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A carrier-mediated prodrug approach to improve the oral absorption of anti-leukemic drug decitabine

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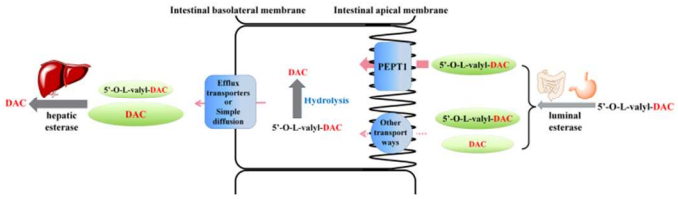
ABSTRACT



Decitabine (5-aza-2'-deoxycytidine, DAC) is a novel DNA methyltransferase (DNMT) inhibitor for the treatment of myelodysplastic syndrome, acute and chronic myeloid leukemia. However, it exhibits a low oral bioavailability (only 9% in mice), because of low permeability across the intestine membrane and rapid metabolism to inactive metabolite. In order to utilize the carrier-mediated prodrug approach for improved absorption of decitabine, a series of amino acid-decitabine conjugates were synthesized to target the intestinal membrane transporter, hPepT1. The Caco-2 permeability of the prodrugs was screened and two L-val (aliphatic, compound 4a) and L-phe (aromatic, compound 4c) prodrugs with higher permeability were selected for further studies. The uptake of Gly-Sar by Caco-2 cells could be competitively inhibited by compound 4a and 4c, with IC_{50} being 2.20 ± 0.28 mM and 3.46 ± 0.16 mM, respectively. The uptake of compound 4a and 4c was markedly increased in the leptin-treated Caco-2 cells compared with the control Caco-2 cells, suggesting hPepT1-mediated transport contributes to oral absorption of compound 4a and 4c. The prodrugs were evaluated for their stability in various phosphate buffers, rat plasma, tissue homogenates and gastrointestinal fluids. Compound 4a and 4c were stable in gastrointestinal tract at pH 6.0 but could be quickly converted into DAC in plasma and tissue homogenates after absorption. The oral absolute bioavailability of DAC was 46.7%, 50.9% and 26.9% after compound 4a, 4c and DAC were orally administered to rats at a dose of 15mg/kg, respectively. The bioavailability of compound 4a and 4c in rats was both reduced to about 32% when orally coadministrated with typical hPepT1 substrate Gly-Sar (150mg/kg).

Overall, compound 4a and 4c can significantly enhance the intestinal membrane permeability of DAC, followed by rapid and mostly bioactivation to parent drug in intestinal and hepatic tissues before entry into systemic circulation, and eventually improve oral bioavailability of DAC in rats. The hPepT1-targeted prodrug strategy is a promising strategy to improve oral bioavailability of poorly absorbed decitabine.

KEYWORDS: decitabine; peptide transporter; prodrug; permeability; oral bioavailability

Introduction

Epigenetic gene silencing marked by aberrant DNA methylation is known to contribute to the malignant transformation of cells [1, 2]. Recently, chemical reversal of gene silencing, called as epigenetic therapy, using DNA-methylation inhibitors, has become an attractive approach for cancer therapy [3, 4]. DNA methyltransferase (DNMT) inhibitors consist of nucleoside inhibitors (azacytidine, decitabine and zebularine) and non-nucleoside inhibitors (procainamide) [5]. Although there are abundant DNMT inhibitors undergoing preclinical and clinical evaluation, the most extensively and advanced investigated drug is 5-aza-2'-deoxycytidine, decitabine. Decitabine (Dacogen; SuperGen, Dublin, Calif) was approved by the FDA for the treatment of myelodysplastic syndrome (MDS) and chronic myelomonocytic leukemia (CMML) in May 2006 [6].

Decitabine exhibits a low oral bioavailability (only 9% in mice) [7], because of low permeability across the intestine membrane and rapid metabolism to inactive metabolite, which necessitates the continuous infusion to maintain therapeutical plasma level clinically [8, 9]. Hence, the development of an oral alternative to intravenous administration of decitabine is needed for both

reducing costs dictated by hospital treatment and improving patient compliance by readily administration route.

