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Synthesis, Transport and Pharmacokinetics of 5′-Amino Acid Ester Prodrugs of 1-β-D-Arabinofuranosylcytosine

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Abstract: Cytarabine (1-β-D-arabinofuranosylcytosine, ara-C, **1**) suffers from low oral bioavailability due to low intestinal membrane permeability and poor metabolic stability, and intravenous infusion is usually adopted as the clinical standard dosing administration. To develop an oral alternative for **1** and utilize the intestinal oligopeptide transporter 1 (PepT1), a series of 5′-amino acid ester derivatives of **1** was synthesized to clarify which modification was the most suitable to increase the oral bioavailability of **1**. Their apical-to-basolateral permeability across Caco-2 cells and the antiproliferative activity with HL-60 cells were screened. 5′-Valyl prodrug **2** demonstrated the highest permeability and was selected for further study. Glycylsarcosine (gly-sar, a typical substrate of PepT1) uptake by Caco-2 cells can be inhibited by **2** in a concentration-dependent manner, and IC_{50} for **2** was 2.18 ± 0.12 mM. The uptake of **2** was markedly increased in the long-term leptin-treated Caco-2 cells compared with the control Caco-2 cells, and was significantly inhibited by the excess of gly-sar, but not by L-valine. A dose-proportional pharmacokinetics was observed in rats when 5, 15, 30 mg/kg doses of **2** (calculated as **1**) were orally administered. The oral absolute bioavailability of **1** was 60.0% and 21.8% after **2** and **1** were orally administered to rats 30 mg/kg, respectively. Following oral administration of 15 mg/kg, the absorption and bioactivation of **2** were extensive and rapid, over 98% of prodrug hydrolysis occurring before appearance in the portal vein. The *in vivo* dispositions of 1-β-D-arabinofuranosyluracil (ara-U), a deaminated product of **1**, were investigated. Oral administration of **2** resulted in an increased **1**/ara-U ratio (2.76) in the blood, much higher than that (1.25) after **1** orally taken. Overall, these results demonstrated that the PepT1-mediated absorption of **2** and the increased metabolic stability resulted in a dramatic increase in the oral bioavailability of **1** in rats and further corroborated the thought that prodrug design strategy targeting intestinal PepT1 was an important and promising strategy to improve oral bioavailability of poorly absorbed drugs.

Keywords: Cytarabine; prodrug; intestinal oligopeptide transporter 1; pharmacokinetics; bioavailability; hepatic portal vein

Introduction

1-β-D-Arabinofuranosylcytosine (cytarabine, ara-C, **1**, Figure 1), a pyrimidine nucleoside analogue, is an attractive therapeutic agent for the treatment of both acute and chronic myeloblastic leukemias.[1,2] It is also therapeutically effective for the solid tumors when combined with other anticancer

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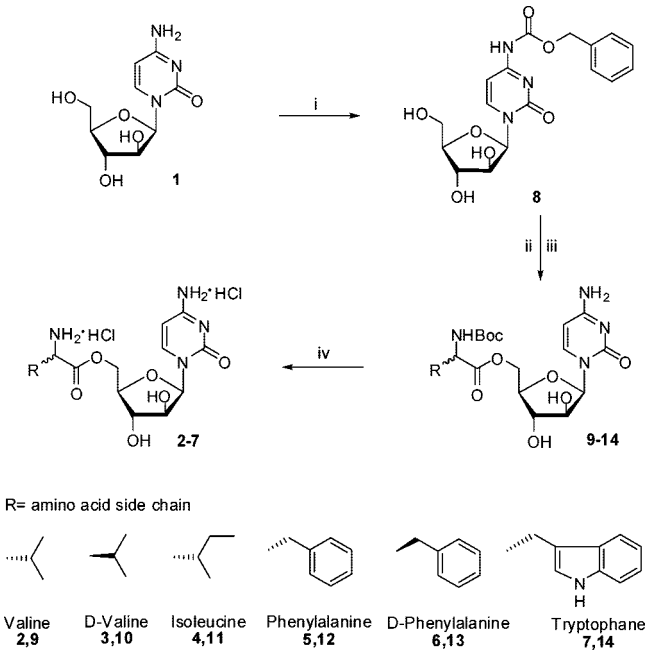

Figure 1. Synthetic route of 5′-amino acid ester prodrugs **2**−**7**. Reagents and conditions: (i) benzyloxycarbonyl chloride, DMA, $NaHCO_3$, rt, overnight; (ii) Boc protected amino acid, CDI, DMAP, TEA, DMF/THF, 80 °C, reflux, 1 h; (iii) Pd/C,75 °C, 3 h; (iv) HCl/EtOH.

agents. [3] *In vivo*, **1** is initially converted into its major active metabolite, 1-β-D-arabinofuranosylcytosine 5′-triphosphate (ara-CTP), via intracellular phosphorylation, and subsequently ara-CTP presumably takes effect both by inhibition of DNA polymerase and by incorporation into DNA,[4,5] resulting in defective ligation or incomplete synthesis of DNA fragments.[6,7] Due to low permeability across the intestinal membrane and rapid deamination to the biologically inactive 1-β-D-arabinofuranosyluracil (ara-U) in intestinal and hepatic cells, **1** has a very short plasma half-life and a very low oral bioavailability (about 20%),[8] which necessitates the use of relatively complex and precise dosing schedules, such as continuous infusion to maintain therapeutical plasma level in human.[9,10] So its clinical utility and patient compliance are severely hampered, and the development of an oral alternative to intravenous administration of **1** is imperative not only for averting the high costs dictated by hospital treatment but also for more patient-friendly delivery.

To date, much effort has been devoted to the design of **1** prodrugs with more favorable oral absorption profiles, but few have led to desired and approved products. For example, L-valine was introduced at the NH_2 of **1** to synthesize N^4-L-valyl-ara-C, but the oral bioavailability of **1** was only 4% after N^4-L-valyl-ara-C was orally administered in rats. The underlying reason was that amide linkage was stable *in vivo* and **1** cannot be released from the prodrug.[11,12] Similar results appeared when the long-chain fatty acids were introduced at the 5′-hydroxyl of **1**.[13]

A prodrug approach targeting gastrointestinal nutrient transporters has been a well-established method to overcome the biopharmaceutical properties of medicinal agents such as permeability and bioavailability, since these transporters play an important role in the oral absorption of nutrients and various therapeutically important drugs.[14−16] In this regard, the intestinal oligopeptide transporter 1, PepT1, is a promising and valuable target protein. The PepT1 was highly expressed in the gastrointestinal tract and can transport di/tripeptides from lumen into cells using an inward H^+-

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electrochemical gradient.[17,18] In addition to dipeptides and tripeptides, β-lactam antibiotics, renin inhibitor, angiotensin-converting enzyme inhibitors and other peptidomimetic agents can also be transported by PepT1. Over the past decade, the human intestinal oligopeptide transporter 1, hPepT1, has been widely explored as a target for increasing the oral bioavailability of poorly absorbed drugs via designing peptidomimetic prodrugs that are recognized and transported by hPepT1 and then hydrolyzed prior to or after the drug delivery to the systemic circulation. For example, acyclovir is an antiviral drug with a low oral bioavailability approximately 15% in human. With the aid of hPepT1-mediated transport, valacyclovir (L-valyl ester of acyclovir) is rapidly absorbed and then almost completely converted into the parent drug by enzymatic hydrolysis, increasing the oral bioavailability of acyclovir 3- to 5-fold.[19−21] Efficacy has also been proved when such approach was extended to LY544344, an *N*-linked alanyl prodrug of LY354740. LY544344 increased the oral bioavailability of LY354740 from 10% to 85% in rats.[22,23] In addition to the increased bioavailability, this prodrug approach is also attractive due to the low potential of side effects after parent drug release, because these promoieties are natural amino acids.[19]

