molecular pharmaceutics

ARTICLE

pubs.acs.org/molecularpharmaceutics

Bifunctional Peptidomimetic Prodrugs of Didanosine for Improved Intestinal Permeability and Enhanced Acidic Stability: Synthesis, Transepithelial Transport, Chemical Stability and Pharmacokinetics

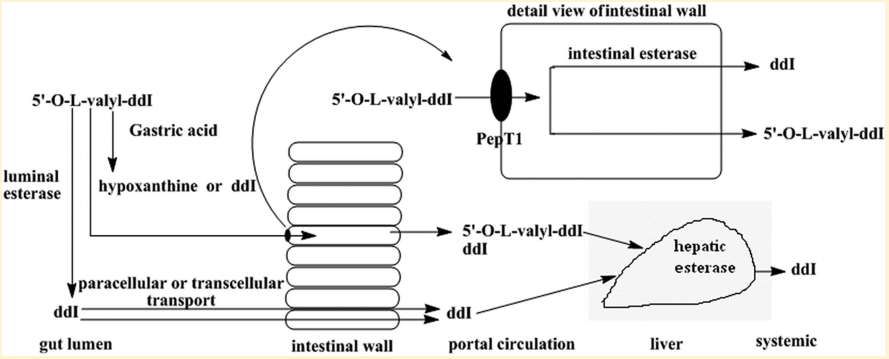
Zhongtian Yan,[†,‡] Jin Sun,[*,†] Yannan Chang,[§] Yanhua Liu,[†] Qiang Fu,[†] Youjun Xu,[§] Yongbing Sun,[†] Xiaohui Pu,[†] Youxi Zhang,[†] Yongkui Jing,[∥] Shiliang Yin,[⊥] Meng Zhu,[†] Yongjun Wang,[†] and Zhonggui He[*,†]

[†]Department of Biopharmaceutics, [⊥]Department of Pharmacology, and [§]Department of Medicinal Chemistry, Shenyang Pharmaceutical University, Shenyang, 110016, China

[‡]Beijing Winsunny Pharmaceutical Co., LTD, Beijing, 101113, China

[∥]Division of Hematology/Oncology, Department of Medicine, Mount Sinai School of Medicine, New York, New York 10029-6547, United States

ABSTRACT:



Five peptidomimetic prodrugs of didanosine (DDI) were synthesized and designed to improve bioavailability of DDI following oral administration via targeting intestinal oligopeptide transporter (PepT1) and enhancing chemical stability. The permeability of prodrugs was screened in Caco-2 cells grown on permeable supports. 5′-*O*-L-Valyl ester prodrug of DDI (compound **4a**) demonstrated the highest membrane permeability and was selected as the optimal target prodrug for further studies. The uptake of glycylsarcosine (Gly-Sar, a typical substrate of PepT1) by Caco-2 cells could be inhibited by compound **4a** in a concentration-dependent manner. The Caco-2 cells were treated with 0.2 nM leptin for enhanced PepT1 expression. The uptake of compound **4a** was markedly increased in the leptin-treated Caco-2 cells compared with the control Caco-2 cells, both of which were obviously inhibited by 20 mM Gly-Sar. The K_m and V_{max} values of kinetic study of compound **4a** transported by PepT1 in Caco-2 cells were 0.91 mM and 11.94 nmol/mg of protein/10 min, respectively. The chemical stability studies were performed in simulated gastric fluid (SGF), phosphate buffers under various pH conditions, rat tissue homogenates and plasma at 37 °C. The concentrations of DDI could not be detected in the two minutes in SGF. But compound **4a** could significantly increase DDI acidic stability, and its $t_{1/2}$ was extended to as long as 36 min in SGF. Compound **4a** was stable in pH 6.0 phosphate buffer but could be quickly transformed into DDI in plasma and tissue homogenates. The oral absolute bioavailability of DDI was 47.2% and 7.9% after compound **4a** and DDI were orally administered to rats at a dose of 15 mg/kg, respectively. The coadministration with antiacid agent could also suggest that compound **4a** was more stable under harsh acidic conditions compared with DDI. Compound **4a** bioavailability in rats was reduced to 33.9% when orally coadministrated with Gly-Sar (100 mg/kg). The In Vivo bioactivation mechanism of compound **4a** was investigated by comparing the levels of DDI and compound **4a** in the jugular and portal veins in rats. The plasma concentration of intact compound **4a** was very low in portal veins and could hardly be detected in the jugular vein. In conclusion, compound **4a** could significantly improve the oral bioavailability of DDI in rats through PepT1-mediated absorption and enhanced acidic stability, followed by rapid and mostly intracellular bioactivation, the majority in the intestinal cells but the minority in the liver. Additionally, the prodrug strategy targeted to intestinal PepT1 could offer a promising strategy to improve oral bioavailability of poorly absorbed didanosine.

KEYWORDS: didanosine, peptide transporter, prodrug, permeability, stability

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) infected by human immunodeficiency virus (HIV) is one of the most

Received: March 4, 2010
Accepted: January 31, 2011
Revised: January 22, 2011
Published: January 31, 2011

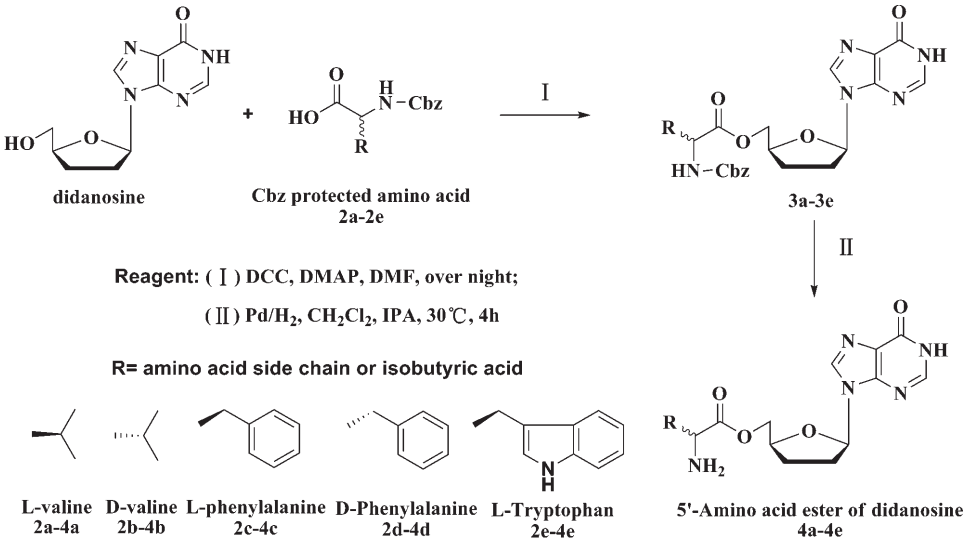


Figure 1. Synthetic scheme and structures of amino acid ester prodrugs of DDI.

important global health problems.[1,2] Didanosine (Videx, 5′-*O*-2′-3′-dideoxydidanosine, DDI) is a very effective antiretroviral drug with specific and prominent anti-HIV action, and is the second anti-HIV drug approved by the FDA.[3,4] It is well tolerated with chronic administration, and its toxicity is rare and usually reversible.[5]

However, DDI suffers from a poor bioavailability (20−40%),[6] which limits its clinical application. Many investigations had focused on the development of DDI prodrugs for increased oral bioavailability. For example, glycerolipidic, dipalmitoylphosphatidyl and adenosine deaminase-activated prodrugs of DDI were designed, but none of them were in routine clinical use as far as we know.[7−12]

In recent years, a number of nutrient transporters, such as oligopeptide, amino acid, bile acid and glucose transporters, have been identified and cloned.[13,14] Prodrugs targeted toward specific membrane transporters were designed to have structural features that allow them to be recognized by the endogenous transporters expressed at the intestinal epithelium.[15−17] Among various membrane transporters, peptide transporters are the most attractive and feasible targets in prodrug design, because of their wide distribution throughout the small intestine, high transport capacity and broad substrate specificity.[18−20] Hu and Amidon[21] have put forward that the prodrug strategy targeting the intestinal peptide carrier system is a practical and effective approach to improve oral absorption of drug with good aqueous solubility but poor membrane permeability. Two representative prodrugs that were applied to PepT1 were valacyclovir (Valtrex; GlaxoSmithKline) and valganciclovir (Valcyte; Roche). They were L-valyl esters prodrug of acyclovir and ganciclovir, both of which have limited and variable oral bioavailability owing to low intestinal permeability. These amino acid ester-based prodrugs could increase the intestinal permeability of their parent drugs by 3−10-fold, and their membrane transport was mediated predominantly by PepT1.[22−25] Both prodrugs were readily bioconverted back to their parent drugs by intracellular hydrolysis during intestinal absorption.[26,27] The prodrug strategy based on PepT1 had also applied to LY544344 (a pipeline drug of Eli Lilly Inc.), an amino acid amide-based prodrug of a metabotropic glutamate receptor 2 agonist, LY354740. LY544344 could significantly increase the oral bioavailability of LY354740 from 10% to 85% in rats through PepT1-mediated transport.[28−30] Previous studies from our laboratory had demonstrated that L-amino acid ester of cytarabine was absorbed primarily through PepT1 transporter and could increase cytarabine oral bioavailability from 21.8% to 60.0%.[31]

DDI was a representative model compound of BCS (biopharmaceutical classification system) class III.[32] The previous studies had shown that DDI absorption was low due to its poor intestinal membrane permeability and acidic instability.[33] Accordingly, we synthesized five 5′-amino acid ester prodrugs of DDI and screened their membrane permeability across Caco-2 cell monolayer. 5′-*O*-L-valyl-didanosine (compound **4a**) was selected for further studies because of its highest membrane permeability. The determination of intestinal permeability and acidic stability of compound **4a** was performed in comparison with the parent drug. Finally, pharmacokinetic studies were evaluated after oral administration of DDI and compound **4a** in Sprague−Dawley rats, respectively. All above results indicated that compound **4a** could enhance oral bioavailability of DDI to a large extent by utilizing intestinal PepT1 mediated transport and increasing the acidic stability of DDI, and had great prospects for further development in the new drug pipeline.

