrates and may facilitate preferential and improved delivery of the drug to cancer cells or tissues overexpressing the hPEPT1 transporter.

In this report, we describe the synthesis of amino acid ester prodrugs of gemcitabine and evaluate their affinity and direct uptake in HeLa/hPEPT1 cells. The promoieties included the aliphatic amino acids L-valine, D-valine, and L-isoleucine, as well as the aromatic amino acids L-phenylalanine and D-phenylalanine. The chemical stability and enzymatic bioconversion of the prodrugs in cell homogenates were also assessed to gain further insight into optimal gemcitabine prodrug design. Select prodrugs exhibiting desirable hPEPT1-mediated uptake, enzymatic bioconversion, and chemical stability profiles were then examined for their ability to evade metabolism by cytidine deaminase compared to gemcitabine.

Materials. Gemcitabine was extracted from the lyophilized powder (Gemzar) supplied by Eli Lilly Pharmaceuticals (Indianapolis, IN). The *tert*-butyloxycarbonyl (Boc) protected amino acids, Boc-L-Val-OH, Boc-D-Val-OH, Boc-L-Ile-OH, Boc-L-Phe-OH, and Boc-D-Phe-OH, 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*-(dimethylamino)pyridine (DMAP), trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA), and all other reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Valacyclovir was a gift from GlaxoSmithKline, Inc. (Research Triangle Park, NC). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA), and cell culture supplies were obtained from Corning (Corning, NY) and Falcon (Lincoln Park, NJ). All other chemicals were either analytical or HPLC grade. Human plasma was obtained from the University of Michigan Hospital Blood Bank, University of Michigan (Ann Arbor, MI), and stored at −80 °C until used.

Prodrug Synthesis. A schematic of the synthetic procedure is shown in Scheme 1. Boc protected amino acids (Boc-L-Val-OH, Boc-D-Val-OH, Boc-L-Ile-OH, Boc-L-Phe-OH, or Boc-D-Phe-OH) (1.0 mmol), DCC (202 mg, 1.0 mmol), and DMAP (12.2 mg, 0.10 mmol) were allowed to react with gemcitabine (**1**) (263 mg,1.0 mmol) in 10 mL of dry *N*,*N*-dimethylformamide (DMF). The reaction mixture was stirred at room temperature for 24 h. The reaction progress was monitored by TLC (ethyl acetate:methanol, 10:1). Each reaction yielded three products as determined by TLC. After 24 h, the reaction mixture was filtered and DMF removed in vacuo at 50−55 °C. The residue was dissolved in ethyl acetate (30 mL) and washed with water (2 × 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 intermediates (**2a**−**4a**, **2b**−**4b**, **2c**−**4c**, **2d**−**4d**, and **2e**−**4e**) observed with TLC were separated and purified using column chromatography with a silica gel column (230−400 mesh, 1 × 18 cm) and serial elution with ethyl acetate:hexane, 1:1−1:0. 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 (6:4). After 4 h the solvent was removed and the residues were reconstituted with water and lyophilized. The TFA salts of amino acid prodrugs of gemcitabine (**5a**−**7a**, **5b**−**7b**, **5c**−**7c**, **5d**−**7d**, and **5e**−**7e**) were obtained as white fluffy solids. The combined yield of gemcitabine prodrugs was approximately 40%.

The purity of gemcitabine extracted from Gemzar and its prodrugs was determined by HPLC. These prodrugs were easily separated from parent drug by HPLC. Electrospray ionization mass spectra (ESI-MS) were obtained on a Thermoquest LCQ electrospray ionization mass spectrometer. The observed molecular weights of all prodrugs were found to be identical to that required by their structure. For prodrugs such as the 3′- and 5′-monoesters with similar molecular weights but different HPLC retention times, structural identity was then confirmed using proton nuclear magnetic resonance spectra (^{1}H NMR). ^{1}H NMR spectra were obtained with a 300 MHz Bruker NMR spectrometer.

Gemcitabine. The purity of the extracted gemcitabine was 99% by HPLC. ^{1}H NMR (D_2O): δ 3.85−3.89 (m, 1H, C4′), 3.92−3.94 (m, 2H, C5′), 4.21−4.25 (m, 1H, C3′), 5.90 (d, 1H, J = 7.86 Hz), 6.04−6.10 (m, 1H, C1′), 7.60 (d, 1H, J = 6.93 Hz).

3′-L-Valyl-gemcitabine (5a): yield 16%; percent purity, 97%; ^{1}H NMR (D_2O) δ 0.92−0.96 [m, 6H, $(CH_3)_2CH$], 2.25−2.33 (m, 1H, $^{\beta}$CH), 3.71−4.32 (m, 4H, $^{\alpha}$CH, C4′, C5′), 5.40−5.49 (m, 1H, C3′), 6.11−6.22 (m, 2H, C1′), 7.82 (d, 1H, J = 7.9 Hz); ESI-MS 363.3 $(M + H)^+$.

5′-L-Valyl-gemcitabine (6a): yield 12%; percent purity, 95%; ^{1}H NMR (D_2O) δ 0.88−0.93 [m, 6H, $(CH_3)_2CH$], 2.22−2.28 (m, 1H, $^{\beta}$CH), 3.97−4.53 (m, 5H, $^{\alpha}$CH, C3′, C4′, C5′), 6.03−6.12 (m, 2H, C1′), 7.82 (d, 1H, J = 7.9 Hz); ESI-MS 363.3 $(M + H)^+$.

3′,5′-L-Divalyl-gemcitabine (7a): yield 11%; percent purity, 98%; ^{1}H NMR (D_2O) δ 0.92−0.96 (m, 12H, CH_3), 2.12−2.34 (m, 2H, $^{\beta}$CH), 3.88−4.55 (m, 5H, $^{\alpha}$CH, C4′, C5′), 5.50−5.63 (m, 1H, C3′), 6.11−6.19 (m, 2H, C1′), 7.66 (d, 1H, J = 6.8 Hz); ESI-MS 460.2 $(M + H)^+$.

3′-D-Valyl-gemcitabine (5b): yield 17%; percent purity, 97%; ^{1}H NMR (D_2O) δ 0.93−0.96 [m, 6H, $(CH_3)_2CH$], 2.32−2.33 (m,1H, $^{\beta}$CH), 3.71−4.31 (m, 4H, $^{\alpha}$CH, C4′, C5′), 5.41−5.45 (m, 1H, C3′), 6.11−6.22 (m, 2H, C1′), 7.84−7.86 (d, 1H, J = 7.7 Hz); ESI-MS 363.4 $(M + H)^+$.