However, little effort has been devoted to design decitabine prodrugs for improving oral absorption so far. Prodrug strategy has been increasingly applied over the past decades in order to overcome the undesired biopharmaceutical properties of parent drugs. With the current rapid development of molecular biology, a range of nutrient transporters have been identified and cloned, such as peptide transporter 1 (PepT1), sodium dependent vitamin C transporter (SVCT), monocarboxylate transporter 1 (MCT1) and human apical sodium-dependent bile acid transporter (hASBT), and so on [10,11]. Meanwhile, transporter targeted prodrugs were developed to have structural features that allow them to be recognized by the endogenous transporters present at the intestinal epithelium [12-14]. Due to wide distribution in the gastrointestinal tract, broad substrate specificity and high transport capacity, peptide transporter has been the most promising and feasible target in the prodrug design among various membrane transporters [15-17]. Two excellent examples of successfully marketed prodrugs targeting PepT1 are valacyclovir (Valtrex; GlaxoSmithKline) and valganciclovir (Valcyte; Roche). These L-valyl ester prodrugs increased the intestinal permeability of their parent drugs by 3-10 fold predominantly through peptide transporter-mediated absorption [18-21].

Based on these considerations, we developed a series of PepT1-targeted prodrugs of DAC and screened their transmembrane permeability in Caco-2 cell monolayer. Two different structural L-val (aliphatic) and L-phe (aromatic) prodrugs with higher permeability were selected for further studies. We have investigated bioactivation of the prodrugs and their competitive inhibition effect on Gly-Sar uptake in Caco-2 cells and direct uptake in leptin-treated Caco-2 cells. We also reported the chemical and enzymatic stability of the prodrugs in various phosphate buffers, rat

plasma, tissue homogenates and gastrointestinal fluids. Finally, pharmacokinetic studies were evaluated after oral administration of DAC, compound **4a** and **4c** in Sprague-Dawley rats, respectively. The combined results of these studies showed the carrier-targeted prodrugs can improve the intestinal membrane transport of decitabine. Meanwhile, the prodrugs can be rapidly and mostly bioactivated to parent drug in intestinal and hepatic tissues before entry into systemic circulation. Eventually, the carrier-targeted prodrug strategy successfully enhances the oral bioavailability of decitabine.

■ Materials and methods

Decitabine (98% purity) was synthesized in Shenyang Pharmaceutical University (Shenyang, China). The carbobenzyloxy (Cbz) protected amino acids Cbz-L-valine, Cbz-D-valine, Cbz-L-phenylalanine, Cbz-L-isoleucine and Cbz-L-tryptophan were obtained from Baosheng Chemicals (Yangzhou, China). Pd/C and N,N'-Dicyclohexylcarbodiimide (DCC) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shenyang, China). 4-(Dimethylamino) pyridine (DMAP) was obtained from Dongyang Tianyu Chemicals Co. Ltd. (Dongyang, China). Leptin was obtained from ProSpec TechnoGene (Rehovot, Israel). Gly-Sar was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of the highest purity available.

Synthesis of 5'-amino acid ester derivatives of DAC

DAC (4.57 g, 20mmol), Cbz-amino acid (40 mmol), DCC (6.18 g, 30 mmol) and DMAP(0.24 g, 0.1 mmol) were dissolved in anhydrous DMF (N,N-dimethylformamide) and stirred at room

temperature for 24 h under a nitrogen atmosphere. After 24 h, the reaction mixture was filtered and DMF was evaporated under reduced pressure. The residue was dissolved in ethyl acetate (50 mL) and washed with water (2 × 35 mL), saturated $NaHCO_3$ (2 × 35 mL) and brine (1 × 30 mL), respectively. The organic layer was 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 petroleum ether: ethyl acetate (1:30- 1:100). The eluent belonging to each intermediate was collected and concentrated. Pure fractions were dissolved in ethyl acetate and isopropanol, and then Pd/C (10%) was added. The reaction mixture was stirred under H_2 at 30 ^{0}C for 4 h and filtered. Compounds **4a**, **4b**, **4c**, **4d** and **4e** were washed with isopropanol and concentrated in vacuo (Scheme 1). Structural identities of all compounds were confirmed by ^{1}H NMR, and electrospray ionization-mass spectrometry.