MATERIALS AND METHODS

Materials. Didanosine was purchased from Wuhan Yuancheng Techonolgy Development Co., Ltd. C (Wuhan, China). The carbobenzyloxy (Cbz) protected amino acids Cbz-L-valine, Cbz-D-valine, Cbz-L-phenylalanine, Cbz-D-phenylalanine, and Cbz-L-tryptophan were obtained from GL Biochem Ltd. (Shanghai, China). *N*,*N*′-Dicyclohexylcarbodiimide (DCC) and Pd/C were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shenyang, China). 4-(Dimethylamino)pyridine (DMAP) was purchased from Dongyang Tianyu Chemicals Co., Ltd. (Dongyang, China). Gly-Sar was obtained from Sigma-Aldrich (St. Louis, MO, USA). Leptin was obtained from ProSpec TechnoGene (Rehovot, Israel). All other chemicals were of analytical grade.

Caco-2 cells were obtained from the American Tissue Culture Collection (Rockville, MD, USA) and were grown routinely on 75 cm^2 culture flasks in DMEM (Dulbecco's modified Eagle's medium, 4500 mg/L glucose) containing 20 mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), supplemented with 10% fetal bovine serum, 1% nonessential 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 to 3 days after incubation. For transport studies, Caco-2 cells were seeded onto polycarbonate membrane at a density about 2×10^5 cells/cm^2 and allowed to grow for 21 to 25 days. For uptake studies, Caco-2 cells were seeded onto 24-well plastic cluster trays at a density about 1×10^5 cells/cm^2 and used 15 days after seeding.

Synthesis of 5′-Amino Acid Ester Derivatives of DDI. Cbz-amino acid (40 mmol) and DCC (20 mmol, 4.12 g) were dissolved in anhydrous DMF (*N*,*N*-dimethylformamide, 20 mL) under a nitrogen atmosphere. The mixture was continuously stirred for 1 h at 0 °C. A solution of DDI (4.72 g, 20 mmol) and DMAP (0.24 g, 0.1 mmol) in DMF (100 mL) was added dropwise to the reaction mixture. The reaction mixture was stirred and allowed to warm up to the room temperature. The reaction was monitored by TLC (thin layer chromatography, 1:9, methanol to dichloromethane). The reaction mixture was filtered and DMF was removed in vacuo at 50−55 °C after 24 h. The residue was dissolved in dichloromethane (50 mL) and washed with water (2×50 mL), saturated $NaHCO_3$ (2×50 mL) and brine (1×20 mL), respectively. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The intermediates (compounds **3a**, **3b**, **3c**, **3d**, and **3e**) were purified using column chromatography with a silica gel column and serial elution with dichloromethane:methanol (1:50−1:20). The eluents belonging to each intermediate were collected and concentrated. Pure intermediates were dissolved in dichloromethane and isopropanol, and then Pd/C (10%) was added. The reaction mixture was stirred under H_2 at 30 °C for 3 h and filtered. Compounds **4a**, **4b**, **4c**, **4d** and **4e** were concentrated and collected in vacuo (Figure 1).

The purity of prodrugs was determined by HPLC (high-performance liquid chromatography). All compounds were characterized by proton nuclear magnetic resonance spectra (^{1}H NMR) and electrospray ionization mass spectra (ESI-MS).

DDI: percent purity, 99%; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.33 (br, 1H), 8.33 (s, 1H), 8.04 (s, 1H), 6.22−6.19 (t, 1H), 5.00 (br, 1H), 4.13−4.09 (m, 1H), 3.60 (m, 1H), 3.53−3.50 (m, 1H), 2.46−2.42 (m, 1H), 2.37−2.33 (m, 1H), 2.07−2.00 (m, 2H); ESI-MS m/z 236.2 $(M + H)^+$.

Compound ***4a*** *(5′-O-L-valyl-didanosine):* yield, 42%; percent purity, 97%; ^{1}H NMR (600 MHz, DMSO-d_6/D_2O) δ 8.25 (s, 1H), 8.06 (s, 1H), 6.23−6.21 (t, 1H), 4.33−4.30 (m, 1H), 4.24−4.17 (m, 1H), 3.08−3.07 (d, 1H, J = 5.4 Hz), 2.51−2.48 (m, 2H), 2.16−2.13 (m, 1H), 2.09−2.06 (m, 1H), 1.79−1.76 (m, 1H), 0.83−0.82 (d, 3H, J = 6.6 Hz), 0.77−0.76 (d, 3H, J = 6.6 Hz); ESI-MS m/z 336.2 $(M + H)^+$.

Compound ***4b*** *(5′-O-D-valyl-didanosine):* yield, 38%; percent purity, 98%; ^{1}H NMR (600 MHz, DMSO-d_6/D_2O) δ 8.27 (s, 1H), 8.06 (s, 1H), 6.24−6.22 (t, 1H), 4.32−4.29 (m, 1H), 4.28−4.25 (m, 1H), 4.22−4.19 (m, 1H), 3.26 (t, 1H), 2.15−2.10 (m, 2H), 1.81−1.78 (m, 1H), 1.04−1.03 (d, 3H, J = 6.6 Hz), 0.83−0.78 (d, 3H, J = 6.6 Hz); ESI-MS m/z 336.1 $(M + H)^+$.

Compound ***4c*** *(5′-O-L-phenylanlanyl-didanosine):* yield, 41%; percent purity, 96%; ^{1}H NMR (600 MHz, DMSO-d_6/D_2O) δ 8.23 (s, 1H), 8.06 (s, 1H), 7.27−7.23 (m, 3H), 7.20−7.12 (m, 2H), 6.22−6.21 (t, 1H), 4.25 (m, 1H), 4.18−4.11 (m, 2H), 3.57−3.54 (t, 1H), 2.80−2.77 (m, 2H), 2.47−2.42 (m, 2H), 2.08 (m, 1H), 2.03−2.02 (m, 1H); ESI-MS m/z 384.2 $(M + H)^+$.

Compound ***4d*** *(5′-O-D-phenylanlanyl-didanosine):* yield, 41%; percent purity, 97%; ^{1}H NMR (600 MHz, DMSO-d_6/D_2O) δ 8.27 (s, 1H), 8.06 (s, 1H), 7.27−7.24 (m, 2H), 7.22−7.20 (m, 1H), 7,16−1.15 (m, 2H), 6.23−6.22 (t,1H), 4.24−4.20 (m, 2H), 4.17−4.16 (m, 1H), 3.87−3.85 (t, 1H), 2.91−2.83 (m, 2H), 2.51−2.46 (m, 2H), 2.11−2.07 (m, 2H); ESI-MS m/z 384.2 $(M + H)^+$.

Compound ***4e*** *(5′-O-L-tryptophan-didanosine):* yield, 35%; percent purity, 95%; ^{1}H NMR (600 MHz, DMSO-d_6/D_2O) δ 10.86 (br, 1H), 8.17 (s, 1H), 8.05 (s, 1H), 7.45−7.4 4 (d, 1H, J = 8.4 Hz), 7.32−7.31 (d, 1H, J = 8.4 Hz), 7.12−7.11 (d, 1H, J = 1.8 Hz), 7.03−7.01 (m, 1H), 6.89−6.87 (m, 1H), 6.20−6.18 (t, 1H), 4.19−4.18 (m, 1H), 4.15−4.12 (m, 1H), 4.07−4.04 (m, 1H), 3.65−3.63 (t, 1H), 2.97−2.95 (m, 2H), 2.51−2.50 (m, 1H), 2.39 (m, 1H), 1.79−1.76 (m, 2H); ESI-MS m/z 423.2 $(M + H)^+$.