5′-D-Valyl-gemcitabine (6b): yield 11%; percent purity, 96%; ^{1}H NMR (D_2O) δ 0.89−0.93 [m, 6H, $(CH_3)_2CH$], 2.22−2.26 (m, 1H, $^{\beta}$CH), 3.98−4.57 (m, 5H, $^{\alpha}$CH, C3′, C4′, C5′), 5.96−6.10 (m, 2H, C1′), 7.65 (d, 1H, J = 8.0 Hz); ESI-MS 363.3 $(M + H)^+$.

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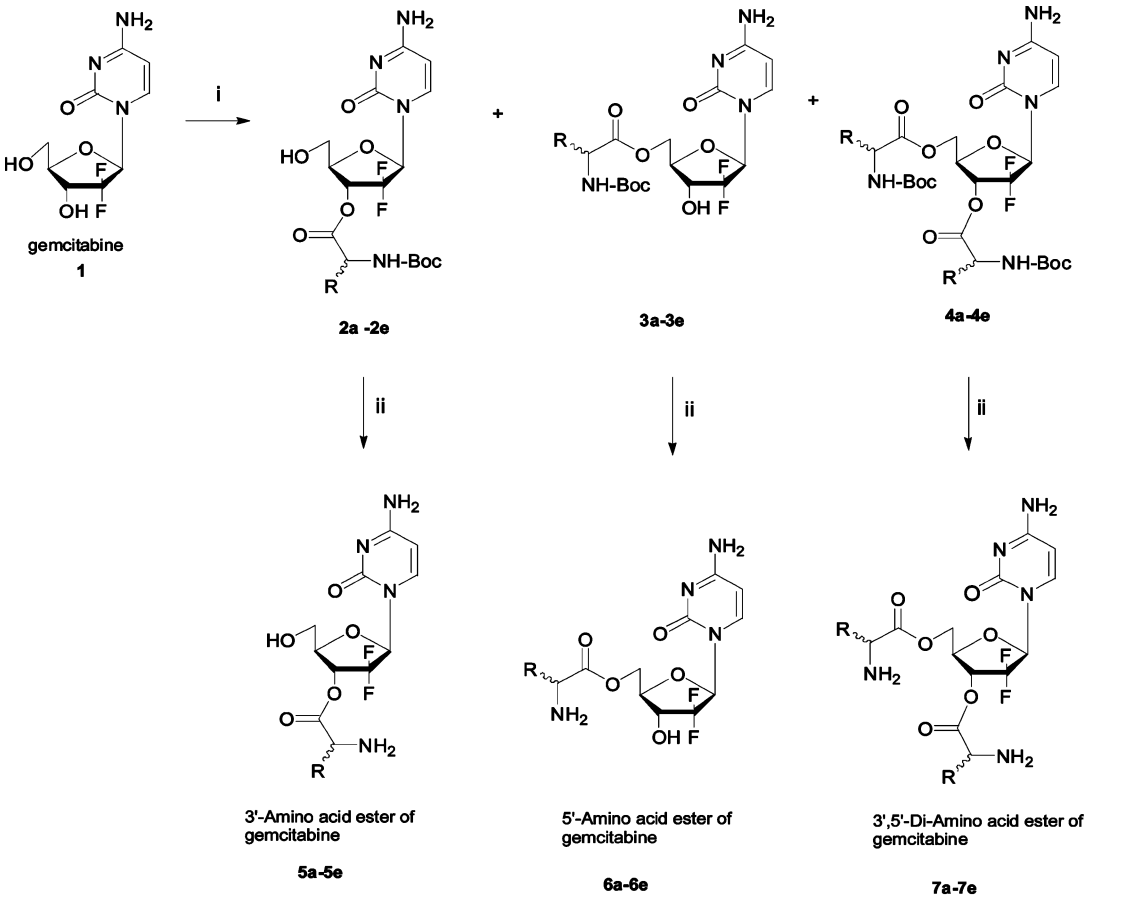
Scheme 1. Synthetic Scheme and Structures of Amino Acid Ester Prodrugs of Gemcitabine



3′,5′-D-Divalyl-gemcitabine (7b): yield 12%; percent purity, 97%; ^{1}H NMR (D_2O) δ 0.93−0.96 (m, 12H, CH_3), 2.15−2.34 (m, 2H, $^{\beta}$CH), 3.89−4.57 (m, 5H, Cα, C4′, C5′), 5.55−5.65 (m, 1H, C3′), 6.11−6.17 (m, 2H, C1′), 7.75 (d, 1H, J = 6.9 Hz); ESI-MS 460.3 $(M + H)^+$.

3′-L-Isoleucyl-gemcitabine (5c): yield 15%; percent purity, 95%; ^{1}H NMR (D_2O) δ 0.82−0.93 (m, 6H, CH_3), 1.23−1.40 (m, 2H), 3.70−4.30 (m, 4H, $^{\alpha}$CH, C4′, C5′), 5.42−5.55 (m, 1H, C3′), 6.15−6.25 (m, 2H, C1′), 7.85−7.87 (d, 1H, J = 6.8 Hz); ESI-MS 377.2 $(M + H)^+$.

5′-L-Isoleucyl-gemcitabine (6c): yield 13%, percent purity, 97%; ^{1}H NMR (D_2O) δ 0.78−0.90 (m, 6H, CH_3), 1.19−1.37 (m, 2H), 3.90−4.54 (m, 5H, $^{\alpha}$C, C3′, C4′, C5′), 6.00−6.20 (m, 2H, C1′, CHCH), 7.65 (d, 1H, J = 6.3 Hz); ESI-MS 377.2 $(M + H)^+$.

3′,5′-L-Diisoleucyl-gemcitabine (7c): yield 12%; percent purity, 92%; ^{1}H NMR (D_2O) δ 0.68−0.94 (m, 12H, CH_3), 1.11−1.34 (m, 2H), 4.11−4.69 (m, 5H, $^{\alpha}$CH, C4′, C5′), 5.51 (m, 1H, C3′), 6.10−6.16 (m, 2H, C1′), 7.68 (d, 1H, J = 6.9 Hz); ESI-MS 490.3 $(M + H)^+$.

3′-L-Phenylalanyl-gemcitabine (5d): yield 14%, percent purity, 91%; ^{1}H NMR (D_2O) δ 3.19−3.22 (m, 2H, $^{\beta}CH_2$), 3.55−3.99 (m, 3H, C4′, C5′), 4.51 (t, 1H, J = 7.4 Hz, $^{\alpha}$CH), 5.26−5.34 (m, 1H, C3′), 6.01−6.13 (m, 2H, C1′), 7.18−7.33 (m, 5H, aromatic protons), 7.79 (d, 1H, J = 7.9 Hz); ESI-MS 411.4 $(M + H)^+$.