DAC: percent purity, 98%; ^{1}H NMR (300 MHz, DMSO-d6) δ 2.16(2H, m), 3.61(2H, m), 3.81(1H, d, J=3.0 Hz), 4.24(1H, brs), 5.05(1H, t, J=5.1 Hz), 5.24(1H, d, J=4.2 Hz), 6.03(1H, t, J=6.3 Hz), 7.50(1H, s) 7.53(1H, s), 8.51(1H, s); ESI-MS m/z 229.3 $(M + H)^+$.

Compound **4a** (5'-O-L-valyl-decitabine): yield, 42%; percent purity, 96%; ^{1}H NMR (600 MHz, DMSO-d6) δ 0.82(3H, d, J=13.2 Hz), 0.87(3H, d, J=13.2 Hz), 1.82(1H, m), 2.21(1H, m), 2.29(1H, m), 3.13(1H, d, J=5.4 Hz), 3.97(1H, m), 4.20-4.27(3H, m), 5.38(1H, d, J=4.2 Hz), 6.03(1H, t, J=6.6 Hz), 7.52(2H, s), 8.29(1H, s); ESI-MS m/z 327.8 $(M + H)^+$.

Compound **4b** (5'-O-D-valyl-decitabine): yield, 38%; percent purity, 98%; ^{1}H NMR (600 MHz, DMSO-d6) δ 0.83(3H, d, J=6.8 Hz), 0.87(3H, d, J=6.8 Hz), 1.82(1H, m), 2.21(1H, m), 2.29(1H, m), 3.13(1H, d, J=5.4 Hz), 3.97(1H, m), 4.20-4.27(3H, m), 6.03(1H, t, J=6.6 Hz), 7.53(1H, s), 7.54(1H, s), 8.30(1H, s); ESI-MS m/z 327.79 $(M + H)^+$

Compound **4c** (5'-O-L-phenylanlanyl-decitabine): yield, 41%; percent purity, 96%; ^{1}H NMR (600 MHz, DMSO-d6) δ 2.19(2H, m), 2.78(1H, m), 2.86(1H, m), 3.60(1H, t, J=6.6 Hz), 3.95(1H, m), 4.11(1H, m), 4.15-4.22(2H, m), 6.02(1H, t, J=6.6 Hz), 7.17-7.26(5H, m), 7.55(2H, s), 8.30(1H, s); ESI-MS m/z 374.97 $(M + H)^+$

Compound **4d** (5'-O-L- isoleucyl-decitabine): yield, 41%; percent purity, 97%; ^{1}H NMR (600 MHz, DMSO-d6) δ 0.81(3H, t, J=7.4 Hz), 0.83(3H, d, J=6.8 Hz), 1.11(1H, m), 1.40(1H, m), 1.57(1H, m), 2.22(1H, m), 2.28(1H, m), 3.19(1H, d, J=5.4 Hz), 3.99(1H, m), 4.01(1H, d, J=6.6 Hz), 4.20-4.26(3H, m), 6.04(1H, t, J=6.6 Hz), 7.53(2H, s), 8.29(1H, s); ESI-MS m/z 341.86 $(M + H)^+$

Compound **4e** (5'-O-L- tryptophyl -decitabine): yield, 35%; percent purity, 95%; ^{1}H NMR (600 MHz, DMSO-d6) δ 2.08(1H, m), 2.13(1H, m), 2.92(1H, dd, J=6.6, 14.4 Hz), 3.01(1H, dd, J=6.6, 14.4 Hz), 3.64(1H, t, J=6.6 Hz), 3.95(1H, q, J=4.2 Hz), 4.06(1H, m), 4.12(1H, m, dd, J=5.4, 6.0 Hz), 4.19(1H, m, dd, J=3.0, 12.0 Hz), 6.01(1H, t, J=6.0 Hz), 6.94(1H, t, J=7.8 Hz), 7.04(1H, t, J=7.8 Hz), 7.13(1H, s), 7.32(1H, d, J=7.8 Hz), 7.47(1H, d, J=7.8 Hz), 7.55(2H, s), 8.30(1H, s); ESI-MS m/z 414.74 $(M + H)^+$

Caco-2 culture.