Caco-2 Permeability. Caco-2 monolayers grown on permeable filter supports (Costar Transwell filter plate 0.4 μm pore size) for 21 to 25 days and having a TEER of exceeding 250 $\Omega \cdot cm^2$ were used for the transepithelial transport studies. HBSS transport buffer consisted of 5.4 mM KCl, 0.5 mM KH_2PO_4, 136 mM NaCl, 0.34 mM $Na_2HPO_4 \cdot 7H_2O$, 5.6 mM D-glucose, 1 mM $CaCl_2$, and 10 mM HEPES. 0.4 mL of donor buffer (containing 0.5 mM test drug, pH 6.0) and 0.6 mL of receiver 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, and 120 min. 200 μL of HBSS was added to basolateral at the sampling time. The apparent permeability (P_{app}) was calculated using the following equation:

$$P_{app} = \frac{(dC_r/dt)V_r}{AC_0} \quad (1)$$

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

Gly-Sar Uptake Inhibition. Drug inhibition effect was studied by inhibiting Gly-Sar uptake.[34,35] After washing the monolayers with HBSS buffer, the cells were incubated with a mixture of 20 μM Gly-Sar and various concentrations of compound **4a** (0.1−40 mM) for 10 min. After incubation, the medium was aspirated, and the cells were rapidly rinsed twice with 1 mL of ice-cold incubation medium. The cells were scraped into a 1.5 mL tube and homogenized with 0.3 mL of water. The homogenates were centrifuged at 2500 g for 10 min, and the supernatants were collected. The content of Gly-Sar in the supernatants was analyzed by UPLC (ultraperformance liquid chromatography). The protein concentration was determined by Coomassie Brilliant Blue assay using a bovine serum albumin as standard. IC_{50} values were determined using nonlinear fitting.

Uptake of Compound 4a by Leptin-Treated Caco-2 Cells. For leptin-treated Caco-2 cells, the cells were treated with 2 nM

leptin from the eighth day to the fifteenth day.[36,37] The uptake procedure and cell samples preparation were described as above. The uptake of Gly-Sar by the leptin-treated and the control Caco-2 cells were performed to evaluate whether the expression of PepT1 in the leptin-treated Caco-2 cells was increased. The uptake mechanism of compound **4a** was studied through the uptake experiment on Caco-2 cells in the absence and presence of Gly-Sar and L-valine. The cell samples were analyzed by HPLC.

Kinetic Analysis of Compound 4a Uptake by Caco-2 Cells. To examine the kinetics in Caco-2 cells, uptake of compound **4a** (0.5 to 5 mM) into Caco-2 cells at pH 6.0 as a function of substrate was measured either in the absence of Gly-Sar or in the presence of 50 mM Gly-Sar. Cell lysates were collected as described above, and compound **4a** was determined by HPLC.

Hydrolysis Stability Studies. *1. Stability in Phosphate Buffer and Simulated Gastric Fluid.* The degradation of compound **4a** was studied in potassium monobasic phosphate buffer solution at 37 °C which was adjusted to the corresponding pH values with hydrochloric acid or sodium hydroxide solution over the range of 1.2−10.7.

2. Stability in Intestinal and Liver Homogenates. The Sprague−Dawley rat was euthanized with sodium pentobarbital, and the liver and intestinal segment were removed and washed with buffer C (10 mM HEPES, 25 mM KCl, and 5 mM $MgCl_2$, pH 7.4) several times to remove blood, then homogenized with a tissue homogenizer and centrifuged at 2500 g at 4 °C for 10 min. The supernatant was collected for stability study. Total protein content was determined with the Coomassie Brilliant Blue assay using bovine serum albumin as standard.[38] Experiment was initiated by addition of drug solutions to the homogenates at 37 °C, where compound **4a** and protein concentrations in the mixture were 80 μg/mL and 200 μg/mL, respectively. At the predetermined time points, 100 μL of sample was collected and added to 300 μL of ice-cold acetonitrile, and then centrifuged at 2500 g for 10 min at 4 °C. The supernatant was analyzed by HPLC.

3. Stability in Rat Plasma. Blood was collected from the rat jugular vein. After centrifugation at 2500 g for 10 min, plasma was collected and stored at −80 °C until further use. One volume of drug stock solution (0.8 mM, 4 °C) was mixed with nine volumes of prewarmed plasma (37 °C) and vortexed. Extraction and analysis methods were similar to those for the homogenate experiment.

The apparent first-order degradation rate constants of compound **4a** at 37 °C were determined by plotting the logarithm of compound **4a** remaining as a function of time. The degradation rate constant (k) was the slope of the plot of log C vs time curve, and the degradation half-life ($t_{1/2}$) was then calculated by 0.693/k.

Acidic Stability Studies. Under the acidic condition, DDI was readily destroyed into the inactive degradation product, hypoxanthine. Compound **4a** and DDI were investigated in simulated gastric fluid (SGF) for comparing their acidic stability. As for **4a** acidic stability study, both concentrations of compound **4a** and DDI were determined. The apparent first-order degradation rate constant of compound **4a** was determined by plotting the logarithm of the sum of compound **4a** and DDI remaining as a function of time. As for DDI stability study, DDI concentration was determined. The apparent first-order degradation rate constant of DDI was determined by plotting the logarithm of DDI concentration remaining as a function of time.

Pharmacokinetic Studies. All animal experiments were performed in accordance with institutional guidelines and approved by the Shenyang Pharmaceutical University Animal Care and Use Committee. Male Sprague−Dawley rats (weighing from 220 to 250 g) were supplied by the Animal Centre of the Shenyang Pharmaceutical University. All the rats were clinically healthy during the period of the experiment. The rats were fasted but had free access to water overnight before administration of DDI or compound **4a**.

The rats were administered by gastric lavage of compound **4a** (5, 15, 30 mg/kg, calculated as DDI) or DDI (30 mg/kg) in aqueous solution. During sampling, rats were anesthetized with ether. For coadministration with antiacid, compound **4a** (15 mg/kg, calculated as DDI) and DDI (15 mg/kg) were administrated to rats alone or with antiacid agent ($CaCO_3$, 500 mg/kg). For coadministration with Gly-Sar, compound **4a** (10 mg/kg, calculated as DDI) was administrated to rats alone or with Gly-Sar (100 kg/mg). Serial blood samples (about 0.2 mL) were obtained at 2, 5, 10, 15, 20, 30, 45, 60, 120, 180, 240, and 360 min, respectively. All samples were placed into heparinized tubes. The rat plasma was centrifugated at 2500 g for 10 min, collected, frozen at −80 °C and analyzed by UPLC.

The systemic and hepatic portal pharmacokinetics of compound **4a** was evaluated in male Sprague−Dawley rats after an oral administration of compound **4a** at 15 mg/kg (calculated as DDI). 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, 15, 25, 30, 45, 60, and 120 min.[39] Plasma was isolated by centrifugation, frozen at −80 °C, and analyzed for DDI and compound **4a** by UPLC.

Development of Analytical Method. *1. HPLC−UV Analysis.* The HPLC analytical method for DDI and amino acid ester prodrugs was carried out on a SHIMADZU liquid chromatography instrument equipped with a SPD-10A UV−vis detector and a LC-10AT pump. The stationary phase, a C_{18} column (5 μm, 200 mm × 4.6 μm, Diamosil, DIKMA) was kept at 40 °C. The mobile phase was a mixture of methanol:0.05 M KH_2PO_4 buffer solution (pH 5.8) = 25:75. The flow rate was 1.0 mL/min, and the wavelength was 250 nm.

2. UPLC−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 testing of Gly-Sar,[40] isoniazid was selected as the internal standard. The protein precipitation was applied to extract Gly-Sar and isoniazid from cell homogenates with acetonitrile. A hydrophilic interaction column (ACQUITY UPLC BEH HILIC, 50 mm × 2.1 mm, 1.7 μm, Waters Corporation, Wexford, Ireland) was used to analyze Gly-Sar and isoniazid. 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 DDI and compound **4a** and lamivudine (the internal standard), oasis HLB cartridges obtained from Waters Corporation (1 cc, 30 mg, Milford, MA, USA) were used to extract analytes from rat plasma.[41] The same chromatographic column as mentioned above was used for analysis. UPLC elution was carried out using a mobile phase consisting of water containing 0.1% formic acid and methanol (85:15, v/v). The ESI source

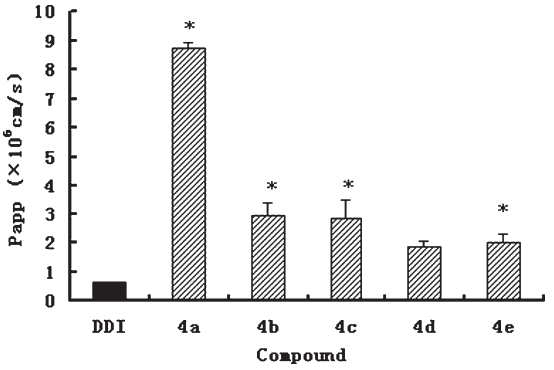


Figure 2. The apical-to-basolateral permeability for the transport of DDI and its 5′-amino acid ester prodrugs in Caco-2 cells (mean ± SD, n = 3). *, $p < 0.05$, versus DDI.