5′-L-Phenylalanyl-gemcitabine (6d): yield 13%; percent purity, 97%; ^{1}H NMR (D_2O) δ 3.15−3.24 (m, 2H, $^{\beta}$CH), 3.81−4.51 (m, 5H, $^{\alpha}$CH, C3′, C4′, C5′), 5.95−6.12 (m, 2H,

C1′), 7.15−7.27 (m, 5H, aromatic protons), 7.51 (d, 1H, J = 7.9 Hz); ESI-MS 411.4 $(M + H)^+$.

3′,5′-L-Diphenylalanyl-gemcitabine (7d): yield 12%; percent purity, 96%; ^{1}H NMR (DMSO-d_6) δ 3.10−3.18 (m, 4H, $^{\beta}$CH), 4.13−4.54 (m, 5H, $^{\alpha}$CH, C4′, C5′), 5.39−5.45 (m, 1H, C3′), 5.90−6.18 (m, 2H, C1′), 7.21−7.30 (m, 10H, aromatic protons), 7.66 (d, 1H, J = 7.8 Hz); ESI-MS 556.6 $(M + H)^+$.

3′-D-Phenylalanyl-gemcitabine (5e): yield 15%; percent purity, 90%; ^{1}H NMR (D_2O) δ 3.18 (m, 2H, $^{\beta}CH_2$), 3.64−4.17 (m, 3H, C4′, C5′), 4.51 (t, 1H, J = 7.4 Hz, $^{\alpha}$CH), 5.36−5.40 (m, 1H, C3′), 6.11−6.16 (m, 2H, C1′), 7.19−7.33 (m, 5H, aromatic protons), 7.83 (d, 1H, J = 8.0 Hz); ESI-MS 411.4 $(M + H)^+$.

5′-D-Phenylalanyl-gemcitabine (6e): yield 12%; percent purity, 96%; ^{1}H NMR (D_2O) δ 3.18 (d, 2H, J = 6.8 Hz, $^{\beta}CH_2$), 4.01−4.70 (m, 5H, $^{\alpha}$CH, C3′, C4′, C5′), 5.98−6.10 (m, 2H, *CH*CH, C1′), 7.13−7.26 (m, 5H, aromatic protons), 7.62 (d, 1H, J = 8.0 Hz); ESI-MS 411.3 $(M + H)^+$.

3′,5′-D-Diphenylalanyl-gemcitabine (7e): yield 12%; percent purity, 96%; ^{1}H NMR (DMSO-d_6) δ 3.11−3.17 (m, 4H, $^{\beta}$CH), 4.16−4.54 (m, 5H, Cα, C4′, C5′), 5.39−5.44 (m, 1H, C3′), 5.90−6.18 (m, 2H, C1′), 7.23−7.30 (m, 10H, aromatic protons), 7.66−7.68 (d, 1H, J = 7.8 Hz); ESI-MS 556.6 $(M + H)^+$.

Cell Culture. HeLa cells (passage 85−90) from American Type Culture Collection (Rockville, MD) were routinely maintained in DMEM containing 10% 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.

[^{3}H]Gly-Sar Uptake Inhibition. HeLa cells were infected with adenovirus containing PEPT1 as described previously.[28] Two days postinfection, cells were washed twice with uptake buffer (pH 6.0, 145 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, and 5 mM MES) and incubated with 10 μmol/L Gly-Sar (9.94 μmol/L Gly-Sar and 0.06 μmol/L [^{3}H]Gly-Sar) and various concentrations (0.1−2 mM) of gemcitabine or its prodrugs in 0.3 mL of uptake buffer for 30 min at room temperature. After 30 min, the drug solution was aspirated and the cells were washed three times with ice-cold uptake buffer and solubilized with 0.1% Triton X-100/0.1 N NaOH. Aliquots of the suspensions were then used for scintillation counting (Beckman LS-9000, Beckman Instruments, Fullerton, CA) and for protein assays. IC_{50} values were determined using nonlinear data fitting (Graph Pad Prism v3.0).

Uptake Studies. Carrier-mediated prodrug transport was screened in HeLa/hPEPT1 cells as described previously.[16] Briefly, at 2 days postinfection, the growth medium was removed and cells were washed once with pH 6.0 uptake buffer and incubated with 0.5 mL of freshly prepared drug solution (1 mM) in uptake buffer. After 45 min, the cells were washed three times with ice-cold pH 6.0 uptake buffer, and 0.3 mL of Milli-Q water containing 0.1% SDS was added into each well. Cell lysate was treated with ice-cold trifluoroacetic acid (final concentration of 5%), vortexed, and centrifuged for 5 min at 3000 rpm. The supernatant was then filtered (0.45 μm) and analyzed by HPLC. Control experiments were performed in normal HeLa cells. The protein amount of each sample was determined with the Bio-Rad DC Protein Assay using bovine serum albumin as a standard.

Hydrolysis Studies. Enzymatic Stability. Confluent Caco-2 cells were washed with phosphate buffer saline (PBS, pH 7.4) and then harvested 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 (10 mM), and resuspended in pH 7.4 phosphate buffer (10 mM) to obtain a final concentration of approximately 4.70×10^6 cells/mL. The cells were then lysed with 1 volume of 0.5% Triton-X 100 solution. The cells were then homogenized by vigorous pipetting, and total protein was quantified with the BioRad DC Protein Assay using bovine serum albumin as a standard. The hydrolysis reactions were carried out in 96-well plates (Corning, Corning, NY). Caco-2 cell suspension (230 μL) was placed in triplicate wells, and the reactions were started with the addition of substrate stock solution (40 μL) in 10 mM pH 7.4 phosphate buffer and incubated at 37 °C. To test stability in human plasma, 230 μL of undiluted human plasma was added to each well in triplicate and 40 μL of substrate stock solution was added to start the reactions, which were conducted at 37 °C for up to 4 h. The final concentration of the test compounds in the reaction mixtures was 400 μM. At various time points, 40 μL aliquots were removed and added to 2 volumes of 10% ice-cold TFA. The mixtures were centrifuged for 10 min at 1800 rcf and 4 °C, and the supernatant was filtered through a 0.45 μm filter. The recovered filtrate was then analyzed by HPLC.

Chemical Stability. The degradation profiles of the prodrugs were determined in pH 7.4 phosphate buffer (10 mM) at 37 °C in order to obtain the contribution of nonenzymatic hydrolysis. The experiments were carried out in triplicate as described above except that each well contained buffer instead of cell homogenate.

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 gemcitabine 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

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

The degradation half-lives were then estimated by the equation

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

(28) Hsu, C. P.; Hilfinger, J. M.; Walter, E.; Merkle, H. P.; Roessler, B. J.; et al. Overexpression of human intestinal oligopeptide transporter in mammalian cells via adenoviral transduction. *Pharm. Res.* **1998**, *15*, 1376−1381.