Caco-2 cells from Institute of Basic Medical Sciences Chinese Academy of Medical Sciences Cell Culture Center (Shanghai, China) were grown routinely on 75 cm^2 culture flasks in DMEM (4500 mg/L glucose) as described by Sun et al.. [22]. For transport experiments, Caco-2 cells were seeded onto polycarbonate membrane at a density about 1×10^5 cells/cm^2 and allowed to grow

for 21 to 25 days. For uptake experiments, Caco-2 cells were seeded onto 24-well plastic cluster trays at the same density but used 15 days after seeding.

Caco-2 permeability.

The Caco-2 permeability studies for the prodrugs and parent drug were performed in triplicate with some modifications as described previously [23]. The transport procedure was described in detail in our previous study [24] and the concentrations of DAC and its prodrugs were analyzed by HPLC.

Gly-Sar uptake inhibition.

Drug inhibition effect was studied by inhibiting Gly-Sar uptake [25, 26]. After washed twice with HBSS buffer, the cells were incubated with 20 μM Gly-Sar along with various concentrations of compound **4a** or **4c** (0.1-40 mM) at 37 ^{0}C for 30 min. After 30 min, the medium was removed and the cells were rapidly rinsed tiwce with 1 mL of ice-cold pH 6.0 uptake buffer. The cells were homogenized and centrifuged at 2500 g for 10 min, and then the supernatants were collected for analysis by UPLC/MS/MS. The protein concentration of each sample was determined by Bicinchoninic Acid assay using a bovine serum albumin as standard [27]. IC_{50} values were determined using nonlinear fitting.

Uptake of compound 4a and 4c by leptin-treated Caco-2 cells.

For leptin-treated Caco-2 cells, the cells were cultured with 2 nM leptin from the eighth day to the fifteenth day [28, 29]. The uptake of prodrugs was calculated as the sum of the amounts of the unchanged form and DAC. The uptake procedure was described as above and the samples were analyzed by HPLC.

Hydrolysis stability studies.

(a) Chemical stability.

The nonenzymatic hydrolysis of compound **4a** and **4c** was determined in different pH phosphate buffers (pH 1.2, 4.5, 6.8, 7.4) at 37 ^{0}C for a period of 4 h. At every time point, 100 μL of the samples was taken and analyzed by HPLC.

(b) Hydrolysis in rat gastric juices and intestinal fluids.

The gastric juices and intestinal fluids for stability studies were collected from a 250 g male Sprague-Dawley rat. The experiments were carried out by adding 200 μL of a stock solution of compound **4a** or **4c** to 1.8 mL of gastric juices or intestinal fluids preheated to 37 ^{0}C, and the concentration of compound **4a** and **4c** in the biological media was about 80 μg/mL and 100 μg/mL, respectively. Hydrolysis of the prodrugs was studied at 37 ^{0}C for a period of 4 h. Samples (100 μL) were taken at various time points and quenched with 300 μL of ice-cold methanol, then centrifuged at 2500g and 4 ^{0}C for 10 min. The supernatants were analyzed by HPLC.

(c) Stability in intestinal and liver homogenates.

The jejunum segment and liver were removed from the euthanized Sprague-Dawley rat and washed with ice-bath buffer C (25 mM KCl, 5 mM $MgCl_2$ and 10 mM HEPES, pH 7.4) several times to remove blood, then homogenized with a tissue homogenizer and centrifuged at 2000g and 4 ^{0}C for 10 min. The resulting supernatant was collected and total protein amount was determined as above [27]. The hydrolysis experiment was carried out by addition of drug solutions to the homogenates at 37 ^{0}C, where compound **4a**, compound **4c** and protein concentrations in the mixture were 80 μg/mL(compound 4a), 150 μg/mL (compound 4c) and 200 μg/mL, respectively. The sample procedure was described as above and supernatant was analyzed using HPLC.

(d) Stability in rat plasma.

Plasma was obtained from the rat jugular vein after centrifugation at 2500 g for 10 min. One volume of drug stock solution (0.8 mg/mL for compound **4a**, 1.5 mg/mL for compound **4c**) was mixed with nine volumes of plasma preheated to 37 ^{0}C. Aliquot samples were collected at various time points (0, 30, 60, 90, 120 min). Extraction and analysis methods were similar to those for gastrointestinal fluids experiment.