Thus, prodrug approach targeting intestinal PepT1 may facilitate intestinal absorption of **1** and allow for improving oral bioavailability. Based on these considerations, we synthesized a series of 5′-amino acid ester derivatives of **1** to clarify which amino acid modification was the most suitable for improving the oral bioavailability of **1** in this study. Caco-2 cell has been shown to express hPepT1, and has been used to characterize the recognition of various peptidomimetics and other substrates by hPepT1. The transport characteristics of 5′-amino acid ester derivatives of **1** across Caco-2 cells monolayer were compared with **1** to screen the target compound with high permeability. We also examined the *in vitro* antiproliferative activity of these prodrugs in HL-60 cells. The uptake mechanism of 5′-L-valyl prodrug **2** (Figure 1) in leptin-treated Caco-2 cells by inhibition study was performed to determine whether the transport of **2** across the intestinal epithelium was mediated by PepT1. Finally, the disposition of **1** and the deaminated product ara-U after oral administration of **1** and prodrug **2** was evaluated in Sprague−Dawley rats. The combined results of these studies showed amino acid ester prodrugs can improve the permeability of **1** across the intestinal epithelial cells and enhance the delivery of **1** *in vivo*.

Materials and Methods

Materials. Cytarabine (**1**, 99.3% purity) was purchased from Surui Chemical Corporation (Suzhou, China). The *tert*-butyloxycarbonyl (Boc) protected amino acids Boc-L-valine, Boc-D-valine, Boc-L-isoleucine, Boc-L-phenylalanine, Boc-D-phenylalanine, and Boc-L-tryptophan were obtained from Baosheng Chemicals (Yangzhou, China). Carbonyldiimidazole (CDI) was purchased from Kaile Chemicals (Shanghai, China). 4-Dimethylamino-pyridine (DMAP) was purchased from Tianyu (Dongyang, China). Benzyloxycarbonyl chloride was purchased from Lanfeng Chemicals (Xuzhou, China). Glycylsarcosine (gly-sar) was purchased from Sigma-aldrich (St. Louis, MO). Leptin was obtained from Prospec Technogene (Israel). All other chemicals used were of the highest purity available.

Synthesis of 5′-Amino Acid Ester Derivatives of 1. To a mixture of **1** (24.32 g, 100 mmol) in *N*,*N*-dimethylacetamide (DMA, 400 mL), $NaHCO_3$ (25.20 g, 300 mmol) and benzyloxycarbonyl chloride (25.59 g, 150 mmol) were added sequentially. The reaction mixture was stirred overnight at room temperature and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (dichloromethane (DCM)/methanol, gradient 5:1) to give N^4-benzyloxycarbonyl-1-β-D-arabinofuranosylcytosine (**8**) (22.64 g, 60%) as a white solid (Figure 1).

A solution of *N*-Boc-protected amino acid (44 mmol), carbonyldiimidazole (CDI, 7.13 g, 44 mmol) and anhydrous tetrahydrofuran (THF, 200 mL) was stirred under argon at 25 °C for 1.5 h and then 40−50 °C for 20 min. This mixture was slowly added to a stirred solution of **8** (15.09 g, 40 mmol), DMAP (0.49 g, 4 mmol), triethylamine (TEA, 100 mL) and anhydrous *N*,*N*-dimethylformamide (DMF, 200 mL). The temperature was maintained up to 80 °C during the addition process, and the reaction mixture was stirred at this temperature for 1 h. The reaction mixture was cooled to room temperature, and TEA and THF were removed under vacuum. The remaining DMF solution was then neutralized to pH 7.5 with acetic acid, and DMF was evaporated under

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reduced pressure. The residue was diluted with ethyl acetate (EtOAc, 500 mL) and successively washed with water and brine. The organic layer was condensed to 30 mL, and then Pd/C (10%) was added. The reaction mixture was stirred under H_2 at 75 °C for 3 h and then filtered. Compounds **9**, **10**, **11**, **12**, **13**, and **14** were collected. Hydrogen chloride gas was bubbled into a clear solution of **9**, **10**, **11**, **12**, **13**, and **14** in alcohol (EtOH, 80 mL), respectively, over a period of 2 h. The reaction temperature was kept below 30 °C for 5 h. Solids were collected by filtration and washed with EtOH and diethyl ether (Et_2O) to give the target compounds **2**, **3**, **4**, **5**, **6**, and **7**. All compounds were characterized by ^{1}H NMR, ^{13}C NMR and electrospray ionization-mass spectrometry.

***N*^{4}-Benzyloxycarbonyl-1-*β*-D-arabinofuranosylcytosine (8):** mp 182−185 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.77(1H, br), 8.04 (1H, d, J = 7.5 Hz), 7.34−7.40 (5H, m), 7.02 (1H, d, J = 7.5 Hz), 6.04 (1H, d, J = 3.6 Hz), 5.48 (2H, m), 5.19 (2H, s), 5.05 (1H, br), 3.82−4.05 (3H, m), 3.61 (2H, m); MS(ESI) m/z 378.1 $(M + H)^+$, 400.1 $(M + Na)^+$, 416.1 $(M + K)^+$.

5′-L-Valyl-cytarabine (2): yield 42%; mp 222−224 °C; purity 99.2% according to HPLC; λ_{max} = 272.0 nm; ^{1}H NMR (600 MHz, DMSO-d_6, δ ppm) 9.93 (1H, br), 8.62−8.75 (4H, 2br), 7.83−7.86 (1H, d, J = 7.8 Hz), 6.18 (1H, d, J = 7.8 Hz), 6.05 (1H, d, J = 3.4 Hz), 5.90 (1H, br), 4.52−4.59 (1H, dd, J = 11.6, J = 8.3), 4.33−4.38 (1H, dd, J = 11.6, J = 3.8), 4.07 (2H, m), 4.00 (1H, m), 3.92 (1H, m), 2.20 (1H, m), 1.00 (6H, m); ^{13}C NMR (600 MHz, DMSO-d_6, δ ppm) 168.9, 159.6, 147.1, 145.8, 92.8, 87.4, 83.1, 76.0, 74.1, 65.2, 57.4, 29.5, 18.4, 17.6; MS(ES+) m/z 343.0 $(M + H)^+$, 684.8 $(2M + H)^+$, 707.0 $(2M + Na)^+$.

5′-D-Valyl-cytarabine (3): yield 40%; mp 218−220 °C; purity 97.3% according to HPLC; λ_{max} = 272.0 nm; ^{1}H NMR (600 MHz, DMSO-$d6$/D_2O, δ ppm) 9.92 (1H, br), 8.69−8.80 (4H, 2br), 7.86−7.87 (1H, d, J = 7.9 Hz), 6.21−6.23 (1H, d, J = 7.9 Hz), 6.05 (1H, d, J = 3.1 Hz), 5.91 (1H, br), 4.44−4.47 (1H, dd, J = 11.5, J = 8.2), 4.34−4.38 (1H, dd, J = 11.5, J = 3.8), 4.10 (2H, m), 4.00 (1H, m), 3.91 (1H, m), 2.21 (1H, m), 1.00 (6H, m); ^{13}C NMR (600 MHz, DMSO-d_6, δ ppm) 168.8, 159.6, 146.9, 146.0, 93.0, 87.5, 82.8, 76.1, 74.2, 65.2, 57.3, 29.5, 18.5, 17.6; MS(ES+) m/z 343.1 $(M + H)^+$, 684.9 $(2M + H)^+$, 707.0 $(2M + Na)^+$.