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.

Preparation of Crude Cytidine Deaminase. Cytidine deaminase was prepared from HeLa cells using a modification of the method described earlier.[29] Briefly, HeLa cells were harvested and homogenized with a glass homogenizer in 4 volumes of 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM $MgCl_2$, and 50 μM potassium phosphate. Cell debris was removed by centrifugation at 10000*g* at 0 °C for 10 min, followed by centrifugation at 90000*g* at 0 °C for 2 h. The supernatant was heated at 70 °C for 5 min, and denatured protein was removed by centrifugation at 20000*g* for 1 h. After 2-fold reduction in volume, the enzyme solution contained 1.2 mg of protein/mL.

Stability Studies with Cytidine Deaminase. The stability of gemcitabine and its prodrugs in the presence of crude cytidine deaminase was determined in order to evaluate the effect of the promoiety on deamination by the enzyme. Briefly, 230 μL portions of crude cytidine deaminase preparation (1.2 mg of protein/mL) were placed in wells of 96-well plates (Corning, Corning, NY) and the reactions were initiated by the addition of substrates (200 μM) in pH 7.4 phosphate buffer and incubated at 37 °C for 30 min. A minimum of three wells were used for each compound tested. At various time points, 40 μL aliquots were removed and added to 2 volumes of 10% ice-cold TFA. The mixtures were vortexed and centrifuged for 10 min at 1800 rcf at 4 °C, and the supernatant was filtered through a 0.45 μm filter. The recovered filtrate was then analyzed by HPLC. The retention times for gemcitabine and its presumed deaminated product, 2′,2′-difluorodeoxyuridine, were 8.4 and 7.7 min, respectively. The total absence of the HPLC peak at 7.7 min following 30 min incubation in the presence of tetrahydrouridine, a specific inhibitor of cytidine deaminase, was used to confirm the HPLC identity of the deaminated product. However, since the pure deaminated product was not available for construction of standard curves, stability profiles in the presence of cytidine deaminase were monitored using the disappearance of gemcitabine or prodrug.

Caco-2 Monolayer Transport and Stability Studies. The Caco-2 permeability of gemcitabine, 5′-L-valyl-gemcitabine, 5′-D-valyl-gemcitabine, and 5′-L-isoleucyl-gemcitabine (80 μM) was evaluated as previously described.[16] Caco-2 cells were plated on 6-well format collagen-coated transwell inserts (Corning, NY, 0.4 μm pore size; area, 4.7 cm^2) at a density of 79,600 cells/cm^2 and maintained in DMEM containing 10% FBS, 1% nonessential amino acids, 1 mM sodium pyruvate, and 1% L-glutamine (Invitrogen, Carlsbad, CA). Plates were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37 °C. The culture medium was replaced every other day. Transport studies were performed 21 days post-seeding. Filter inserts were rinsed with HEPES buffer pH 7.4 (5 mM D-glucose, 5 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4) and allowed to equilibrate at 37 °C for 15 min. Gemcitabine and its prodrugs (0.8 mM) were dissolved in MES buffer, pH 6.0 (5 mM D-glucose, 5 mM MES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4). Experiments were initiated by replacing the apical (1.5 mL) buffer with test solution and the basolateral buffer with fresh HEPES, pH 7.4 (2.6 mL). Two hundred microliter aliquots of the basolateral receiver solution were withdrawn at predetermined intervals and replaced with fresh HEPES pH 7.4 buffer. The epithelial integrity of representative cell monolayers was assessed by monitoring transepithelial resistance and flux of the paracellular marker, ^{14}C-mannitol. The apparent permeability (P_{app}) was calculated using the following equation:

$$P_{app} = \frac{V_r}{AC_0}\frac{dC_r}{dt}$$

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

The stability of the prodrugs in donor solutions bathing the apical side and in receiver solution at the end of the transport experiments (120 min) was assessed with HPLC to determine the mode and extent of degradation of the prodrugs when in contact with Caco-2 monolayers and to determine metabolism of the prodrugs following passage across Caco-2 cells.

HPLC Analysis. The concentrations of gemcitabine and its amino acid ester prodrugs were determined on a Waters HPLC system (Waters Inc., Milford, MA). The HPLC system consisted of two Waters pumps (model 515), a Waters autosampler (WISP model 712), and a Waters UV detector (996 photodiode array detector). The system was controlled by Waters Millennium 32 software (Version 3.0.1). Samples were injected onto a Waters Xterra C_{18} reversed-phase column (5 μm, 4.6 × 250 mm) equipped with a guard column. The compounds were eluted using a gradient method. Table 1 lists the solvent compositions, retention times, and detection wavelengths for gemcitabine and its amino acid ester prodrugs examined in this study. The aqueous mobile phase (solvent A) was 0.1% (v/v) HFBA in distilled water, and the organic mobile phase (solvent B) was 0.1% (v/v) HFBA in acetonitrile. Standard curves generated for gemcitabine and each of its prodrugs were utilized for quantitation of integrated area under peaks.

Results

Synthesis of Gemcitabine Prodrugs. The aliphatic amino acids L-Val-OH, D-Val-OH, and L-Ile-OH and the aromatic amino acids L-Phe-OH and D-Phe-OH were selected as

(29) Wentworth, D. F.; Wolfenden, R. On the interaction of 3,4,5,6-tetrahydrouridine with human liver cytidine deaminase. *Biochemistry* **1975**, *14*, 5099−5105.

Table 1. HPLC Methods and Retention Times for Gemcitabine (Gem) Prodrugs

prodrug	gradient description (% solvent B)[a]	λ (nm)	retention time (min)			
			Gem	Gem prodrug		
				5′	3′	3′,5′
L-Val-Gem	0−38% in 15 min	274	9.6	14.7	15.2	16.5
D-Val-Gem	0−38% in 15 min	274	9.6	14.7	15.0	16.5
L-Ile-Gem	0−38% in 15 min	274	9.6	15.8	16.0	17.5
L-Phe-Gem	0−40% in 21 min	274	9.6	18.4	19.0	21.1
D-Phe-Gem	0−40% in 21 min	274	9.6	18.4	18.7	21.5

[a] Solvent A: 0.1% (v/v) heptafluorobutyric acid in distilled water. Solvent B: 0.1% (v/v) heptafluorobutyric acid in acetonitrile. Flow rate: 1 mL/min.

promoieties for synthesis of gemicitabine prodrugs (Scheme 1). Amino acid ester prodrugs of gemcitabine were synthesized as described in Scheme 1. The reaction of Boc protected amino acids with gemcitabine resulted in production of three intermediates as observed by TLC. The expected intermediates were the Boc protected, 3′-amino acid ester, 5′-amino acid ester, and 3′,5′-amino acid diester, prodrugs of gemicitabine. Following purification by column chromatography, the Boc group was removed by treatment of the pure intermediates with TFA. After removal of excess TFA, the resulting residues were 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 gemcitabine were obtained as white fluffy powders. The yield for each amino acid prodrug analogue was approximately 10−15%, and with the exception of the 3′-L-phenylalanyl, 3′-D-phenylalanyl, and 3′,5′-diisoleucyl prodrugs, the purity of all prodrugs was at least 95% as determined by HPLC. The impurities were generally the other known amino acid prodrug analogues or the parent drug. The identity of the prodrugs was confirmed by electrospray ionization mass spectrometry (ESI-MS) and proton nuclear magnetic resonance (^{1}H NMR). The molecular weights and ^{1}H NMR spectra for all prodrugs were in agreement with those required by their structures.