Rate constants of hydrolysis were determined by pseudo-first-order kinetic models.

Pharmacokinetic studies.

Animal experiments were performed in accordance with the guide for the care and use of laboratory animals of Shenyang Pharmaceutical University. Male Sprague-Dawley rats (weighing from 220 to 250 g) were used in these studies. The rats were fasted but had free access

to water for 12 h before administration. The rats were administered by gastric lavage of compound **4a**, **4c** (15 mg/kg, calculated as DAC) or DAC (15 mg/kg) in aqueous solution. For coadministration with Gly-Sar, compound **4a** or **4c** (15 mg/kg, calculated as DAC) was administrated to rats alone or with Gly-Sar (150 kg/mg). At predetermined time points, 200 μL of blood sample was collected and placed into heparinized tubes containing the deaminase inhibitor, tetrahydrouridine (0.1 mM). The rat plasma was centrifugated at 2500 g for 10 min, collected, frozen at -80 ^{0}C and analyzed by HPLC/MS/MS.

Analytical method.

(a) HPLC analysis.

The HPLC analytical method for DAC and amino acid ester prodrugs was conducted on a SHIMADZU liquid chromatography instrument equipped with a LC-10AT pump and a SPD-10A UV-vis detector. A C_{18} column (5 μm, 200 mm × 4.6 μm, Diamosil, DIKMA) was used for sample analysis. The mobile phase was a mixture of methanol: 0.05 M KH_2PO_4 buffer solution (pH 5.8) = 22:78. The flow rate was 1.0 mL/min, and the wavelength was 242 nm. The temperature of the column was set at 25 ^{0}C.

(b) HPLC/MS/MS analysis. The analytes were determined by a Waters ACQUITY Xevo TQD system, which consisted of an ACQUITY HPLC system and an ACQUITY triple-quadrupole tandem mass spectrometer with an electrospray ionization (ESI) interface. The ESI source was operated in positive mode.

For determination of Gly-Sar [30], isoniazid was selected as the internal standard. A simple protein precipitation was utilized to extract Gly-Sar and the internal standard 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 applied 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 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 DAC, compound **4a** and **4c**, Waters Oasis MCX cation-exchange solid-phase extraction cartridges (Waters Corporation; Milford, MA) were used to extract analytes from rat plasma. The HPLC system used a Waters Symmetry C18 column (150 mm × 4.6 mm, 3.5 μm, Waters Corp., Milford, MA). HPLC elution was carried out using a gradient of water containing 0.1% formic acid together with 0.1% ammonium hydroxide and acetonitrile containing 0.1% formic acid, and the flow rate was 0.2 mL/min [31].

Data analysis.

(a) Caco-2 monolayer permeability

The apparent permeability (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 dCr/dt is the rate of change of concentration in the receiver solution, Vr is the receiver volume, A is the surface area of the monolayer, C_0 is the concentration of the

DAC or prodrug in the donor solution.