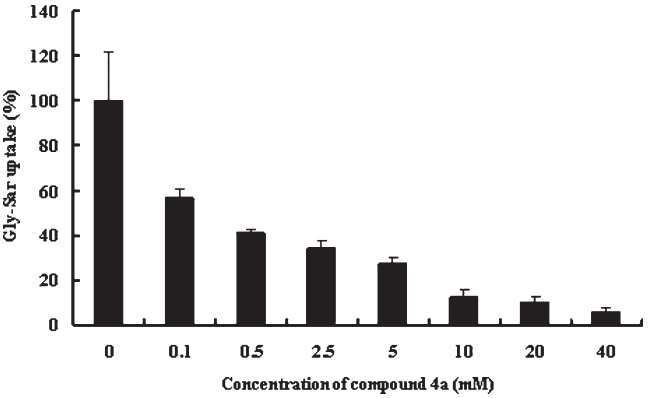


Figure 3. Inhibition of compound **4a** on the uptake of Gly-Sar on Caco-2 cells. The cells were incubated at 37 °C for 10 min with 20 μM Gly-Sar, pH 6.0, in the presence of various concentrations of compound **4a** (0.1−40 mM). After the incubation, the concentration of Gly-Sar in cells was determined by UPLC−MS/MS. Results are the mean ± SEM of three experiments.

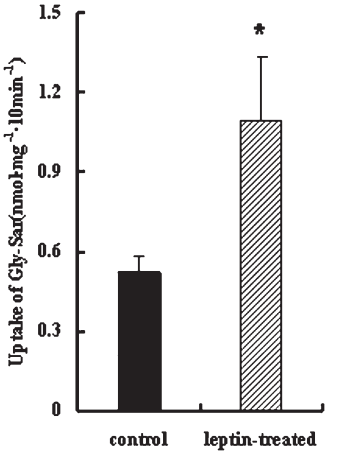


Figure 4. Uptake of Gly-Sar by the 7-day leptin-treated Caco-2 and the control Caco-2 cells. Leptin-treated Caco-2 and control Caco-2 cells were incubated at 37 °C for 10 min with 20 μM Gly-Sar, pH 6.0, respectively. The amounts of Gly-Sar in cell homogenates were measured by UPLC−MS/MS. *, $p < 0.05$, versus the control Caco-2 cells. Results are the mean ± SEM of three experiments.

was set in positive ionization mode. Cone voltage was 10, 20, and 22 V and collision energy was 10, 12, and 12 eV for DDI, compound **4a** and lamivudine, respectively. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 236 to 137 for DDI, m/z 336 to 200 for compound **4a**, m/z 230 to 112 for lamivudine.

Pharmacokinetic Data Analysis. Noncompartmental pharmacokinetic analysis was conducted to calculate the area under the curve (AUC). The peak plasma concentration (C_{max}) and the time to reach peak plasma concentration (T_{max}) of the different dosage forms were determined by a visual inspection of the experimental data. The AUC was estimated according to the linear trapezoidal rule. The statistical differences were tested using a one-tailed Student t test at the $p < 0.05$ level.

RESULTS AND DISCUSSION

Synthesis of 5′-Amino Acid Ester Derivatives of DDI. The aromatic amino acids (L-phe, D-phe and L-tryptophan) and the aliphatic amino acids (L-val and D-val) were selected as promoieties for the synthesis of didanosine prodrugs. Amino acid ester-based prodrugs of DDI were synthesized as described in Figure 1. The reaction was observed and detected by TLC. One of the simplest methods for esterification of the hydroxymethyl DDI was to use the symmetrical anhydride of the protected amino acid in the presence of DMAP. Symmetrical anhydrides were generated under nitrogen atmosphere using two equivalents of Cbz-protected amino acid and one equivalent of DCC in DMF.[42] The Cbz-protected group was chosen because strict acidic conditions would result in the degradation of DDI when removing the Boc-protected group.

Screening Caco-2 Permeability of DDI Peptidomimetic Prodrugs. The apical to basolateral permeability of DDI and five prodrugs was evaluated. As shown in Figure 2, all prodrugs were found to be more permeable than DDI. Consistent with the previous studies,[22−25,31] compound **4a** (5′-*O*-L-valyl prodrug) exhibited the highest permeability and could be efficiently transported across the Caco-2 monolayer. This was likely due the possibility that L-valine might have the optimal combination of chain length and branch at the β-C of the amino acid for PepT1 recognition and binding affinity. The stereochemical preference in the transport of prodrugs containing L- and D-promoieties across Caco-2 cells was also observed. Compared with the D-counterpart, the L-configuration was more permeable. This was consistent with the previous reports for amino acid esters of acyclovir and AZT.[43,44] Therefore, we selected compound **4a** as the optimal target compound for further studies.

Effect of Compound 4a on Gly-Sar Uptake by Caco-2 Cells. To assess the interaction of compound **4a** with PepT1, the inhibitory effect of compound **4a** (0.1−40 mM) on the uptake of 20 μM Gly-Sar by Caco-2 cells was examined. The initial rate at time point 10 min for the uptake of Gly-Sar was selected as the literature reported.[31] Seen from Figure 3, the uptake of Gly-Sar was inhibited by compound **4a** in a concentration-dependent manner, with IC_{50} being 0.27 ± 0. 07 mM.

Uptake of Gly-Sar by Leptin-Treated Caco-2 Cells. The stable transfected hPepT1/HeLa and hPepT1/MDCK cells were usually used 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 straightforward to obtain these PepT1-transfected cell systems. It has been reported that treatment with leptin on Caco-2 could significantly increase PepT1 activity and protein expression In Vivo and in vitro, and

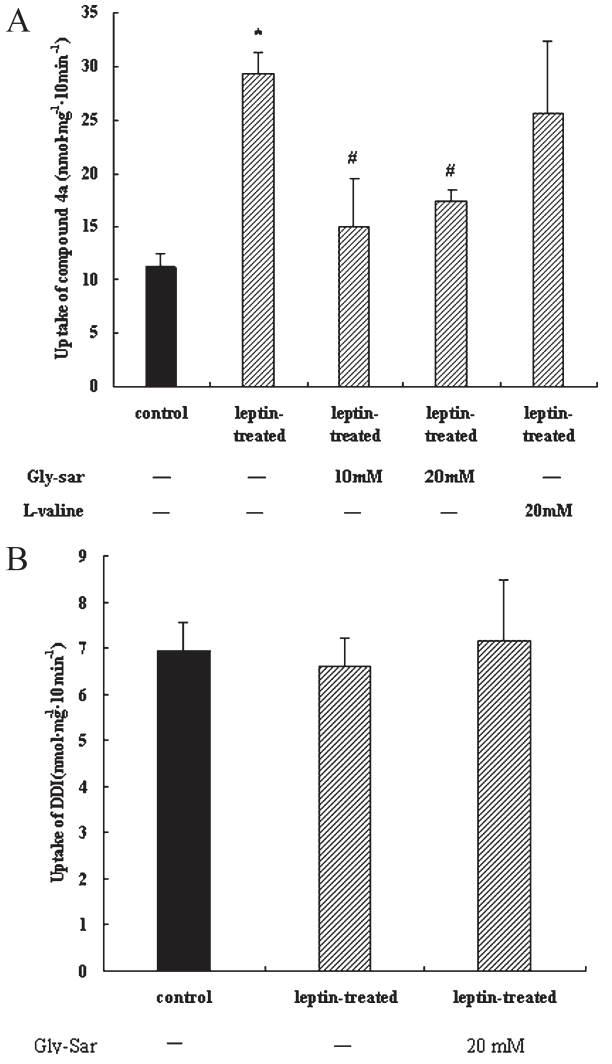


Figure 5. Uptake of compound **4a** (A) and DDI (B) in the 7-day leptin-treated Caco-2 and the control Caco-2 cells. The Caco-2 cells were incubated at 37 °C for 10 min with 0.5 mM compound **4a** or DDI, at pH 6.0, in the absence and presence of Gly-Sar and L-valine. After incubation, the amounts of compound **4a** or DDI in the cell homogenates were determined by HPLC. Results are the mean ± SEM of three experiments. *, $p < 0.05$, compared with the control Caco-2 cells; #, $p < 0.05$, versus the leptin-treated Caco-2 cells without inhibitors.

the expression of PepT1 protein was 2.1-fold augmentation when a 0.2 nM leptin treatment was applied on the apical sides of Caco-2 cells for 7 days.[36,45,46]

In this study, the uptake of Gly-Sar by the leptin-treated Caco-2 cells was determined to judge whether this treatment was successful by comparison with the control Caco-2 cells. The uptake of Gly-Sar was 1.10 nmol/mg of protein/10 min in the leptin-treated Caco-2 cells, 1.95-fold greater than the control Caco-2 cells (Figure 4), which indicates significantly increased expression of PepT1 in the leptin-treated Caco-2 cells.