Uptake Inhibition Studies. IC_{50} values of the amino acid ester prodrugs of gemcitabine for PEPT1 determined using inhibition of Gly-Sar uptake in HeLa/hPEPT1 cells are summarized in Table 2. All prodrugs displayed increased affinity for hPEPT1 compared to the parent drug gemcitabine, which exhibited no apparent affinity in the concentration range tested. Table 2 also lists the IC_{50} value for valacyclovir, the valine ester prodrug of acyclovir, a known hPEPT1 substrate that was used as a positive control. With a few exceptions, all prodrugs tested exhibited affinity for hPEPT1 that was comparable to or better than that of valacyclovir ($IC_{50} \sim 0.45$ mM). Further, the effect of stereochemistry of the promoiety and the site of esterification on uptake inhibition appears to be negligible (Table 2). The significantly higher affinity for hPEPT1 exhibited by the 5′-L-valyl- and 5′-L-isoleucyl-gemcitabine prodrugs (IC_{50} values $\sim$ 0.14−19 mM) is noteworthy.

Table 2. Inhibition of [^{3}H]Gly-Sar Uptake in HeLa/hPEPT1 Cells by Gemcitabine Prodrugs

prodrug	IC_{50} (mM) av ± SEM[a]
3′-*O*-L-valyl (**5a**)	0.33 ± 0.04
5′-*O*-L-valyl (**6a**)	0.14 ± 0.01
3′,5′-*O*-L-valyl (**7a**)	0.32 ± 0.01
3′-*O*-D-valyl (**5b**)	0.39 ± 0.03
5′-*O*-D-valyl (**6b**)	0.33 ± 0.05
3′,5′-*O*-D-valyl (**7b**)	0.36 ± 0.04
3′-*O*-L-isoleucyl (**5c**)	0.41 ± 0.01
5′-*O*-L-isoleucyl (**6c**)	0.19 ± 0.02
3′,5′-di-*O*-L-isoleucyl (**7c**)	0.40 ± 0.01
3′-*O*-L-phenylalanyl (**5d**)	0.45 ± 0.01
5′-*O*-L-phenylalanyl (**6d**)	0.61 ± 0.01
3′,5′-di-*O*-L-phenylalanyl (**7d**)	0.28 ± 0.03
3′-*O*-D-phenylalanyl (**5e**)	0.47 ± 0.03
5′-*O*-D-phenylalanyl (**6e**)	0.66 ± 0.03
3′,5′-di-*O*-D-phenylalanyl (**7e**)	0.42 ± 0.02
gemcitabine (**1**)	no inhibition[b]
valacyclovir	0.45 ± 0.02

[a] Mean and standard error of the mean derived from three separate experiments. [b] No inhibition over concentration range 0.1−2 mM.

Direct Uptake in HeLa/hPEPT1 Cells. Enhanced hPEPT1-mediated uptake in HeLa/hPEPT1 cells was observed only with 5′-L-valyl, 5′-L-phenlylalanyl, and 5′-L-isoleucyl-gemcitabine prodrugs (11.3-fold, 6.9-fold, and 5.6-fold, respectively), compared to that in control HeLa cells (Table 3). The other prodrugs tested either showed little or no enhancement of uptake in HeLa/hPEPT1 cells or in a manner similar to gemcitabine exhibited no direct uptake in either HeLa or HeLa/hPEPT1 cells (Table 3). Valacyclovir, used as a positive control, was transported 4.3-fold higher in HeLa/hPEPT1 compared to that in HeLa cells and was consistent with previous findings.

Stability Studies. Nonenzymatic Hydrolysis in Phosphate Buffer. The estimated half-lives ($t_{1/2}$), obtained from linear regression of pseudo-first-order plots of prodrug concentration vs time for gemcitabine prodrugs in 100 mM phosphate buffer, pH 7.4, at 37 °C are listed in Table 4. The mass balance for prodrug disappearance and parent drug appearance was excellent (>98%). The structure of the promoiety influenced the rate of hydrolysis of the prodrugs. Thus, the stability of the prodrugs in pH 7.4 was in the order isoleucyl $\approx$ valyl $\gg$ phenyalanyl ester prodrugs (Table 4). Table 4 also lists the $t_{1/2}$ value for the reference control amino acid ester prodrug, valacyclovir, in phosphate buffer, 7.4. It is clear from the results shown in Table 4 that only the 5′-valyl- and 5′-isoleucyl-gemcitabine prodrugs exhibit chemical stability comparable to that of the reference prodrug. The stereochemistry of the amino acid promoiety did not influence the rate of hydrolysis of the ester prodrugs of gemcitabine in phosphate buffer. Thus, hydrolysis rates ($t_{1/2}$) of both L and D forms of the valyl and phenylanalyl ester prodrugs were similar. The site of esterification significantly influenced the rate of hydrolysis of amino acid ester prodrugs of gemcitabine. In general, the stability of the prodrugs was in