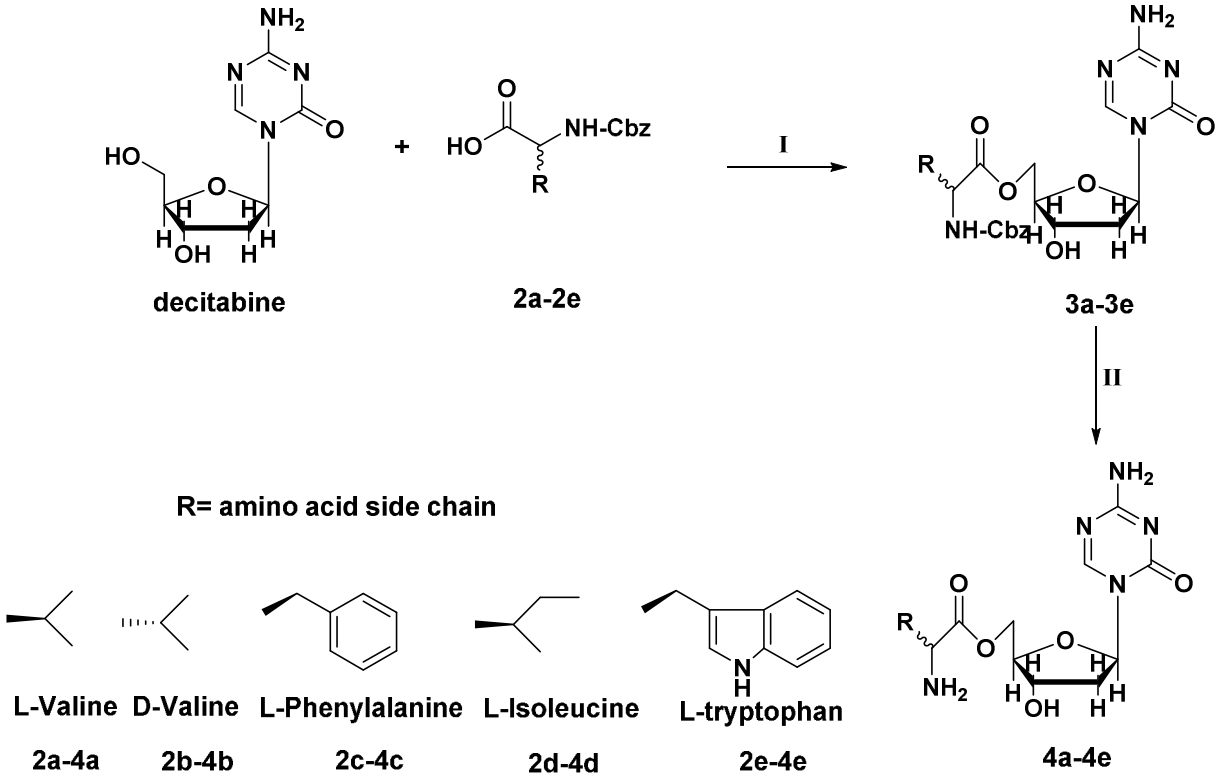
(b) Pharmacokinetics

Noncompartmental pharmacokinetic analysis was conducted to calculate the plasma pharmacokinetic parameters. Area under curve (AUC) was calculated using the linear trapezoidal rule. The peak plasma concentration (C_{max}) and the time to reach peak concentration levels (T_{max}) were obtained from the time versus plasma concentration profile. 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 DAC.

The aliphatic amino acids (L-val, D-val and L-ile) and the aromatic amino acids (L-phe and L-tryptophan) were selected as promoieties for the synthesis of decitabine prodrugs. Amino acid ester prodrugs of DAC are synthesized as described in Scheme 1. The hydroxymethyl group of DAC was coupled with the Cbz-protected amino acid in the presence of DCC and DMAP. The desired intermediates were the Cbz protected 5'-amino acid ester prodrugs of decitabine. Following purification by column chromatography, the Cbz group of the desired intermediate was removed by catalytic hydrogenation with Pd/C [32]. The Cbz-protected group was chosen because mild condition in deprotection reaction could avoid the degradation of DAC and the cleavage of ester bond.



Scheme 1. Synthetic route of 5’ -amino acid ester prodrugs **4a**-**4e**. Reagents and conditions: (i) DCC, DMAP, DMF, rt, overnight; (ii) Pd/H_2, EtOAc, iPrOH, 30 ^{0}C, 4h.

Screening Caco-2 permeability of DAC peptidomimetic prodrugs.

The Caco-2 permeability in the apical to basolateral (AP to BL) direction of DAC and five prodrugs was determined. As shown in Figure 1, all prodrugs were found to be more permeable than DAC. Compound **4c** (5’-O-L-phenylanlanyl-decitabine) was approximately 4-fold higher than that of DAC. Compound **4a** (5’-O-L-valyl- decitabine) and compound **4d** (5’-O-L-isoleucyl-decitabine) exhibited high permeability with little difference and could be efficiently transported across the Caco-2 monolayer. After transporting across the cell monolayers, all the

prodrugs were partially hydrolyzed to DAC and were detected in the receiver compartment. Two prodrugs with higher permeability, an alphatic (L-val) and an aromatic (L-phe), were chosen for further studies to see the effect of amino acid promoieties on cell uptake and oral absorption of the prodrugs.