5′-L-Isoleucyl-cytarabine (4): yield 35%; mp 220−221 °C; purity 96.8% according to HPLC; λ_{max} = 272.0 nm; ^{1}H NMR (600 MHz, DMSO-d_6, δ ppm) 9.93 (1H, br), 8.65−8.77 (4H, 2br), 7.83−7.86 (1H, d, J = 7.8 Hz), 6.17−6.19 (1H, d, J = 7.8 Hz), 6.05 (1H, d, J = 3.2 Hz), 5.91 (2H, br), 4.44−4.47 (1H, dd, J = 11.6, J = 8.4), 4.34−4.38 (1H, dd, J = 11.6, J = 3.5), 4.07−4.10 (2H, m), 4.00 (1H, m), 3.97 (1H, m), 1.95 (1H, m), 1.50 (1H, m), 1.27 (1H, m), 0.92−0.93 (3H, d), 0.88−0.90 (3H, m); ^{13}C NMR (600 MHz, DMSO-d_6, δ ppm) 168.6, 159.3, 146.8, 145.5, 92.6, 87.3, 82.9, 75.9, 73.9, 64.9, 55.9, 35.9, 25.2, 14.1, 11.4; MS(ES+) m/z 357.1 $(M + H)^+$, 713.1 $(2M + H)^+$, 735.1 $(2M + Na)^+$.

5′-L-Phenylalanyl-cytarabine (5): yield 31%; mp 152−154 °C; purity 95.7% according to HPLC; λ_{max} = 272.0 nm; ^{1}H NMR(600 MHz, DMSO-d_6, δ ppm) 9.98 (1H, br), 8.75−8.80 (4H, 2br), 7.77−7.79 (1H, d, J = 7.7 Hz), 7.26−7.32 (5H, m) 6.19−6.20 (1H, d, J = 7.7 Hz), 6.01−6.02 (1H, d, J = 3.2 Hz), 5.91 (2H, br), 4.32−4.36 (2H, m), 4.27 (1H, m), 3.97−4.05 (3H, m), 3.11−3.19 (2H, m); ^{13}C NMR (600 MHz, DMSO-d_6, δ ppm) 168.7, 159.3, 146.7, 145.7, 134.6, 129.4, 128.5, 127.2, 92.7, 87.1, 82.6, 75.8, 74.0, 65.0, 53.2, 35.7; MS(ES+) m/z 391.3 $(M + H)^+$, 413.3 $(M + Na)^+$.

5′-D-Phenylalanyl-cytarabine (6): yield 33%; mp 150−153 °C; purity 97.5% according to HPLC; λ_{max} = 272.0 nm; ^{1}H NMR (600 MHz, DMSO-d_6, δ ppm) 9.98 (1H, br), 8.80 (4H, br), 7.80−7.81 (1H, d, J = 7.8 Hz), 7.26−7.34 (5H, m) 6.19−6.20 (1H, d, J = 7.8 Hz), 6.00−6.01 (1H, d, J = 3.5 Hz), 5.91 (2H, br), 4.40 (1H, dd, J = 11.6, J = 8.4), 4.28 (1H, m), 4.20 (1H, dd, J = 11.6, J = 3.5), 3.95−4.07 (3H, m), 3.21−3.24 (1H, dd, J = 14.0, J = 5.7), 3.10−3.14 (1H, dd, J = 14.0, J = 7.7); ^{13}C NMR (600 MHz, DMSO-d_6, δ ppm) 169.0, 159.5, 146.9, 146.0, 134.7, 129.5, 128.7, 127.3, 92.9, 87.3, 82.6, 76.0, 74.2, 65.2, 53.4, 35.9; MS(ES+) m/z 391.2 $(M + H)^+$, 413.2 $(M + Na)^+$.

5′-L-Tryptophyl-cytarabine (7): yield 37%; mp 157−159 °C,; purity 95.5% according to HPLC; λ_{max} = 272.0 nm; ^{1}H NMR (600 MHz, DMSO-d_6) δ 3.46 (2H, m), 4.00−4.25 (5H, m), 4.36−4.37 (2H, m), 5.91 (1H, br), 6.04 (1H, d, J = 2.9), 6.16 (1H, d, J = 7.8), 7.00 (1H, m), 7.10 (1H, m), 7.26 (1H, s), 7.37 (1H, m), 7.57 (1H, m), 7.81 (1H, d, J = 7.8), 8.65 (1H, br), 9.88 (1H, br), 11.1 (1H, s); ^{13}C NMR (600 MHz, DMSO-d_6, δ ppm) 169.3, 159.5, 146.9, 146.0, 134.7, 129.5, 128.7, 127.3, 92.9, 87.2, 82.7, 76.1 (*C*-2′), 74.3, 65.3, 52.9, 35.9; MS(ES+) m/z 430.4 $(M + H)^+$, 452.3 $(M + Na)^+$.

Cell Proliferation Assays. The human leukemic cell line, HL-60, purchased from American type Culture Collection, ATTC (Rockville, MD), was cultured in RPMI-1640 medium (Gibco, New York, NY) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 1 mmol/L L-glutamine. Cell growth inhibition was performed as reported previously.[24] Briefly, cells in logarithmic growth were seeded at 4×10^4 cells/mL and were treated with compound **1** and prodrugs **2**−**7** at different concentrations for 3 days. Cell viability was determined after staining with trypan blue. Trypan blue-stained (nonviable) cells and total cell number were measured with the aid of a hematocytometer. The growth rates of cells after treatments with these compounds at different concentrations were compared with that of control cells. The half-growth inhibitory concentration (GI_{50}) was obtained by regression analysis of the concentration−response data. The data was derived from three independent tests.

Caco-2 Culture. Caco-2 cells were obtained from the American Tissue Culture Collection (Rockville, MD). Caco-2 cells were grown routinely on 75 cm^2 culture flasks in DMEM (4500 mg/L glucose) containing 20 mmol/L HEPES, supplemented with 10% fetal bovine serum, 1% nonessential

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amino acids, 4 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 1.0 mmol/L sodium pyruvate at 37 °C in an atmosphere of 5% CO_2 and 90% humidity. The medium was replaced every 2−3 days after incubation.

Caco-2 Permeability. Transcellular transport studies were performed in triplicate with some modifications as described previously.[25] Uptake buffer was the Hanks' balanced salt solution (HBSS) which was supplemented with 20 mmol/L HEPES. HBSS contained 8.0 g/L NaCl, 0.05 g/L Na_2HPO_4, 0.4 g/L KCl, 0.06 g/L KH_2PO_4, 1.0 g/L D-glucose, 0.35 g/L $NaHCO_3$, 0.1 g/L $CaCl_2$, 0.14 g/L $MgSO_4$. Briefly, Caco-2 cells were seeded onto polycarbonate filter inserts (0.6 cm^2 growth area, 0.4 μm pore size, Millipore, MA) at a density of 1×10^5 cells/cm^2 and allowed to grow for 21−26 days. The integrity of the monolayer was determined by measuring the TEER (transepithelial electric resistance) using the Millicell ERS system (Millipore). TEER values >200 Ω·cm^2 were used for the study. Sodium fluorescein was used as the paracellular leakage marker, and the permeability coefficient of sodium fluorescein $<0.7 \times 10^{-6}$ cm/s was acceptable.[26−29] 0.4 mL of uptake buffer (containing 0.5 mM test drug, pH 6.0) and 0.6 mL of uptake buffer (pH 7.4) were added to the apical side and the basolateral side of the monolayer, respectively. Samples (200 μL) were taken from the basolateral side at 15, 30, 45, 60, 90, 120 min. The samples were deproteinated with acetonitrile and stored under −80 °C until analysis by HPLC. Because amino acid prodrugs of **1** were partially metabolized to **1** and various amino acids within Caco-2 cells, the amount of prodrugs transported was calculated as the sum of the amounts of the unchanged form and **1**.