Table 3. Direct Uptake of Gemcitabine Prodrugs in HeLa/hPEPT1 and HeLa Cells (Mean ± SD, $n = 3$)

prodrug	HeLa/hPEPT1 nmol/mg/45min	HeLa nmol/mg/45min	hPEPT1/control
3′-*O*-L-valyl (**5a**)	1.12 ± 0.07	1.01 ± 0.03	1.11 ± 0.03
5′-*O*-L-valyl (**6a**)	2.14 ± 0.05	0.18 ± 0.01	11.25 ± 0.35
3′,5′-di-*O*-L-valyl (**7a**)	1.76 ± 0.09	1.52 ± 0.05	1.15 ± 0.17
3′-*O*-D-valyl (**5b**)	0.81 ± 0.02	0.76 ± 0.03	1.06 ± 0.04
5′-*O*-D-valyl (**6b**)	1.14 ± 0.04	0.72 ± 0.04	1.58 ± 0.04
3′,5′-di-*O*-D-valyl (**7b**)	1.11 ± 0.08	0.98 ± 0.06	1.13 ± 0.07
3′-*O*-L-isolecucyl (**5c**)	1.03 ± 0.11	0.94 ± 0.06	1.09± 0.10
5′-*O*-L-isolecucyl (**6c**)	1.22 ± 0.05	0.21 ± 0.01	5.64 ± 0.17
3′,5′-di-*O*-L-isolecucyl (**7c**)	1.16 ± 0.13	1.09 ± 0.03	1.06 ± 0.06
3′-*O*-L-phenylalanyl (**5d**)	*a*	*a*	
5′-*O*-L-phenylalanyl (**6d**)	0.71 ± 0.02	0.10 ± 0.01	6.92 ± 0.02
3′,5′-di-*O*-L-phenylalanyl (**7d**)	*a*	*a*	
3′-*O*-D-phenylalanyl (**5e**)	*a*	*a*	
5′-*O*-D-phenylalanyl (**6e**)	*a*	*a*	
3′,5′-di-*O*-D-phenylalanyl (**7e**)	*a*	*a*	
gemcitabine (**1**)	*a*	*a*	
valacyclovir	2.51 ± 0.28	0.59 ± 0.06	4.25 ± 0.17

[a] No detectable uptake.

Table 4. Estimated Half-Lives (Expressed as Mean ± SEM) of the Hydrolytic Degradation of Gemcitabine Prodrugs in pH 7.4 Phosphate Buffer, Caco-2 Cell Homogenates, and Human Plasma ($n = 3$)

	$t_{1/2}$ (min)		
prodrug	buffer pH 7.4	Caco-2 cell homogenates	human plasma
3′-*O*-L-valyl (**5a**)	64.0 ± 1.4	5.0 ± 0.1	5.4 ± 0.1
5′-*O*-L-valyl (**6a**)	416.0 ± 8.5	7.1 ± 0.6	56.4 ± 2.9
3′,5′-di-*O*-L-valyl (**7a**)	55.0 ± 2.7	0.9 ± 0.0	2.0 ± 0.1
3′-*O*-D-valyl (**5b**)	74.0 ± 1.2	23.2 ± 0.2	5.99 ± 0.0
5′-*O*-D-valyl (**6b**)	424.0 ± 1.2	37.4 ± 1.4	58.1 ± 2.1
3′,5′-di-*O*-D-valyl (**7b**)	52.0 ± 1.1	10.3 ± 0.7	2.1 ± 0.1
3′-*O*-L-isoleucyl (**5c**)	66.0 ± 0.2	10.6 ± 0.3	8.0 ± 0.1
5′-*O*-L-isoleucyl (**6c**)	452.0 ± 9.6	75.3 ± 2.8	99.2 ± 1.6
3′,5′-di-*O*-L-isoleucyl (**7c**)	61.0 ± 0.5	2.1 ± 0.0	2.64 ± 0.0
3′-*O*-L-phenylalanyl (**5d**)	39.0 ± 0.1	0.8 ± 0.0	5.7 ± 0.1
5′-*O*-L-phenylalanyl (**6d**)	200.0 ± 1.9	3.2 ± 0.1	8.4 ± 0.2
3′,5′-di-*O*-L-phenylalanyl (**7d**)	38.0 ± 0.2	0.6 ± 0.0	0.7 ± 0.0
3′-*O*-D-phenylalanyl (**5e**)	39.0 ± 0.7	8.8 ± 0.1	7.7 ± 0.2
5′-*O*-D-phenylalanyl (**6e**)	204.0 ± 3.5	11.4 ± 0.2	34.8 ± 1.1
3′,5′-di-*O*-D-phenylalanyl (**7e**)	28.0 ± 0.6	8.3 ± 0.6	2.4 ± 0.2
valacyclovir	1029.0 ± 11.4	10.3 ± 0.3	312.0 ± 24.6

the order 5′-monoesters > 3′-monoesters ≈ 3′,5′-diesters. Thus, the 3′-monoester and 3′,5′-diester prodrugs were hydrolyzed five to seven times faster than 5′-monoester prodrugs in pH 7.4 phosphate buffer at 37 °C. These results are contrary to those observed with amino acid ester prodrugs of floxuridine.[30]

Enzymatic Bioconversion in Caco-2 Cell Homogenates. The enzymatic bioconversion of the amino acid ester prodrugs of gemcitabine was determined in Caco-2 cell homogenates and in human plasma at 37 °C. The estimated half-lives ($t_{1/2}$), obtained from linear regression of pseudo-first-order plots of prodrug concentration vs time, are listed in Table 4. The corresponding values for the reference prodrug, valacyclovir, are also listed in Table 4. The hydrolysis rates of the gemcitabine prodrugs and valacyclovir in plasma were roughly three times higher in plasma compared to that in phosphate buffer, pH 7.4. However, the profiles were highly correlated ($r^2 = 0.95$ including valacyclovir; 0.88 excluding it), suggesting similarity of degradation pathways in the two media. The hydrolysis rates of the prodrugs in Caco-2 cell homogenates were not as well correlated with those in buffer ($r^2 = 0.31$), suggesting highly specific enzymatic action. Thus, contrary to buffer media, (a) the effect of structure of promoiety on stability was in

(30) Vig, B. S.; Lorenzi, P. J.; Mittal, S.; Landowski, C. P.; Shin, H. C.; et al. Amino acid ester prodrugs of floxuridine: synthesis and effects of structure, stereochemistry, and site of esterification on the rate of hydrolysis. *Pharm. Res.* **2003**, *20*, 1381−1388.

Table 5. Transport and Stability of Gemcitabine and Select Prodrugs in Caco-2 Cell Monolayers[a]

compound	permeability[b] ($\times 10^6$, cm/s)	stability[c] AP donor	stability[c] BL receiver
gemcitabine	0.99 ± 0.04	98.5 ± 4.0	87.2 ± 3.2
5′-*O*-L-valyl-gemcitabine	3.45 ± 0.17	87.8 ± 7.3	9.1 ± 1.3
5′-*O*-D-valyl-gemcitabine	0.76 ± 0.03	97.4 ± 9.7	88.6 ± 2.0
5′-*O*-L-isoleucyl-gemcitabine	1.89 ± 0.25	91.0 ± 9.0	46.0 ± 2.9

[a] All values expressed as mean ± SD of triplicate determinations at 37 °C. [b] Drug or prodrug concentration, 800 μM. [c] Percent prodrug remaining in donor or receiver solution at 120 min.

the order isoleucyl > valyl ≫ phenylalanyl; and (b) the stereochemistry of the promoiety affected the stability of the gemcitabine prodrugs in a profound manner (D-valyl and D-phenylalanyl prodrugs were roughly 4- to 14-fold more stable in Caco-2 cell homogenates than the corresponding L-analogue). Further, the effect of site of esterification was found to be dependent on the promoiety structure. Thus, compared to buffer, the preference for 5′-monoesters over 3′-monoesters or 3′,5′-diesters in Caco-2 cell homogenates was attenuated for L-valyl, D-valyl, and D-phenylalanyl prodrugs and not significantly different for L-isoleucyl and L-phenylalanyl prodrugs.