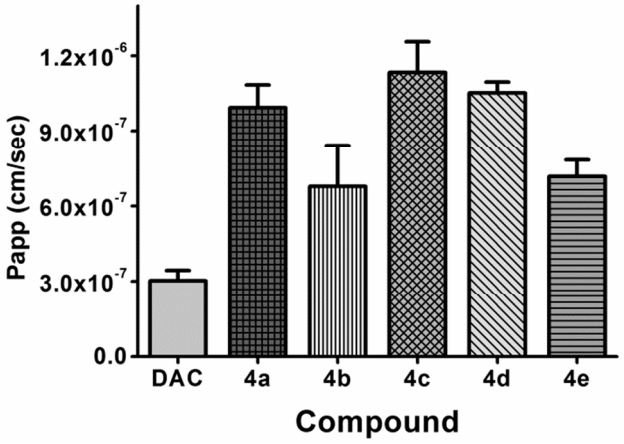


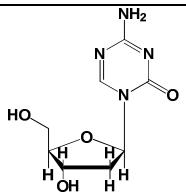
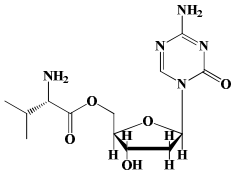
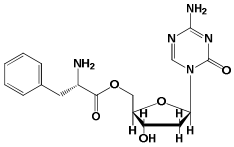
Figure 1. The apical-to-basolateral permeability for the transport of DAC and its 5'-O-amino acid ester prodrugs in Caco-2 cells (mean ± SD, n = 3).

Effect of compound 4a and 4c on Gly-Sar uptake by Caco-2 cells.

To assess the interaction of compound **4a** and **4c** with PepT1, the inhibitory effect of compound **4a** and **4c** (0.1-40 mM) on the uptake of 20 μM Gly-Sar by Caco-2 cells was examined. As shown in Table 1, the uptake of Gly-Sar was significantly inhibited by compound **4a** and **4c**, with IC_{50} being 2.20 ± 0.28 mM and 3.46 ± 0.16 mM, respectively. But decitabine exhibited no inhibitory effect on the uptake of Gly-Sar in the concentration range tested (0.1-40 mM). The aliphatic prodrug compound **4a** (5'-O-L-valyl-decitabine) showed higher affinity than the

aromatic prodrug compound **4c** (5'-O-L-phenylanlanyl-decitabine), but the difference was not significant.

Table 1. Gly-Sar uptake inhibition results in Caco-2 cells

Compound name	Structure	MW	(IC_{50})(mM) Mean±SD[a]
DAC		228.21	No Inhibition[b]
5'-O-L-valyl-decitabine(**4a**)		327.34	2.20±0.28
5'-O-L-phenylanlanyl-decitabine(**4c**)		375.38	3.46±0.16

[a] Mean and SD from 3 experiments. [b] No inhibition over concentration range of 0.1-40 mM.

Uptake of Gly-Sar by leptin-treated Caco-2 cells.

The stably transfected hPepT1/MDCK and hPepT1/HeLa 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 treatment with leptin on Caco-2 could significantly increase PepT1 activity and 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 a successive 7 days [33, 34]. In our study, the uptake of Gly-Sar by the leptin-treated and control Caco-2 cells was determined to evaluate whether this treatment was successful. As shown in Figure 2, the uptake of Gly-Sar was 1.05 nmol/mg of protein/30 min in the leptin-treated Caco-2 cells, 1.9-fold greater than the control, demonstrating enhanced expression of PepT1 in the leptin-treated Caco-2 cells.

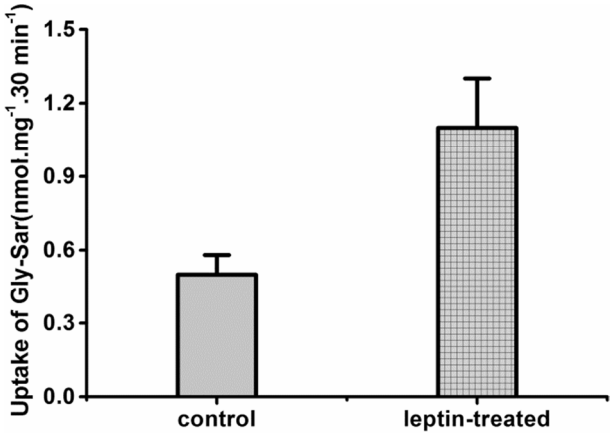


Figure 2. Uptake of Gly-Sar by the 7-day leptin-treated Caco-2 and the control Caco-2 cells. Leptin-treated Caco-2 and control Caco-2 cells were incubated at 37 ^{0}C for 30 min with 20 μM Gly-Sar, pH 6.0, respectively. The amounts of Gly-Sar in cell homogenates were measured by UPLC-MS/MS. The results are expressed as mean ± SD (n = 3).