Gly-sar Uptake Inhibition. The uptake inhibition of gly-sar in Caco-2 cells was measured as described previously.[30] Caco-2 cells were seeded at a density of 1×10^5 cells/cm^2 in 24-well plastic cluster trays and used 15 days after seeding. The cells were incubated with 10 μM gly-sar along with various concentrations (0.1−20 mM) of **2** for 10 min. The cells were washed three times with 1.5 mL of ice-cold uptake buffer (pH 6.0), and then transferred to a 1.5 mL tube and homogenized in 0.3 mL water. The homogenates were centrifuged at 2500*g* for 10 min, and the supernatants were collected. The content of gly-sar in the supernatant was analyzed by HPLC/MS/MS. The protein concentration was determined by Coomassie Brilliant Blue assay using a bovine serum albumin as standard. IC_{50} values were determined using nonlinear data fitting.

Uptake of 2 by Leptin-Treated Caco-2 Cells. Caco-2 cells were seeded at a density of 1×10^5 cells/cm^2 in 24-well plastic cluster trays. The medium was changed on the third day after seeding and every day thereafter. From the eighth day to the fifteenth day, the cells were treated with 0.2 nM leptin.[31,32] The uptake of gly-sar by the leptin-treated and the control Caco-2 cells was performed to evaluate whether the expression of PepT1 in the leptin-treated Caco-2 cells was increased. The uptake procedure was described as above.

The uptake of **1** and **2** by the leptin-treated and the control Caco-2 cells in the absence and presence of inhibitor such as gly-sar and L-valine was evaluated to study the uptake mechanism of **2**. The samples were analyzed by HPLC.

Stability and Hydrolysis Studies. (a) Stability in Rat Plasma. A stock solution of **2** was aliquoted, dried under N_2 flow, and reconstituted to 80 μg/mL with plasma previously preheated to 37 °C. Aliquot samples were collected at different time points 0, 30, 70, 100, 120 min. Samples were quenched in 5-fold volumes of ice-cold methanol and centrifuged at 2500*g* for 5 min. Supernatant was removed and analyzed by HPLC. Rate constants of hydrolysis were determined by pseudo-first-order kinetic models.

(b) Stability in Intestinal and Liver Homogenates. [33] Liver and jejunum segment were obtained from a male Sprague−Dawley rat. Tissue samples were placed on ice in buffer C (10 mM HEPES, 25 mM KCl, and 5 mM $MgCl_2$, pH 7.4). The tissue was homogenized with a tissue homogenizer and then centrifuged at 2000*g* and 4 °C for 10 min. The resulting supernatant was collected for metabolism study. Total protein content was determined with the Coomassie Brilliant Blue assay using a bovine serum albumin as standard. Reactions were initiated by addition of drug

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solutions to the homogenates at 37 °C, where **2** and protein concentrations in the reaction mixture were 80 μg/mL and 200 μg/mL, respectively. At the desired time points, aliquots (100 μL) were removed and added to 300 μL of ice-cold acetonitrile, and then centrifuged at 2500*g* for 5 min at 4 °C. The supernatant was removed and analyzed by HPLC.

(c) Chemical Stability. The nonenzymatic hydrolysis of **2** in different pH phosphate buffers (pH 1.2, 4.5, 6.8, 7.4) was determined as described above.

(d) Hydrolysis in Rat Gastric Juices and Intestinal Fluids. The rat gastric juices and intestinal fluids were collected from a 250 g male Sprague−Dawley rat. The pH of the gastrointestinal fluids was determined with a pH meter. The experiments were initiated by adding 200 μL of a stock solution of **2** to 2 mL of gastric juices or intestinal fluids preheated to 37 °C, and the concentration of **2** in the biological media was about 80 μg/mL. The mixture was stirred and incubated for 24 h at 37 °C. At the predetermined time points, samples (200 μL) were taken and quenched with 600 μL of ice-cold acetonitrile, then centrifuged at 2500*g* and 4 °C for 10 min. The supernatants were analyzed by HPLC.

Pharmacokinetic Studies. (a) Rat Pharmacokinetic Dose Response. Male Sprague−Dawley rats with body weights ranging from 220 to 260 g were housed in a temperature-controlled room for at least one week before pharmacokinetic study. All animal experiments in the present study were approved by the University Committee on Use and Care of Animals, Shenyang Pharmaceutical University. The rats ($n = 4$ per treatment) were administered by gavage of **2** (5, 15, 30 mg/kg, calculated as **1**) or **1** (30 mg/kg) in aqueous solution. Serial blood samples (0.2 mL) were obtained from orbital plexus at 5, 15, 30, 45 min and 1, 1.5, 2, 3, 4, 6, 8, 12, 24 h after oral administration separately. The solution of **1** was also intravenously administered to 4 rats at 8 mg/kg. Samples (0.2 mL) were collected at 5, 10, 15, 30 min and 1, 2, 3, 4, 5, 6, 8, 10, 12 h after administration. During sampling, rats were anesthetized with ether. All samples were placed into heparinized tubes containing the deaminase inhibitor, tetrahydrouridine (0.1 mM). After centrifugation at 800*g* and 4 °C for 10 min, plasma was collected and frozen at −80 °C until **1**, **2** and ara-U were determined by HPLC/MS/MS.

(b) Rat Portal Vein Pharmacokinetics. The systemic and hepatic portal pharmacokinetics of **1** and **2** were evaluated in male Sprague−Dawley rats with body weights ranging from 220 to 260 g ($n = 4$) after an oral administration of **2** at 15 mg/kg (calculated as **1**). A lateral incision in the abdominal cavity was made to allow access to the hepatic portal vein. Portal vein and jugular samples (0.1 mL) were withdrawn simultaneously at 5, 20, 45 min and 1, 2, 3, 4, 6, 8, 10 h. Plasma was isolated by centrifugation, frozen at −80 °C, and analyzed for **1** and **2** by HPLC/MS/MS.

Analytical Method. (a) HPLC Analysis. HPLC analysis of **1** and amino acid ester prodrugs was carried out on a SHIMADZU liquid chromatography instrument equipped with a SPD-10A UV−vis detector, an LC-10AT pump, a manual injection valve, an Anastar data workstation and a C_{18} column (5 μm, 20 cm × 0.46 cm, Diamosil, DIKMA). The analytes were eluted by 5 mM NaH_2PO_4 buffer solution: methanol:formic acid (70:30:0.1%, v/v/v) with a flow rate of 1.0 mL/min and a wavelength of 272 nm. The temperature of the column was set at 40 °C.

(b) HPLC/MS/MS Analysis. The analytes were determined by a Waters ACQUITY TQD system using a Waters ACQUITY UPLC system coupled to a Waters ACQUITY triple-quadrupole tandem mass spectrometric detector with an electrospray ionization (ESI) interface.

For determination of gly-sar, gly-sar and the internal standard isoniazid were extracted from cell homogenates using a simple protein precipitation procedure with acetonitrile. A hydrophilic interaction column (ACQUITY UPLC BEH HILIC, 50 mm × 2.1 mm, 1.7 μm) was used to retain and separate analytes from endogenous materials. The elution was carried out using a gradient of acetonitrile and water containing 0.1% formic acid. The ESI source was set in positive ionization mode. The MS/MS transitions monitored were *m*/*z* 147 to 90 (collision energy 11 eV) for gly-sar and *m*/*z* 138 to 121 (collision energy 14 eV) for isoniazid.