Caco-2 Permeability and Prodrug Stability. The apparent permeabilities of gemcitabine and select prodrugs in the apical-to-basolateral (AP to BL) direction are shown in Table 5. The permeabilities of 5′-L-valyl and 5′-L-isoleucyl-gemcitabine were roughly 3.5-fold and 1.9-fold higher than that of gemcitabine. The permeability of 5′-D-valyl-gemcitabine was much lower than its L-analogue and similar to that of gemcitabine itself.

The extent of prodrug degradation to the parent drug in Caco-2 monolayer transport experiments is also summarized in Table 5. The results indicate that ester hydrolysis of the prodrugs is not extensive when in contact with the apical side of the monolayers (<12% at 120 min). The fate of the prodrug following transport across the monolayers was, however, dramatically dependent on the amino acid promoiety and its stereochemistry. Thus, 5′-L-valyl-gemcitabine was almost extensively hydrolyzed (~90%) whereas its D-analogue exhibited much greater stability following transport. In comparison, the extent of hydrolysis of 5′-L-isoleucyl-gemcitabine following transport appears to be intermediate (~50%).

Deamination by Cytidine Deaminase. The disappearance of gemcitabine in the presence of crude cytidine deaminase as a function of incubation time is shown in Figure 1A. Figure 1B shows the percent of initial gemcitabine and its 5′-L-valyl, 5′-D-valyl, and 5′-L-isoleucyl prodrugs remaining after incubation with crude cytidine deaminase for 30 min. No deaminated product was observed with the three prodrugs. The significant relative stability of the three prodrugs compared to the parent gemcitabine indicates clearly the resistance of the prodrugs to deamination by cytidine deaminase.

Discussion

In recent years, much effort has been devoted to the rational design of prodrugs for enhanced therapeutic efficacy. Such studies are based on the rationale of targeted delivery to specific tissues or cells by targeting specific converting enzymes or membrane transporters in the target site or by bioevasion of metabolizing enzymes in first-pass organs such as the liver. Prodrugs targeted to oligopeptide transporters such as PEPT1 have been explored to improve oral bioavailability of a variety of nucleoside agents. Oligopeptide transporters are especially attractive targets since they are highly expressed in the gastrointestinal tract with diverse substrate specificity and high substrate capacity.[14,18,19,22,23] The successful clinical utility of oral valacyclovir and valganciclovir further suggests the potential of amino acids as promoieties. Recent reports on the functional expression of oligopeptide transporters such as PEPT1 in cancer cells provide additional impetus and promise for prodrugs targeted to this transporter. In this study, we report the results of the synthesis of amino acid ester prodrugs of gemcitabine and their suitability as substrates of the PEPT1 transporter. We also describe the chemical stability and bioconversion of these prodrugs and the ability of select gemcitabine prodrugs to evade metabolism by cytidine deaminase in vitro that suggests their potential in enhancing gemcitabine therapeutic action following oral administration.

All gemcitabine prodrugs examined exhibited high affinity for the PEPT1 transporter while the parent drug gemcitabine did not. These results are consistent with earlier findings with amino acid ester prodrugs of nucleoside agents such as acyclovir, AZT, ganciclovir, and floxuridine[16,17,31] and corroborate the hypothesis that amino acid ester prodrugs would exhibit enhanced affinity for hPEPT1. It is noteworthy that the 5′-L-valyl- and the 5′-L-isoleucyl-gemcitabine prodrugs exhibited a 2- to 3-fold higher affinity than valacyclovir. Direct uptake studies indicate that PEPT1-mediated transport occurred only with a few of the prodrugs, indicating that competitive inhibition studies alone are not sufficient to establish transport. Thus, only the 5′-L-valyl- and 5′-L-isoleucyl-gemcitabine prodrugs exhibited significant enhancement of transport in HeLa/hPEPT1 cells compared to control HeLa cells. The transport of the other valyl and isoleucyl ester prodrug analogues of gemcitabine in HeLa/hPEPT1 cells was not significantly different from that in control HeLa cells. The preference for the 5′-monoester and

(31) Faria, T. N.; Timoszyk, J. K.; Stouch, T. R.; Vig, B. S.; Landowski, C. P.; Amidon, G. L.; Weaver, C. D.; Wall, D. A.; Smith, R. L. A Novel High-Throughput PepT1 Transporter Assay Differentiates between Substrates and Antagonists. *Mol. Pharm.* **2004**, *1*, 67−76.