Uptake of Compound 4a and DDI by Leptin-Treated Caco-2 Cells. To further confirm whether the transport of compound **4a** was mediated by PepT1, the uptake was carried out on the leptin-treated Caco-2 cells and the control Caco-2 cells. As shown in Figure 5A, uptake of compound **4a** by the leptin-treated Caco-2 cells was 2.35 times greater than that of the control Caco-2 cells, and it could be inhibited by the excess of

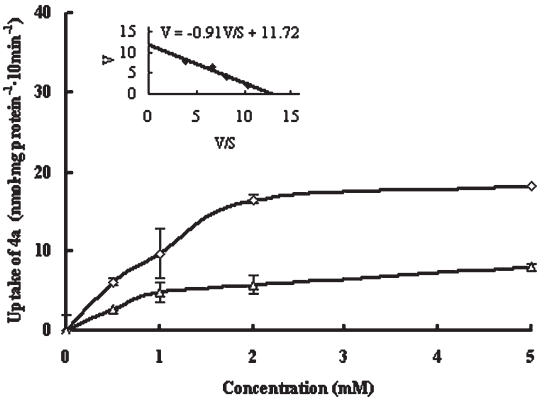


Figure 6. Concentration-dependent uptake of compound **4a** from the apical side in Caco-2 cells. The Caco-2 cells were incubated at 37 °C for 10 min with various concentrations of compound **4a** in the absence (◆) or in the presence (■) of Gly-Sar (50 mM). Each point represents the mean ± SEM of three independent monolayers. Inset: Eadie−Hofstee plot of saturable compound **4a** uptake, *V*, uptake rate (nmol/mg of protein/10 min); *S*, compound **4a** concentration (mM).

Table 1. The Hydrolysis Stability Results of 4a (80 μg/mL) at 37°C in Rat Tissue Homogenates, Plasma and Intestinal Fluids (Protein Concentration, 200 μg/mL)

incubation media	$t_{1/2}$ (min)
intestinal homogenates	121
hepatic homogenates	2
rat plasma	31
intestinal fluids	231

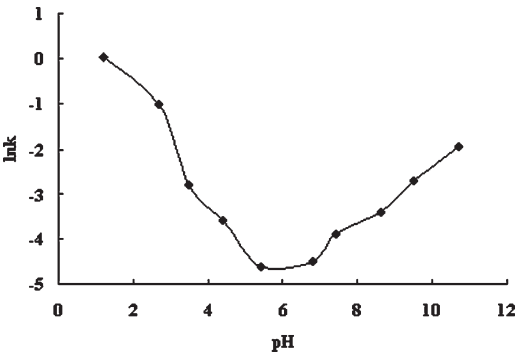


Figure 7. The hydrolysis trend of **4a** in different pH potassium monobasic phosphate buffer solutions at 37 °C, which was adjusted to the corresponding pH values with hydrochloric acid or sodium hydroxide solution over the range of 1.2−10.7.

Gly-Sar, but not by L-valine. This was probably due to that fact that Gly-Sar is a substrate of PepT1, but L-valine is not. In the case of DDI (Figure 5B), there was no significant uptake difference between the leptin-treated and the control Caco-2 cells. Additionally, the uptake of DDI was not inhibited by 20 mM Gly-Sar. Both results indicated that DDI is not a substrate of PepT1.[32,33]

Kinetic Study of Compound 4a Uptake by Caco-2 Cells. We performed a kinetic analysis of compound **4a** uptake from the apical side in the Caco-2 cells. Transformation form of the transport rates according to Eadie−Hofstee (inset) revealed K_m and V_{max} values of 0.91 mM and 11.72 nmol/mg of protein/10 min, respectively (Figure 6). The K_m value of

Table 2. The Acidic Stability Results of DDI and Compound 4a in the Simulated Gastric Fluid

compound	$t_{1/2}$ (min)
DDI	<2
4a	36

compound **4a** was similar to those of Gly-Sar (1 mM) and cafadroxil (1.1 mM), suggestive of the relative low affinity but high capacity (V_{max}) of intestinal PepT1.[47,48] The relatively high K_m value of compound **4a** is essential to the high-capacity PepT1-mediated transport and reproducible oral absorption performances at a wide range of orally administered doses. Figure 6 also showed that the concentration-dependent uptake of compound **4a** was also markedly inhibited by 50 mM Gly-Sar.

Stability Studies. The hydrolysis stability experiments were performed in pH 1.2−10.7 phosphate buffers, rat plasma and tissue homogenates at 37 °C. The estimated half-life ($t_{1/2}$) obtained from linear regression of pseudo-first-order plots of compound **4a** concentration vs time is listed in Table 1. It was easily observed in Figure 7 that the hydrolysis stability of compound **4a** was notably influenced by the pH value of phosphate buffer. Compound **4a** was more stable at about pH 6.0. When the pH < 6.0, it displayed H^+ catalyzed hydrolysis, and when the pH > 6.0, it displayed OH^- catalyzed hydrolysis. The $t_{1/2}$ values of compound **4a** in the hepatic homogenates and plasma were much shorter than those in the intestinal homogenates and buffer solutions (Table 1). In order to ensure the activity of intestinal enzymes in intestinal homogenates, the positive control experiment was carried out. With the classic drug aspirin being the model drug, its $t_{1/2}$ value was determined to be 49 min in intestinal homogenates (the protein concentration, 200 μg/mL). So we concluded that the activity of intestinal enzymes had been maintained following intestinal homogenate preparation in this study.

As for the acidic stability, the $t_{1/2}$ of DDI was very short in SGF and its concentrations could not be detected in two minutes. But compound **4a** could increase DDI stability in SGF, and its $t_{1/2}$ was as long as 36 min (Table 2). Compound **4a** delayed the chemical degradation of DDI in vitro, which achieved one aim of the designed peptidomimetic prodrug. That is to say, compound **4a** was much more stable under acidic conditions compared to DDI, beneficial to prevention of DDI degradation and PepT1-mediated intestinal membrane transport. Followed by intestinal membrane transport, compound **4a** would enter intestine, liver and plasma, where it was rapidly converted to DDI for bioactivation and clinical effect.

An ideal prodrug should exhibit good chemical stability before the PepT1-mediated transport and must be enzymatically converted to active parent drug following transport across the biological membrane. Usually, the amide bond was stable against the enzymatic hydrolysis and then had low bioactivation rate; however the ester bond could be rapidly hydrolyzed by esterases.[31,49] For example, amino acid amide-based prodrug based on PepT1 should be analyzed with caution case by case. The above-mentioned amide-based prodrug LY544344 was easily converted into the active parent drug In Vivo, while amide-based N4-L-valyl-cytarabine exhibited very low oral bioavailability of only 4%, owing to the facts that the amide linkage was stable In Vivo and the active parent drug could not be released from the prodrug. In this study, ester-based 5′-*O*-L-valyl-didanosine could quickly release the parent drug In Vivo at a bioconversion rate a lot higher than in vitro, suggesting that compound **4a** has suitable in vitro chemical stability and In Vivo bioactivation behavior.

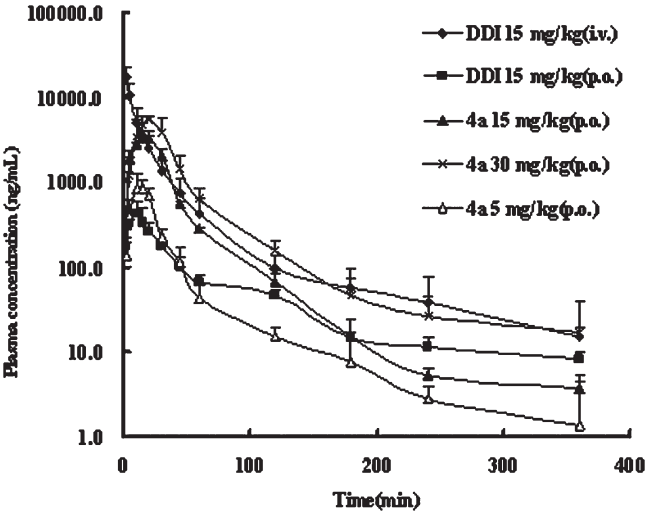


Figure 8. Mean plasma concentration−time profiles of DDI and compound **4a** in Sprague−Dawley rats (n = 4): DDI following intravenous (◆) and oral (■) administration of DDI at 15 mg/kg; compound **4a** after oral administration of compound **4a** at 5 (△), 15 (▲), and 30 mg/kg (×) (calculated as DDI).

Pharmacokinetics in Rats. Figure 8 showed the mean plasma DDI concentration versus time profile after oral administration of DDI and compound **4a** in rats, respectively. And the main pharmacokinetic parameters are shown in Table 3. Since compound **4a** 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. Therefore, we mainly focused on the pharmacokinetic performances of DDI after oral administration of compound **4a**. Following iv injection of DDI to rats (15 mg/kg), the mean AUC was 40.2 μg·h/mL. As observed from Table 3, AUC for DDI after compound **4a** (15 mg/kg, calculated as DDI) and DDI (15 mg/kg) oral administration was 18.9 and 3.2 μg·h/mL, respectively. Thus, the absolute bioavailability of DDI following oral administration of compound **4a** and DDI was 47.1% and 7.9%, respectively. The bioavailability of prodrug-based compound **4a** was nearly 4-fold higher than that of DDI, suggesting that compound **4a** has great prospects for further study during the new drug development pipeline and that the prodrug strategy targeting intestinal PepT1 has a great potential to improve oral bioavailability of poorly absorbed drugs.