For analysis of **1** and **2**,[34] the two analytes and lamivudine (the internal standard) were extracted from rat plasma by Waters Oasis MCX cation-exchange solid-phase extraction cartridges (Waters Corporation; Milford, MA). The UPLC system used an ACQUITY UPLC BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm, Waters Corp., Milford, MA). UPLC elution was carried out using a gradient of water containing 2% methanol and methanol containing 0.1% formic acid. The ESI source was set in positive ionization mode. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of *m*/*z* 244 to 112 for **1** (collision energy 15 eV), *m*/*z* 343 to112 for **2** (collision energy 20 eV), *m*/*z* 230 to 112 for lamivudine (collision energy 15 eV).

For analysis of ara-U, rat plasma was deproteinized with acetonitrile to extract ara-U and the internal standard isoniazid. An ACQUITY UPLC BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm, Waters Corp., Milford, MA) was applied. The mobile phase was water and acetonitrile with a gradient for elution. The ESI was set in negative mode for ara-U analysis and in positive mode for isoniazid analysis. The transition of 243.12 to 199.9 for ara-U (collision energy 19 eV) and 138 to 121 for isoniazid(collision energy 14 eV) was used for MRM quantification.

All HPLC/MS/MS methods were validated to meet the requirement of bioanalytical methodologies, providing good recovery, accuracy and precision. Quantification was achieved by constructing a calibration curve by weighted linear

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regression of the ratio of the analyte peak areas to that of the added internal standard.

Data Analysis. (a) The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = dC_r/dt \times V_r \times 1/A \times 1/C_0 \quad (1)$$

where V_r was the receiver volume, A was the surface area of the monolayer, C_0 was the concentration of prodrug in the donor solution, and dC_r/dt was the rate of concentration change in the receiver solution.

(b) Plasma pharmacokinetic parameters were calculated by noncompartmental analysis. Rat pharmacokinetic parameters were determined from the mean concentration value at each time point. Area under curve (AUC) was calculated using the linear trapezoidal rule, and the C_{max} and T_{max} were observed from the profiles.

(c) Statistical Analysis. The statistical differences were tested using a one-tailed Student t test at the $p < 0.05$ level.

Results

Synthesis of 5′-Amino Acid Ester Derivatives of 1. Initial attempts to synthesize the prodrug were based on the one-pot conversion as described previously.[35−37] However, these attempts were unsuccessful, presumably because of insufficient reaction selectivity and the very low solubility of **1** in organic solvents. To avoid potential side reactions and improve solubility, the exocyclic amine moiety of **1** was protected with the benzyloxycarbonyl group (CBZ), and carbonyldiimidazole (CDI) was employed to activate the carbonyl group of Boc protected amino acids and 4-dimethylamino-pyridine (DMAP) as catalyst (Figure 1). All prodrug structures were confirmed by ESI-MS and NMR.

Cell Proliferation Assays. GI_{50} values for compound **1** and the prodrugs **2**−**7** measured in cell proliferation studies with HL-60 cells are summarized in Table 1.

The GI_{50} values for all prodrugs were in the range of 0.016−0.021 mM, and the GI_{50} for compound **1** was 0.02 mM. Therefore, all prodrugs exhibited similar *in vivo* antiproliferative activity to the parent drug.

Caco-2 Permeability. Apical to basolateral permeability was evaluated for **1** and six prodrugs (Figure 2A). All prodrugs tested were more permeable than **1**. 5′-Valyl prodrug **2** exhibited the highest permeability followed by 5′-L-isoleucyl prodrug **4**, and the permeability of **2** was 11 times higher than the parent drug. Therefore, **2** may be an attractive candidate for further study.

Table 1. The Antiproliferative Effects of Compound **1** and the Amino Acid Ester Prodrugs in HL-60 Cells[a]

compound	GI_{50} (mM)
cytarabine (**1**)	0.02 ± 0.001
5′-L-valyl-cytarabine (**2**)	0.016 ± 0.003
5′-D-valyl-cytarabine (**3**)	0.02 ± 0.005
5′-L-isoleucyl-cytarabine (**4**)	0.021 ± 0.003
5′-L-phenylalanyl-cytarabine (**5**)	0.017 ± 0.002
5′-D-phenylalanyl-cytarabine (**6**)	0.016 ± 0.001
5′-L-tryptophyl-cytarabine (**7**)	0.019 ± 0.004

[a] Mean ± SD, $n = 3$.

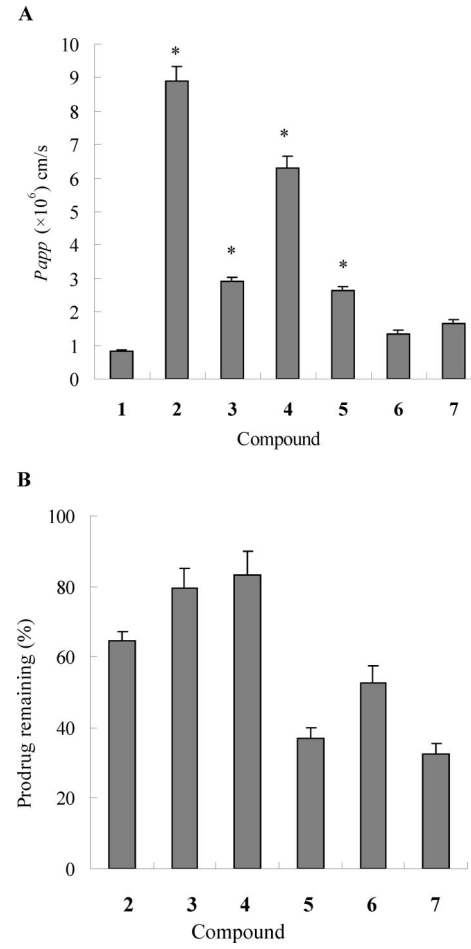

Figure 2. (A) The apical-to-basolateral permeability (P_{eff}) for the transport of compound **1** and 5′-amino acid ester prodrugs in Caco-2 cells (mean ± SD, $n = 3$). *, $p < 0.05$, compared with compound **1**. (B) Percent prodrug remaining intact in basolateral side at 120 min in apical-to-basolateral permeability studies (mean ± SD, $n = 3$).

The extent of prodrug hydrolysis in the Caco-2 monolayer transport experiment is summarized in Figure 2B. The fate of the prodrug after transport across the monolayer was highly dependent on the amino acid promoiety and its

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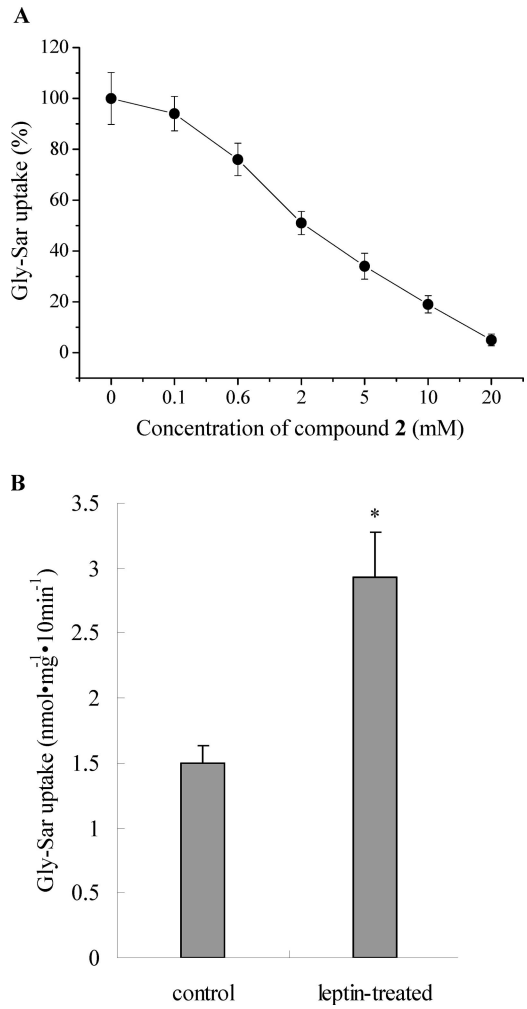