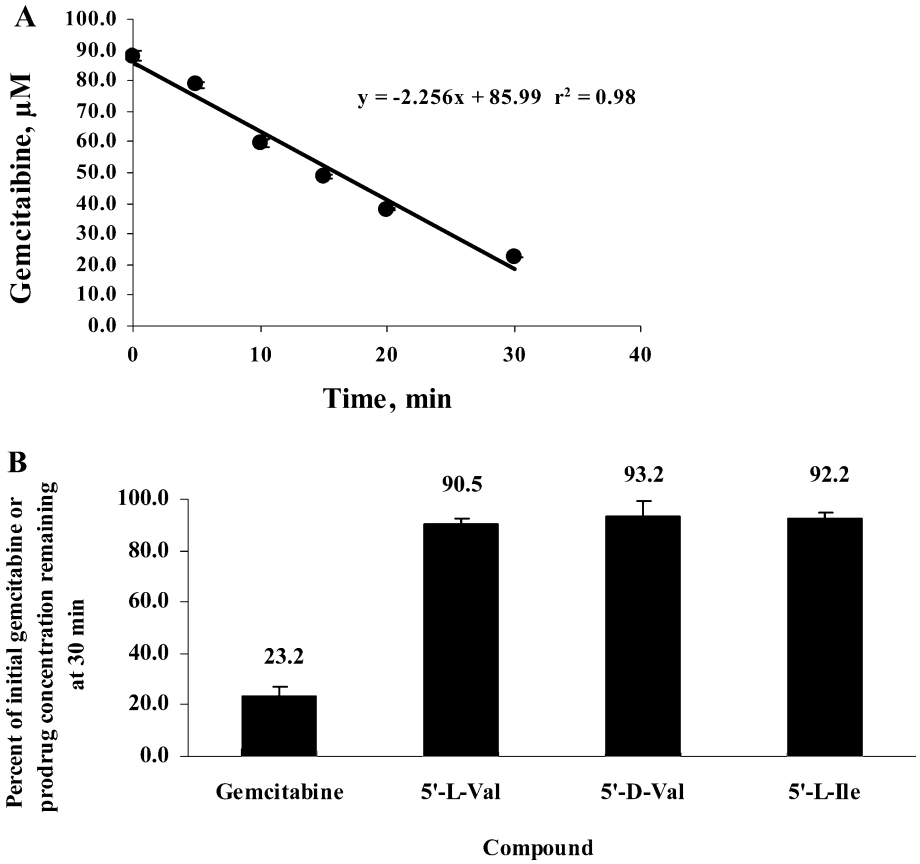


Figure 1. Stability of gemcitabine and its prodrugs in the presence of crude cytidine deaminase. (A) Concentration profile of the disappearance of gemcitabine in the presence of cytidine deaminase. (B) Percent gemcitabine or prodrug remaining following incubation with crude cytidine deaminase for 30 min. The results are expressed as mean ± SEM ($n = 3$).

the L-configuration (5′-L-valyl prodrug exhibits a 7-fold enhancement over its D-counterpart) is consistent with those reported for acyclovir[16] and AZT[16] and with di- and tripeptides.[32]

An ideal prodrug should exhibit good chemical stability but must be enzymatically converted to active parent drug following transport across the biological membrane. The greater stability of amino acid ester prodrugs of gemcitabine containing the aliphatic valyl or isoleucyl promoieties at pH 7.4 compared to the aromatic phenylalanyl prodrugs may be due to the electron-donating alkyl side chains of valine and isoleucine. The site of esterification also appears to be important in determining ester bond chemical stability. The relative instability of the 3′-monoesters compared to 5′-monoesters (5- to 7-fold lower stability) may be attributable to the strong electron withdrawing effects of the two fluorine atoms in the 2′ position of deoxyribose sugar, which renders the 3′-ester linkage more labile to nucleophilic (S_N2, base-catalyzed) attack on the carbonyl carbon. The diester prodrugs were the least stable among all prodrugs, a finding similar to that observed with amino acid prodrugs of floxuridine.[30] Further, as expected, the stereochemistry of the promoiety did not influence the chemical stability of amino acid ester prodrugs of gemcitabine. The chemical stabilities of the 5′-valyl and 5′-isoleucyl prodrugs in phosphate buffer, pH 7.4, were far superior to the other gemcitabine prodrugs and compared favorably with the chemical stability of valacyclovir.

The enzymatic stability of the gemcitabine prodrugs in Caco-2 cell homogenates, a suitable surrogate representing bioconversion in the intestine,[30,33] revealed that the rates of hydrolysis were affected by the structure, stereochemistry, and site of esterification of the promoiety. The hydrolysis rates were on average 30-fold higher in Caco-2 cell homogenates than in pH 7.4 buffer, suggesting the predominant contribution of enzymatic bioconversion of the prodrugs. Individual prodrug converting enzymes expressed in Caco-2 cells, such as BPHL, have been reported to hydrolyze the amino acid ester prodrugs valacyclovir and valganciclovir.[34] The finding that aromatic amino acid ester (L-Phe and D-Phe) prodrugs were less stable than aliphatic amino acid ester (L-Val and D-Val, L-Ile) prodrugs indicates higher compatibility

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with the enzyme(s) responsible for their hydrolysis. The finding that L-amino acid ester prodrugs hydrolyzed faster than the D-amino acid ester is consistent with previous studies with amino acid ester prodrugs of floxuridine.[30] The esterification site also appears to have an effect on the enzymatic stability of the prodrugs. Thus, 3′-monoester prodrugs of gemcitabine were less stable than the 5′-monoester prodrugs in Caco-2 homogenates (most dramatically evident with L-isoleucyl prodrugs; the 5′-monoester is 7-fold more stable than the 3′-monoester). The preferential hydrolysis of 3′-monoester prodrugs of gemcitabine compared to the 5′-monoesters in Caco-2 cell homogenates is contrary to the hydrolysis profiles of these gemcitabine prodrug analogues reported with BPHL.[35] BPHL exhibited preference for 5′-monoesters over 3′-monoesters,[35] suggesting that additional enzymes may be involved in hydrolysis of amino acid ester prodrugs of gemcitabine in Caco-2 cell homogenates.[36,37]

Further, the stability of the prodrugs was investigated in human plasma. The prodrugs were all less stable in plasma compared to buffer. However, the instability in plasma was significantly less than that observed in Caco-2 homogenates.

The dramatic resistance of the 5′-L-valyl and 5′-L-isoleucyl prodrugs of gemcitabine to deamination by cytidine deaminase in vitro, compared to the substantial degradation of gemcitabine itself, is highly significant. Indeed, the total absence of deaminated products of the two prodrugs coupled with the observation of their modest degradation (∼7−10%) suggests that the other 5′-monoester prodrugs could be resistant to deamination as well. The total resistance to deamination suggests that the disposition of gemcitabine following oral administration would then be determined, to a large extent, by the rate of activation of the prodrug following transport across the intestine. Thus, it is expected that the 5′-L-valyl-gemcitabine prodrug may not be as beneficial since it is rapidly converted to gemcitabine following intestinal transport. The 5′-isoleucyl prodrug, however, appears to be quite promising on the basis of its much slower bioconversion to the parent drug.

In conclusion, the structure, stereochemistry, and site of esterification influenced PEPT1-mediated transport and bioconversion of amino acid ester prodrugs of gemcitabine. Prodrugs of aliphatic amino acids in the L-configuration were preferred by the hPEPT1 transporter with the 5′-L-valyl- and 5′-L-isoleucyl-gemcitabine prodrugs exhibiting enhanced transport in cells overexpressing PEPT1 comparable to that of valacyclovir. Further, the chemical stability and rapid enzymatic bioconversion characteristic of the 5′-L-valyl-gemcitabine prodrug suggests its potential in enhancing oral absorption of gemcitabine. However, the rapid bioconversion may prevent the exploitation of the prodrug's metabolic bioevasive capabilities. On the other hand, the relatively slow bioconversion of the 5′-L-isoleucyl-gemcitabine prodrug in Caco-2 cell homogenates and in human plasma and its remarkable resistance to cytidine deaminase deactivation may allow a longer systemic circulation half-life and facilitate targeting of cells overexpressing the hPEPT1 transporter. On the basis of these in vitro results, studies to characterize bioactivation of the prodrug and its bioevasion of cytidine deaminase in vivo are merited.

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