The bioavailability of DDI was increased significantly when DDI was orally coadministrated with antiacid agent. Antacid combination of DDI increased the bioavailability by 115.8%, while **4a** (antacid combination) only increased by 30.1% (Figure 9). The large increase in the bioavailability of DDI could be due to the neutralization of the gastric acid by the antiacid agent and lowered degradation rate of DDI. The In Vivo results confirmed the in vitro result that DDI was relatively instable under acidic conditions compared to **4a** and thus the effect of antiacid agent on the oral absorption of DDI was more significant than for compound **4a**.

Compound **4a** was found to be the substrate of PepT1 in vitro. So we designed the coadministration with the Gly-Sar for the purpose to clarify the role of PepT1 in the oral absorption of compound **4a** In Vivo. When oral coadministration was with PepT1 typical substrate, Gly-Sar (100 mg/kg), the absolute

Table 3. Pharmacokinetic Parameters of DDI, Following Oral Administration of DDI (15 mg/kg) and Compound 4a (5 mg/kg, 15 mg/kg and 30 mg/kg, Calculated as DDI) Respectively to Sprague−Dawley Rats, Respectively (Mean ± SD, n = 4)

	dose				
	4a			DDI	
PK Params of DDI	5 mg/kg (po)	15 mg/kg (po)	30 mg/kg (po)	15 mg/kg (po)	15 mg/kg (iv)
AUC_{0-t} (μg·h/mL)	4.0 ± 0.5	18.9 ± 1.7	32.4 ± 3.7	3.2 ± 0.5	40.2 ± 10.7
AUC/dose	0.8 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	0.2 ± 0.04	2.68 ± 0.07
C_{max} (μg/mL)	0.9 ± 0.3	3.5 ± 0.3	6.0 ± 0.5	0.5 ± 0.1	16.1 ± 5.1
T_{max} (min)	12.5 ± 2.9	18.3 ± 2.9	21.3 ± 6.3	10 ± 4.1	0
$t_{1/2}$ (min)	90.3 ± 75.9	36.2 ± 5.1	46.3 ± 14.9	110.2 ± 56.4	65.5 ± 21.3

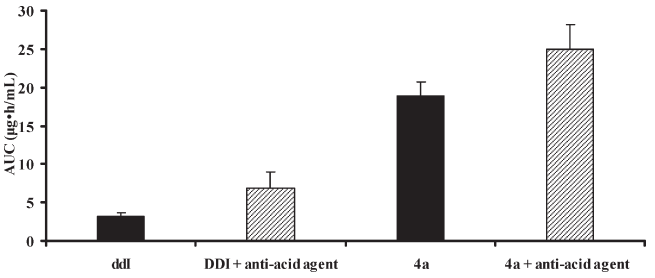


Figure 9. The effect of antiacid agent ($CaCO_3$) on the AUC of DDI and **4a** following oral administration of DDI, DDI with antiacid, **4a** and **4a** with antiacid (15 mg/kg, mean ± SD, n = 4).

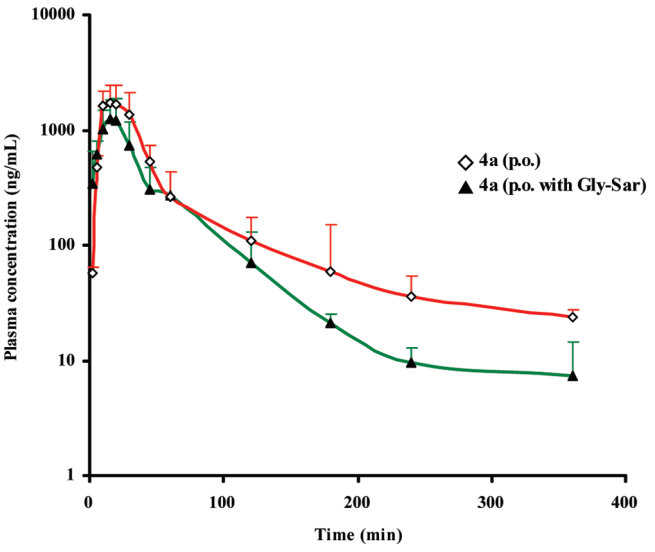


Figure 10. Mean plasma concentration−time profiles of DDI after oral administration of **4a** (10 mg/kg, calculated as DDI dose) to Sprague−Dawley rats and inhibitory effects of Gly-Sar on **4a** absorption (n = 4).

bioavailability of DDI following oral administration of compound **4a** (10 mg/kg, calculated as DDI dose) decreased from 44.6% to 33.9%, respectively (Figure 10). The results showed the competitive inhibition of Gly-Sar on the PepT1-mediated transport of compound **4a** indeed occurred in the In Vivo conditions. Although the inhibitory effect (27.4%) was not so significant, the result did reflect the inhibitory tendency of Gly-Sar on the oral absorption of compound **4a**. For this reason, there exist potential PepT1-mediated drug−drug interactions when compound **4a** is orally coadministrated with some drugs of PepT1 substrate,

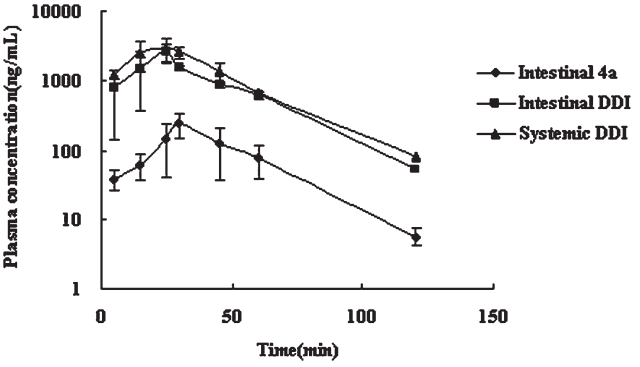


Figure 11. Mean plasma concentration−time curves of compound **4a** and DDI after oral administration of compound **4a** (15 mg/kg, calculated as DDI dose) in Sprague−Dawley rats (mean ± SD, n = 4): (■) and (▲) for concentration of DDI in the hepatic portal vein and systemic circulation, respectively; (◆) for concentration of intact compound **4a** in the hepatic portal vein.

such as β-lactam antibiotics, angiotensin-converting enzyme inhibitors, etc.

In Vivo bioactivation mechanism study of compound **4a** was carried out in Sprague−Dawley rats with cannulated jugular and portal veins and was elucidated by comparing the levels of DDI and compound **4a** in jugular and portal veins (Figure 11). Following oral administration, compound **4a** was rapidly absorbed from the gastrointestinal tract. Portal and jugular vein samples were collected simultaneously from rats after a 15 mg/kg oral dose of compound **4a**. The portal vein samples revealed that the majority of compound **4a** bioactivation occurred in the intestinal cells and then the minority in the liver before reaching the systemic circulation, with C_{max} value for DDI in portal vein nearly 90% of DDI in jugular vein. In the systemic circulation, no intact compound **4a** could be detected. This result was the same as in our previous study of amino acid ester-based valylcytarabine, with over 98% of prodrug hydrolysis occurring before appearance in the portal vein.[31] Both bioactivation studies indicated that the amino acid ester-based prodrugs could easily be converted into the parent drug In Vivo, especially before entering the liver. The relatively quick hydrolysis rate may be unfavorable to further improve the oral bioavailability of prodrug targeted to PepT1, due to relatively rapid degradation rate, especially before the gastrointestinal membrane active transport. So we planned to synthesize the dipeptide ester-based prodrug of DDI targeted to PepT1 in the next study, to further increase the in vitro chemical stability in the gastrointestinal tract and to enhance the binding affinity with PepT1.

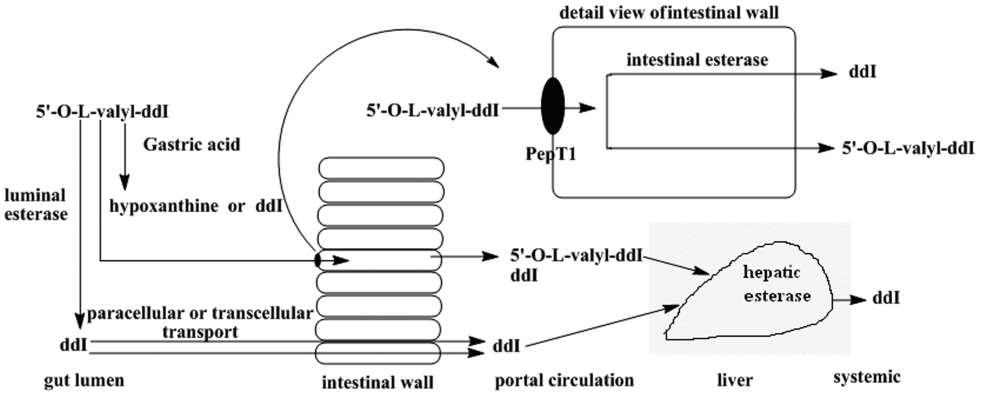


Figure 12. Schematics of In Vivo fate 5′-*O*-L-valyl-DDI following oral administration.