Figure 3. (A) Inhibition of compound **2** on the gly-sar uptake by Caco-2 cells. The cells were incubated at 37 °C for 10 min with 10 μM gly-sar, pH 6.0, in the presence of various concentrations of compound **2** (0.1−20 mM). After the incubation, the concentration of gly-sar in cells was determined by HPLC/MS/MS. (B) Uptake of gly-sar by the 7-day leptin-treated Caco-2 and the control Caco-2 cells. Leptin-treated Caco-2 and control Caco-2 cells were incubated at 37 °C for 10 min with 5 μM gly-sar, pH 6.0, respectively. The amounts of gly-sar in cell homogenates were measured by HPLC/MS/MS. *, $p < 0.05$, significantly different from the control. Data are presented as mean ± SD, $n = 3$.

stereochemistry. 5′-L-Isoleucyl prodrug **4** was the most stable (about 84% of prodrug remaining).

Effect of 2 on Gly-sar Uptake by Caco-2 Cells. To assess the interaction of **2** with PepT1, the inhibitory effect of **2** (0.1−20 mM) on 10 μM gly-sar uptake by Caco-2 cells was examined. The initial rate time point (10 min) for the uptake of gly-sar was selected because the accumulation of gly-sar was linear up to 20 min after incubation in Caco-2 cells (data not shown). As shown in Figure 3A, gly-sar uptake was inhibited by **2** in a concentration dependent manner, and the IC_{50} was 2.18 ± 0.12 mM.

Table 2. The Stability Results of **2** at 37 °C in Phosphate Buffers of Different pH, in Rat Tissue Homogenates, Plasma, Gastric and Intestinal Fluids (Mean ± SD, n=3)

medium	pH	$t_{1/2}$
phosphate buffer	1.2	37.3 ± 1.6 h
	4.5	19.4 ± 0.8 h
	6.8	8.7 ± 0.2 h
	7.4	4.1 ± 0.1 h
intestinal homogenates	7.4	46 ± 3.2 min
hepatic homogenates	7.4	34 ± 2.8 min
rat plasma		84 ± 5.3 min
gastric fluid	1.23	28.4 ± 1.2 h
intestinal fluid	6.67	7.5 ± 0.4 h

Uptake of Gly-sar by Leptin-Treated Caco-2 Cells. To construct a cell system highly expressing PepT1, Caco-2 cells were treated by 0.2 nM leptin for a successive 7 days. The uptake of gly-sar by the leptin-treated and control Caco-2 cells was evaluated to determine whether the long-term treatment with leptin can increase the expression of PepT1 in Caco-2 cells. As shown in Figure 3B, the uptake of gly-sar was markedly increased in the leptin-treated Caco-2 cells compared with the control Caco-2 cells, and a 1.95-fold increase was observed. Therefore, in this study, a long-term treatment with 0.2 nM leptin can increase the expression of PepT1 in Caco-2 cells because gly-sar was a typical substrate of PepT1.

Uptake of 2 and 1 by Leptin-Treated Caco-2 Cells. To confirm the transport of **2** was mediated by PepT1, the uptake of **2** and **1** by the leptin-treated and control Caco-2 cells was measured. As shown in Figure 4A, uptake of **2** by the leptin-treated Caco-2 cells was 2.35 times greater than the control Caco-2 cells, and it can be inhibited by the excess of gly-sar, but not by L-valine. In contrast, as far as the uptake of **1** was concerned, there was no significant difference between the leptin-treated and control Caco-2 cells (Figure 4B).

Stability Studies. The experiments were performed at 37 °C in different pH phosphate buffers, rat plasma, tissue homogenates and gastrointestinal fluids. The estimated half-lives ($t_{1/2}$) obtained from linear regression of pseudo-first-order plots of **2** concentration vs time are listed in Table 2. It can be observed that the chemical hydrolysis of **2** was a pH-dependent process and compound **2** was more stable at acidic pH than at neutral and alkaline pH. The $t_{1/2}$ of **2** in the intestinal and hepatic homogenates was short, which suggested the first-pass metabolism may be an important route for the bioactivation of **2** to parent drug. The degradation of **2** was very slow in the rat gastric fluids, but was more rapid than in the phosphate buffer at the same pH, indicative of a catalytic effect of enzymes arising from rat gastrointestinal tract. In a similar fashion, **2** was hydrolyzed to parent drug with a $t_{1/2}$ of 7.5 h in the rat intestinal fluids.

Pharmacokinetics in Rats. The pharmacokinetic (PK) performance of **1** and **2** after oral administration in rats was evaluated to determine whether an amino acid ester prodrug strategy could improve oral absorption of **1** *in vivo*. The aqueous solutions of **1** (30 mg/kg) and **2** (5, 15, 30 mg/kg,

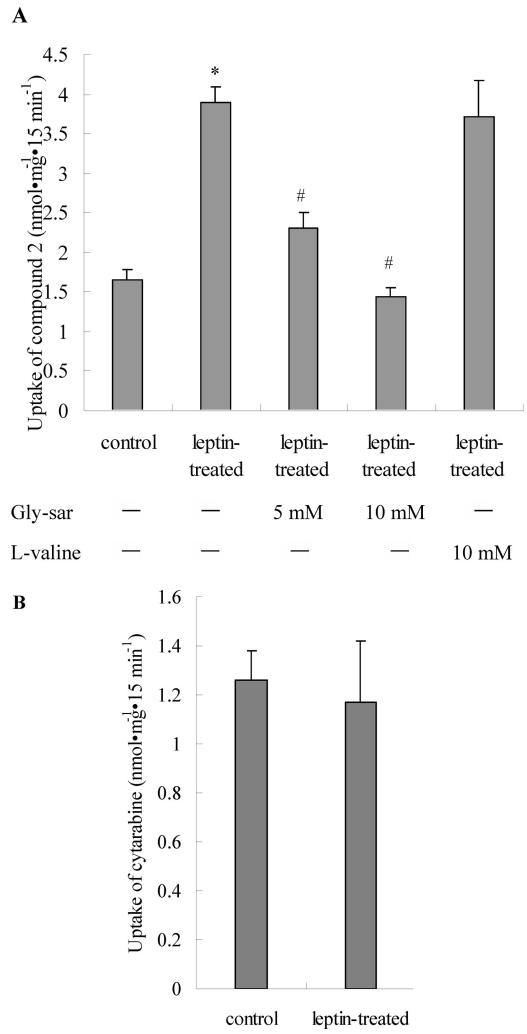

Figure 4. Uptake of compound **2** (A) and **1** (B) in the 7-day leptin-treated Caco-2 cells and the control Caco-2 cells. The Caco-2 cells were incubated at 37 °C for 15 min with 0.5 mM compound **2** or **1**, pH 6.0, in the absence and presence of the inhibitor, such as gly-sar and L-valine. After incubation, the amounts of **1** and **2** in the cell homogenates were determined by HPLC. *, $p < 0.05$, compared with the control; #, $p < 0.05$, compared with the leptin-treated Caco-2 cells without inhibitors. Data are expressed as mean ± SD, $n = 3$.

calculated as **1**) were orally administered to rats, respectively. The solution of **1** was also intravenously administered to the rats at a dose of 8 mg/kg. PK parameters of **1** are presented in Table 3. Plasma concentration−time profiles of **1** and **2** are shown in Figure 5.

Since **2** was rapidly hydrolyzed into the parent drug and its concentration in plasma was very low, the relevant AUC and elimination half-life values could not be accurately determined. Consequently, we mainly focused on the pharmacokinetic performances of **1** after oral administration of **2**. In rats orally administered with **2**, systemic exposure (AUC) of **1** and peak plasma concentration (C_{max}) increased in a dose-proportional manner from 5 mg/kg to 30 mg/kg. Following a single iv dose of **1** to rats at 8 mg/kg, the mean

Table 3. Pharmacokinetic Parameters of **1**, Following Oral Administration of **1** and **2** (Calculated as **1**) to Sprague−Dawley rats ($n = 4$)[a]

	dose			
	2			**1**
PK parameters of **1**	5 mg/kg	15 mg/kg	30 mg/kg	30 mg/kg
AUC_{0-t} (μg·h/mL)	10.49	28.89	53.75	19.53
AUC/dose	2.05	1.93	1.79	0.65
C_{max} (μg/mL)	3.54	9.90	17.16	5.00
T_{max} (h)	1.0	0.75	0.75	1.5
$t_{1/2}$ (h)	1.84	2.31	4.31	3.94

[a] AUC_{0-t}: area under the plasma concentration−time profiles from time 0 to the last time point. $t_{1/2}$: elimination half-life. C_{max}: peak plasma concentration. T_{max}: time to reach peak plasma concentration.

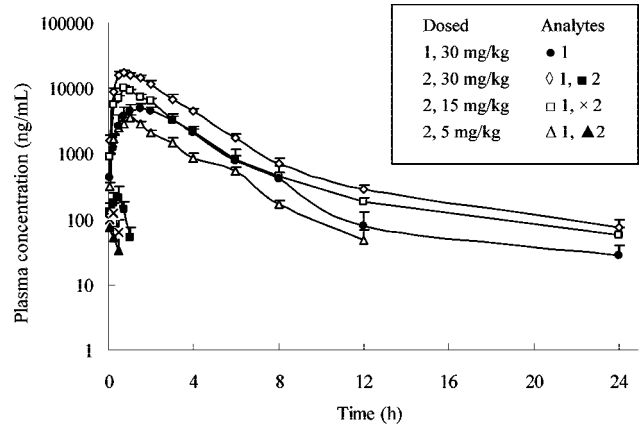

Figure 5. Mean (±SD) plasma concentration−time profiles of **1** and **2** in Sprague−Dawley rats ($n = 4$): (●) **1** following oral administration of **1** at 30 mg/kg; (Δ; □; ◇) **1** and (▲; ×; ■) **2** after oral administration of 5′-valyl prodrug **2** at 5, 15, 30 mg/kg (calculated as **1**), respectively.

AUC was 23.89 μg·h/mL. As observed from Table 3, AUC for **1** after **2** and **1** oral administration (all 30 mg/kg calculated as **1**) was 53.75 and 19.53 μg·h/mL, respectively. Thus, the absolute bioavailability of **1** following oral administration of **2** and **1** at 30 mg/kg was 60.0% and 21.8%, respectively. The prodrug-based improvement in oral availability of **1** was significant, with a 1.75-fold increase in **2** dosing group (30 mg/kg) compared with oral administration of **1**.

The disposition of the inactive metabolite ara-U in the blood was evaluated in **1** and **2** oral dosing group (30 mg/kg) to clarify whether amino acid ester prodrug could improve the metabolic stability of **1** *in vivo* (Figure 6). As observed from Table 4, AUC for ara-U was 15.58 versus 19.46 μg·h/mL, and the AUC ratio of **1**/ara-U was 1.25 versus 2.76 after **1** and **2** were orally administered (30 mg/kg calculated as **1**), respectively. So, the metabolism of **1** to ara-U by cytidine-deoxycytidine (Cyd-dCyd) deaminase was reduced after the prodrug **2** was orally administered.

Portal vein pharmacokinetic studies were conducted to evaluate the role of presystemic activation in the disposition

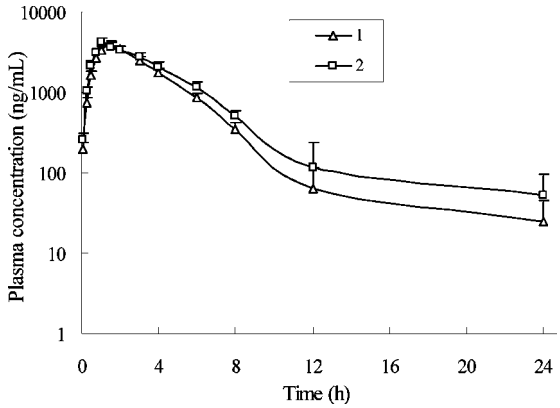

Figure 6. Mean (±SD) plasma concentration−time profiles of ara-U in Sprague−Dawley rats (n = 4) after oral administration of **1** (Δ) and **2** (□) at 30 mg/kg (calculated as **1**), respectively.

Table 4. Pharmacokinetic Parameters of Ara-U, Following Oral Administration of Compounds **1** and **2** to Sprague−Dawley Rats (n = 4) at 30 mg/kg (Calculated as **1**)[a]

PK parameters	AUC_{0-t} (μg·h/mL)	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (μg /mL)
1	15.58	3.82	1.5	3.86
2	19.46	4.37	1.0	4.26

[a] AUC_{0-t}: area under the plasma concentration-time profiles from time 0 to the last time point. $t_{1/2}$: elimination half-life. C_{max}: peak plasma concentration. T_{max}: time to reach peak plasma concentration.

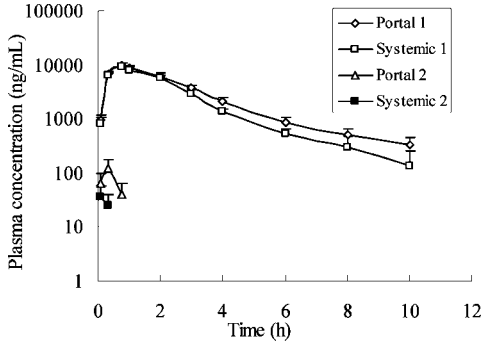

Figure 7. Mean (±SD) hepatic portal and systemic plasma concentration−time profiles of **1** and **2** in Sprague−Dawley rats (n = 4) after a 15 mg/kg oral administration of **2** (calculated as **1**). (◇) and (□) for concentration of **1** in the hepatic portal vein and systemic circulation, respectively (Δ) and (■) for **2** in the hepatic portal vein and systemic circulation, respectively.

of **2** (Figure 7). Portal vein and jugular samples were collected simultaneously from rats after a 15 mg/kg oral dose of **2**. The portal vein study revealed that the majority of **2** bioactivation occurred before reaching the portal circulation, with C_{max} value for **2** less than 1.2% for **1**. In the systemic circulation, C_{max} for **2** dropped to approximately 0.3% of C_{max} for **1**, indicating further conversion of **2** during transit to the systemic circulation and a role of hepatic and/or plasma peptidase in the bioactivation of **2**.

Discussion

Strategies based on targeting a specific intestinal transporter by designing prodrugs may be used to improve oral bioavailability of some poorly permeant drugs. The intestinal peptide transporter PepT1 has a broad spectrum of substrates. PepT1 not only serves to mediate the absorption of di/tripeptides but also functions in the transport of exogenous peptidomimetic compounds, which makes PepT1 an important target for prodrug design.[17,18] The present study demonstrated PepT1 was involved in the disposition of amino acid ester prodrugs of compound **1**.