The purpose of designing DDI prodrugs was based on two points. One was to enhance its membrane permeability through PepT1-mediated membrane transport, while the other was to improve DDI chemical stability under gastric acidic conditions. In vitro results showed that compound **4a** was a substrate of PepT1 and could increase DDI chemical stability in SGF. As for the In Vivo experiments, multiple-dose pharmacokinetic and inhibition study of Gly-Sar confirmed that compound **4a** was the substrate of PepT1 and could significantly enhance the bioavailability of DDI. The combination experiment with antacid agent further confirmed the increased acidic stability of DDI by compound **4a**.

We summarized and delineated the In Vivo fate of the prodrug **4a**, as shown in Figure 12. The prodrug compound **4a** was degraded to a lesser extent by gastric acid and luminal esterases after oral administration. And the undegraded compound **4a** transported the intestinal membrane effectively mediated by intestinal PepT1 and then most of **4a** was bioactivated by the intestinal cellular esterase into the parent drug. The remaining prodrug compound **4a** was further hydrolyzed by the hepatic esterase. In this way, no intact form of the compound **4a** entered the systemic circulation and thus the parent drug DDI was effectively delivered to the blood via the bifunctional peptidomimetic prodrug of DDI.

CONCLUSIONS

Five amino acid ester prodrugs of DDI were synthesized with the purpose to enhance the intestinal membrane transport mediated by PepT1, and finally to increase the oral bioavailability of DDI. The permeability across Caco-2 cells was evaluated, and 5′-*O*-L-valyl ester prodrug of DDI (compound **4a**) demonstrated the highest Caco-2 membrane permeability. The Caco-2 cells inhibition and induction experiment proved that PepT1 was involved in the intestinal membrane transport of compound **4a**, with K_m and V_{max} values being 0.91 mM and 11.94 nmol/mg of protein/10 min, respectively. Also **4a** could significantly enhance the acidic stability of DDI in vitro and In Vivo. Together, compound **4a** showed almost 5-fold increase in the oral bioavailability of DDI, and demonstrated rapid and extensive bioactivation following oral administration in rats. Such an approach would allow the reintroduction of DDI through the suitable prodrug form as a standard agent for the treatment of AIDS.

AUTHOR INFORMATION

Corresponding Author

*Postal address: Mailbox 59#, Department of Biopharmaceutics, School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China (Z.H.). Tel/fax: +86-24-23986321. E-mail: sunjin66@21cn.com (J.S.), hezhonggui@gmail.com (Z.H.).

ACKNOWLEDGMENT

We thank Yue Li for her expert animal experiment technology. We are also grateful to financial support from the National Natural Science Foundation of China (No. 30973588, 30973655). We are grateful for financial support from Key Project for Drug Innovation (No. 2010ZX09401-304, 2009ZX09301-012) from the Ministry of Science and Technology of China.

REFERENCES

(1) AIDS Epidemic Update 2007, World Health Organization. http://www.who.int/hiv/epiupdates/en/index.html (accessed November 2009).

(2) Richman, D. D.; Margolis, D. M.; Delaney, M.; Greene, W. C.; Hazuda, D.; Pomerantz, R. J. The challenge of finding a cure for HIV infection. *Science* **2009**, *323*, 1304–7.

(3) Perry, C. M.; Nobel, S. Didanosine-An updated review of its use in HIV infection. *Drugs* **1999**, *58*, 1099–135.

(4) http://www.fda.gov/drugs/default.htm (accessed November 2009).

(5) Beach, J. W. Chemotherapeutic agents for human immuno deficiency virus infection: mechanism of action, pharmacokinetics, metabolism, and adverse reactions. *Clin. Ther.* **1998**, *20*, 2–25.

(6) Balimane, P. V.; Sinko, P. J. Involvement of multiple transporters in the oral absorption of nucleoside analogues. *Adv. Drug Delivery Rev.* **1999**, *39*, 183–209.

(7) Manouilov, K. K.; Xu, Z. S.; Boudinot, F. D.; Schinazi, R. F.; Chu, C. K. Lymphatic targeting of anti-HIV nucleosides: distribution of 2′,3′-dideoxyinosine after intravenous and oral administration of dipalmitoylphosphatidyl prodrug in mice. *Antiviral Res.* **1997**, *34*, 91–9.

(8) Singhal, D.; Ho, N. F.; Anderson, B. D. Absorption and intestinal metabolism of purine dideoxynucleosides and an adenosine deaminase-activated prodrug of 2′,3-dideoxyinosine in the mesenteric vein cannulated rat ileum. *J. Pharm. Sci.* **1998**, *87*, 569–77.

(9) Anderson, B. D.; Morgan, M. E.; Singhal, D. Enhanced oral bioavailability of DDI after administration of 6-Cl-ddP, an adenosine

deaminase-activated prodrug, to chronically catheterized rats. *Pharm. Res.* **1995**, *12* (8), 1126–33.

(10) Kawaguchi, T.; Hasegawa, T.; Seki, T.; Juni, K.; Morimoto, Y.; Miyakawa, A.; Saneyoshi, M. Prodrugs of 2′,3′-dideoxyinosine (DDI) improved oral bioavailability via hydrophobic esters. *Chem. Pharm. Bull* **1992**, *40* (5), 1338–40.

(11) Sriram, D.; Yogeeswari, P.; Babu, N. R.; Kurre, P. N. Synthesis and in vitro anti-HIV activities of didanosine prodrugs. *J. Enzyme Inhib. Med. Chem.* **2007**, *22* (1), 51–5.

(12) Lalanne, M.; Paci, A.; Andrieux, K.; Dereuddre-Bosquet, N.; Clayette, P.; Deroussent, A.; Re, M.; Vassal, G.; Couvreur, P.; Desmaele, D. Synthesis and biological evaluation of two glycerolipidic prodrugs of didanosine for direct lymphatic delivery against HIV. *Bioorg. Med. Chem. Lett.* **2007**, *17* (18), 2237–40.

(13) Sai, Y.; Tsuji, A. Transporter-mediated drug delivery:recent progress and experimental approaches. *Drug Discovery Today* **2004**, *9* (16), 712–20.

(14) Fei, Y. J.; Kanai, Y.; Nussberger, S.; Ganapathy, V.; Leibach, F. H.; Romero, M. F.; Singh, S. K.; Boron, W. F.; Hediger, M. A. Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature* **1994**, *368* (6471), 563–6.

(15) Balakrishnan, A.; Polli, J. E. Apical sodium dependent bile acid transporter (ASBT, SLC10A2): a potential prodrug target. *Mol. Pharmaceutics* **2006**, *3* (3), 223–30.

(16) Yang, C.; Tirucherai, G. S.; Mitra, A. K. Prodrug based optimal drug delivery via membrane transporter/receptor. *Expert Opin. Biol. Ther.* **2001**, *1*, 159–75.

(17) Han, H. K.; Amidon, G. L. Targeted prodrug design to optimize drug delivery. *AAPS PharmSci* **2002**, *1*, 48–58.

(18) Walter., E.; Kissel., T.; Amidon, G. L. The intestinal peptide carrier: Apotential transport system for small peptide derived drugs. *Adv. Drug Delivery Rev.* **1996**, *20* (26), 33–58.

(19) Rubio-Aliaga, I.; Daniel, H. Mammalian peptide transporters as targets for drug delivery. *Trends Pharmacol. Sci.* **2002**, *23* (9), 434–40.

(20) Nielsen, C. U.; Brodin, B.; Jorgensen, F. S.; Frokjaer, S.; Steffanse, B. Human peptide transporters: therapeutic applications. *Expert Opin. Ther. Pat.* **2002**, *12* (9), 1329–50.

(21) Hu, M.; Amidon, G. L. Passive and carrier-mediated intestinal absorption components of captopril. *J. Pharm. Sci.* **1988**, *77*, 1007–11.

(22) Balimane, P. V.; Tamai, I.; Guo, A.; Nakanishi, T.; Kitada, H.; Leibach, F. H.; Tsuji, A.; Sinko, P. J. Direct evidence for peptide transporter (PepT1)-mediated uptake of a nonpeptide prodrug, valacyclovir. *Biochem. Biophys. Res. Commun.* **1998**, *250*, 246–51.

(23) de Vrueh, R. L.; Smith, P. L.; Lee, C.-P. Transport of L-valine-acyclovir via the oligopeptide transporter in the human intestinal cell line, Caco-2. *J. Pharmacol. Exp. Ther.* **1998**, *286*, 1166–70.

(24) Guo, A.; Hu, P.; Balimane, P. V.; Leibach, F. H.; Sinko, P. J. Interactions of a nonpeptidic drug, valacyclovir, with the human intestinal peptide transporter (hPEPT1) expressed in a mammalian cell line. *J. Pharmacol. Exp. Ther.* **1999**, *289*, 448–54.