1 is a highly hydrophilic molecule and cannot easily penetrate cell membranes. A series of amino acid ester prodrugs were synthesized with the aim to select a target compound to improve the oral bioavailability of the parent drug. The transport experiment across Caco-2 cells was carried out to evaluate their ability to transport across intestinal membranes. Consistent with previous findings (Figure 2A),[21,35] 5′-L-valyl prodrug **2** was the most efficiently transported across Caco-2 monolayer and exhibited the highest permeability, followed by 5′-L-isoleucyl derivative. This was probably due to the possibility that L-valine may have the optimal combination of chain length and branch at the β-C of the amino acid for the intestinal absorption.[14] The stereochemical preference in transport of prodrugs containing L- and D-promoieties across Caco-2 cells was also observed, which suggested that the transport process may be mediated by an active oligopeptide transporter.[14] Therefore, compound **2** was selected as a target compound for further study.

The uptake of gly-sar, a typical substrate of PepT1, by Caco-2 can be inhibited by compound **2** in a concentration-dependent manner, and the IC_{50} was 2.18 ± 0.12 mM, which suggested **2** competed with gly-sar to interact with PepT1 (Figure 3A). To confirm this result and understand the possible contribution of PepT1 to intestinal absorption of compound **2**, the uptake of **2** was performed in the long-term leptin-treated Caco-2 cells. The stably transfected hPepT1/HeLa and hPepT1/MDCK cells were usually utilized to evaluate the functional contribution of PepT1 to the uptake and transport of a potential substrate of PepT1 in many studies. But it was not easy to obtain these PepT1-transfected cell systems. It has been reported that the expression of PepT1 can be increased after a long-term treatment with leptin on Caco-2, and the possible reason was that a long-term treatment can activate the transcription of PepT1 gene and/or enhance PepT1 mRNA stability to reconstitute cytoplasmic pool of PepT1.[31,32] Hindlet reported the expression of PepT1 protein was 2.1- and 2.5- fold augmentation when a 0.2 nM leptin-treatment was applied on the apical and basolateral sides of Caco-2 cells for 7 days, respectively.[31] In this study, the uptake of gly-sar by the leptin-treated Caco-2 cells was compared with the control Caco-2 cells to determine whether this treatment was successful. The uptake of gly-sar was 2.93 nmol/mg protein/10 min in leptin-treated Caco-2 cells, 1.95-fold greater than the control Caco-2 cells, confirming that the expression of PepT1 in the leptin-treated Caco-2 cells was increased to a certain extent in the present experiment (Figure 3B). The uptake of compound **2** by the leptin-treated Caco-2 cells was 2.35-

fold greater than by the control Caco-2 cells (Figure 4A). This result can be explained by the higher expression of PepT1 in the leptin-treated Caco-2 cells. Furthermore, the uptake inhibition of **2** in the leptin-treated Caco-2 cells was characterized by the excess of gly-sar, but not L-valine (Figure 4B). There are multiple transporters present in the Caco-2 cells, such as sodium-glucose transporter 1 (SGLT-1), proton-coupled amino acid transporter (hPAT1)[38] and so on. Perhaps these transporters also contributed to the uptake of **2.** But we can conclude that PepT1-mediated uptake was a chief pathway to intestinal absorption of compound **2** *in vivo*.

The pharmacokinetic study was performed to examine the PepT1-mediated absorption and elucidate the primary site of **2** bioactivation into the parent drug **1** in rats. A dose-proportional pharmacokinetics of **1** at doses of 5−30 mg/kg of **2** was observed, suggesting the high capacity of PepT1-mediated transportation. Compound **2** was rapidly absorbed and extensively converted to **1** after oral administration to rats, as shown in Figure 5. T_{max} for **1** decreased from 1.5 h after orally administered **1** to 0.75 or 1 h after orally administered **2**, and there was a relatively minute amount of prodrug **2** in the blood. The oral bioavailability of **1** after orally administered **2** was about 60%, based on intravenous administration of **1**. The bioactivation of **2** to **1** occurred primarily within intestinal cells after oral dosing, as evidenced by the small amount of **2** appearing in the portal vein (Figure 7). This was consistent with the *in vitro* stability result, and the $t_{1/2}$ of compound **2** in rat jejunal homogenates was as short as 46 min. The fraction of **2** that passed unchanged through the intestinal epithelium was further hydrolyzed in liver and blood, as demonstrated by the lower concentration and rapid clearance of the prodrug in the systemic circulation. Therefore, ester bond was preferred for **1** to amide linkage because N^4-L-valyl-ara-C was rather stable in the systemic circulation and the parent drug cannot be rapidly released.[12] Compound **2** was stable in rat gastric fluids, but approximately 25% hydrolyzed when incubated in rat intestinal fluids for 3 h. So it can be inferred that a certain amount of compound **2** may be hydrolyzed to **1** during the rat small intestinal transit. Due to the low permeability of **1**, it was difficult for **1** to move across the intestinal epithelium. So, the luminal hydrolysis was probably one of the reasons why a higher oral bioavailability (>60%) could not be obtained after **2** oral dosing. At this point, compound **2** was very similar to valacyclovir, and the luminal degradation of valacyclovir is one of the reasons leading to about 54% of acyclovir bioavailability after valacyclovir is orally administered.[39] The next step for us is to modestly increase the chemical and metabolic stability of prodrugs in the rat gastrointestinal tract, such as by synthesizing dipeptide prodrugs of **1**, for further increase in oral bioavailability.

Cyd-dCyd deaminase catalyzes **1** to the inactive metabolite ara-U *in vivo*, which is another reason for the poor oral bioavailability of **1** except for low permeability.[39] It has been reported that the ester derivatives of some compounds can increase the metabolic stability of the parent drugs, such as L-Asp-BDCRB[33] and 5′-L-phenylalanyl-L-tyrosyl-floxuridine.[41] Therefore, the concentration of ara-U in plasma was determined to judge whether amino acid ester prodrug could improve the metabolic stability of **1** *in vivo*. Oral administration of **2** resulted in an increased **1**/ara-U ratio in the blood much grater than that after **1** was orally taken. So the metabolic degradation of **1** to ara-U can be modulated by amino acid ester prodrug. A possible explanation for this finding is that the saturation of the deaminase hydrolysis of **1** appeared in the small intestinal and hepatic cells when a larger amount of **1** was absorbed following oral administration of compound **2**.

Conclusion

A series of amino acid ester prodrugs of **1** were synthesized with the aim to enhance the oral bioavailability of **1**. The permeability across Caco-2 cells and *in vitro* antiproliferative effect in HL-60 cells were evaluated. 5′-*O*-L-Valyl ester prodrug, **2**, was selected as a target compound for further study. **2** exhibited nearly ideal drug properties, such as high solubility and permeability, good oral bioavailability, rapid and extensive bioactivation, and very low concentrations of prodrug in the systemic circulation. The present study also corroborated the idea that amino acid ester prodrug targeting intestinal PepT1 is an important and promising strategy to improve oral bioavailability of poorly absorbed drugs.[42]

Abbreviations Used

PepT1, the intestinal oligopeptide transporter 1; hPepT1, the human intestinal oligopeptide transporter 1; gly-sar, glycylsarcosine; HPLC/MS/MS, high-performance liquid chromatography/tandem mass spectrometry; DMF, *N*,*N*-dimethylformamide; THF, tetrahydrofuran; CDI, carbonyldiimidazole; DMAP, 4-dimethylamino-pyridine; DMA, *N*,*N*-dimethylacetamide; EtOAc, ethyl acetate; Et_2O, diethyl ether; EtOH, alcohol; DCM, dichloromethane.

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