(25) Sugawara, M.; Huang, W.; Fei, Y. J.; Leibach, F. H.; Ganapathy, V.; Ganapathy, M. E. Transport of valganciclovir, a ganciclovir prodrug, via peptide transporters PEPT1 and PEPT2. *J. Pharm. Sci.* **2000**, *89*, 781–9.

(26) Han, H.; de Vrueh, R. L.; Rhie, J. K.; Covitz, K. M.; Smith, P. L.; Lee, C. P.; Oh, D. M.; Sadee, W.; Amidon, G. L. 5′-Amino acid esters of antiviral nucleosides, acyclovir, and AZT are absorbed by the intestinal PEPT1 peptide transporter. *Pharm. Res.* **1998**, *15* (8), 1154–9.

(27) Kim, I.; Chu, X. Y.; Kim, S.; Provoda, C. J.; Lee, K. D.; Amidon, G. L. Identification of a human valacyclovirase: biphenyl hydrolase-like protein as valacyclovir hydrolase. *J. Biol. Chem.* **2003**, *278*, 25348–56.

(28) Bueno, A. B.; Collado, I.; de Dios, A.; Domingguez, C.; Martin, J. A.; Martin, L. M.; Martinez-Grau, M. A.; Montero, C.; Pedregal, C.; Catlow, J.; Coffey, D. S.; Cley, M. P.; Dantzig, A. H.; Lindstrom, T.; Monn, J. A.; Jiang, H. Y.; Schoepp, D. D.; Stratford, R. E.; Tabas, L. B.; Tizaano, J. P.; Wright, R. A.; Herin, M. F. Dipeptides as effective prodrugs of unnatural amino acid (+)-2-aminobicyclo-[3.1.0]hexane-2,6-dicarboxylic acid (LY354740), a select group Π metabotropic glutamate receptor agonist. *J. Med. Chem.* **2005**, *48* (16), 5305–20.

(29) Perkins, E. J.; Abraham, T. Pharmacokinetics, metabolism, and excretion of the intestinal peptide transporter 1 (SLC15A1)-targeted prodrug (1S, 2S, 5R, 6S)-2-[(2′S)-(2-amino)propionyl]aminobicyclo-[3.1.0.]hexen-2, 6-dicarboxylic acid (LY544344) in rats and dogs: assessment of first-pass bioactivation and dose linearity. *Drug Metab. Dispos.* **2007**, *35* (10), 1903–9.

(30) Eriksson, A. H.; Varma, M. V.; Perkins, E. J.; Zimmerman, C. L. The intestinal absorption of a prodrug of the mGlu2/3 receptor agonist LY354740 is mediated by PEPT1: in situ rat intestinal perfusion studies. *J. Pharm. Sci.* **2009**, *99* (3), 1574–81.

(31) Sun, Y. B.; Sun, J.; Shi, S. L.; Jing, Y. K.; Yin, S. L.; Chen, Y.; Li, G..; Xu, Y. J.; He, Z. G. Synthesis, transport and pharmacokinetics of 5′-amino acid ester prodrugs of 1-beta-D-arabinofuranosylcytosine. *Mol. Pharmaceutics* **2009**, *6* (1), 315–25.

(32) Pretorius, E.; Bouic, P. J. Permeation of four oral drugs through human intestinal mucosa. *AAPS PharmSciTech* **2009**, *10* (1), 270–5.

(33) Sinko, P. J.; Hu, P.; Waclawski, A. P.; Patel, N. R. Oral absorption of anti-AIDS nucleoside analogues. 1. Intestinal transport of didanosine in rat and rabbit preparations. *J. Pharm. Sci.* **1995**, *84* (8), 959–65.

(34) Tsuda, M.; Terada, T.; Irie, M.; Katsura, T.; Niida, A.; Tomita, K.; Fujii, N.; Inui, K.-i. Midodrine, and its amino acid derivatives transport characteristics of a novel peptide transporter 1 substrate, antihypotensive drug. *J. Pharmacol. Exp. Ther.* **2006**, *318* (1), 455–60.

(35) Irie, M.; Terada, T.; Sawada, K.; Saito, H.; Inui, K.-I. Peptide Transporter in Caco-2 Cells Recognition and Transport Characteristics of Nonpeptidic Compounds by Basolatera. *J. Pharmacol. Exp. Ther.* **2001**, *298* (2), 711–7.

(36) Hindlet, P.; Bado, A.; Farinotti, R.; Buyse, M. Long-term effect of leptin on H^+-coupled peptide cotransporter 1 activity and expression In Vivo: evidence in leptin-deficient mice. *J. Pharmacol. Exp. Ther.* **2007**, *323* (1), 192–201.

(37) Nduati, V.; Yan, Y. T.; Dalmasso, G.; Driss, A.; Sitaraman, S.; Merlin, D. Leptin transcriptionally enhances peptide transporter (hPepT1) expression and activity via Camp-response elementbingind pritein Cdx2 transportion factors. *J. Biol. Chem.* **2007**, *282* (2), 1359–73.

(38) Lorenzi, P. L.; Landowski, C. P.; Song, X. Q.; Borysko, K. Z.; Breitenbach, J. M.; Kim, J. S.; Hilfinger, J. M.; Townsend, L. B.; Darch, J. C.; Amidon, G. L. Amino acid ester prodrugs of 2-Bromo-5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole enhance metabolic stability in vitro and In Vivo. *J. Pharmacol. Exp. Ther.* **2005**, *314* (2), 883–90.

(39) Anand, B. S.; Katragadda, S.; Mitra, A. K. Pharmacokinetics of novel dipeptide ester prodrugs of acyclovir after oral administration: intestinal absorption and liver metabolism. *J. Pharmacol. Exp. Ther.* **2004**, *311*, 659–67.

(40) Sun, Y. B.; Sun, J.; Liu, J. F.; Yin, S. L.; Chen, Y.; Zhang, P.; Pu, X. H.; Sun, Y. H.; He, Z. G. Rapid and sensitive hydrophilic interaction chromatography/tandem mass spectrometry method for the determination of glycyl-sarcosine in cell homogenates. *J. Chromatogr., B* **2009**, *877* (7), 649–52.

(41) Yan, Z. T.; Sun, J.; Wang, J. L.; Xu, Y. J.; Chang, Y. N.; Meng, P.; Zhu, M.; Fu, Q.; Sun, Y. B.; He, Z. G. Simultaneous determination of didanosine and its amino acid prodrug, valdidanosine by hydrophilic interaction chromatography coupled with electrospray ionization tandem mass spectrometry: application to a pharmacokinetic study in rats. *J. Chromatogr., B* **2009**, *878* (1−3), 466–70.

(42) Nashed, Y. E.; Mitra, A. K. Synthesis and characterization of novel dipeptide ester prodrugs of acyclovir. *Spectrochim. Acta, Part A* **2003**, *59* (9), 2033–9.

(43) Tamura, K.; Lee, C.; Smith, P.; Borchardt, R. Metabolism, uptake, and transepithelial transport of the stereoisomers of Val-Val-Val in the human intestinal cell line, Caco-2. *Pharm. Res.* **1996**, *13*, 1663–7.

(44) Han, H.; de Vrueh, R. L.; Rhie, J. K.; Covitz, K. M.; Smith, P. L.; Lee, C. P.; Oh, D. M.; Sadee, W.; Amidon, G. L. 5′-Amino acid esters of antiviral nucleosides, acyclovir, and AZT are absorbed by the intestinal PEPT1 peptide transporter. *Pharm. Res.* **1998**, *15*, 1154–9.

(45) Buyse, M.; Berlioz, F.; Guilmeau, S.; Tsocas, A.; Voisin, T.; Peranzi, G.; Merlin, D.; Laburthe, M.; Lewin, M. J.; Roze, C.; Bado, A. PepT1-mediated epithelial transport of dipeptides and cephalexin is enhanced by luminal leptin in the small intestine. *J. Clin. Invest* **2001**, *108* (10), 1483–94.

(46) Hindlet, P.; Bado, A.; Kamenick, P.; Deloménie, C.; Bourasset, F.; Nazaret, C.; Farinotti, R.; Buyse, M. Reduced intestinal absorption of dipeptides via PepT1 in mice with diet-induced obesity is associated with leptin receptor down-regulation. *J. Biol. Chem.* **2009**, *284* (11), 6801–8.

(47) Irie, M.; Terada, T.; Tsuda, M.; Katsura, T.; Inui, K. Prediction of glycylsarcosine transport in Caco-2 cell lines expressing PEPT1 at different levels. *Pflugers Arch.* **2006**, *452*, 64–70.

(48) Zhang, Q. H.; Liu, Q.; Wu, J. J.; Wang, C. Y.; Peng, J. Y.; Ma, X. C.; Liu, K. X. PEPT1 involved in the uptake and transepithelial transport of cefditoren In Vivo and in vitro. *Eur. J. Pharmacol.* **2009**, *612* (1−3), 9–14.

(49) Cheon, E.; Han, H. Pharmacokinetic characteristics of L-valyl-ara-C and its implication on the oral delivery of ara-C. *Acta Pharmacol. Sin.* **2007**, *28* (2), 268–72.