Uptake of compound 4a, 4c and DAC by leptin-treated Caco-2 cells.

To confirm whether the transport of compound **4a** and **4c** was mediated by PepT1, the uptake was conducted on the leptin-treated and the control Caco-2 cells. Increased hPepT1-mediated

uptake in the leptin-treated Caco-2 cells was observed in the case of compound **4a** (1.8-fold) and **4c** (1.9-fold), compared to those of the control Caco-2 cells (Fig. 3). In contrast, there was no significant difference between the leptin-treated and control Caco-2 cells for DAC. The results indicate that DAC is not a substrate of PepT1.

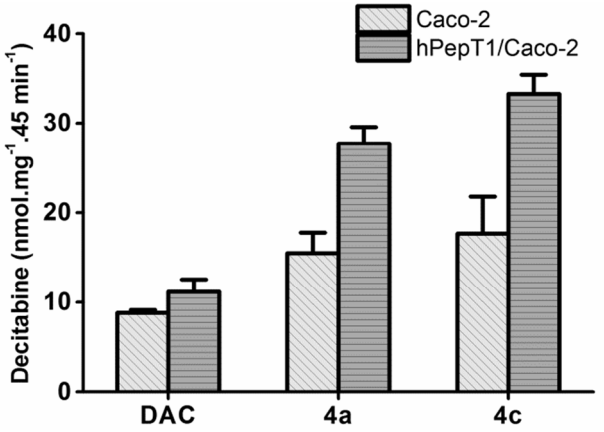


Figure 3. Comparison of decitabine prodrugs uptake in the 7-day leptin-treated Caco-2 and the control Caco-2 cells. The Caco-2 cells were incubated at 37 ^{0}C for 45 min with 0.5 mM compound **4a**, **4c** or DAC at pH 6.0. After incubation, the amounts of compound **4a**, **4c** or DAC in the cell homogenates were determined by HPLC. The uptake of prodrugs was calculated as the sum of the amounts of the unchanged form and DAC. The results are expressed as mean ± SD (n = 3).

Table 2. The stability results of compound **4a** and **4c** at 37 ^{0}C in phosphate buffers of different pH values, rat tissue homogenates, plasma, gastric and intestinal fluids (protein concentration, 200 μg/mL)

media	$t_{1/2}$(min)	
	compound **4a**	compound **4c**

pH 1.2	228	>240
pH 4.5	180	>240
pH 6.8	143	192
pH 7.4	221	154
gastric fluid	211	>240
intestinal fluid	173	95
intestinal homogenates	61	7
hepatic homogenates	5	2
rat plasma	86	N.D.

N.D., not detected.

Stability studies.

The stability experiments were performed at 37 ^{0}C in various phosphate buffers of different pH values, rat plasma, tissue homogenates and gastrointestinal fluids. The estimated half-lives ($t_{1/2}$) obtained from linear regression of pseudo-first-order plots of compound **4a** and **4c** concentration vs time are shown in Table 2. It could be observed that the hydrolysis stability of compound **4a** and **4c** was significantly influenced by the pH value of phosphate buffer. The L-phenylanlanyl prodrug was shown to be somewhat more stable than the L-valyl prodrug in phosphate buffer.

Additionally, $t_{1/2}$ values of compound **4a** and **4c** in the hepatic/intestinal homogenates and plasma were much shorter than those in the gastrointestinal fluids and buffer solution (Table 2). Intestinal enzymes in intestinal homogenates had been proved to remain active during the experimental conditions in the previous study using a positive control aspirin [24]. Furthermore, the L-phenylanlanyl prodrug was shown to be more unstable than the L-valyl prodrug in hepatic/intestinal homogenates and plasma, especially in rat plasma, and the intact L-phenylanlanyl prodrug could not be detected.

Pharmacokinetics in rats.

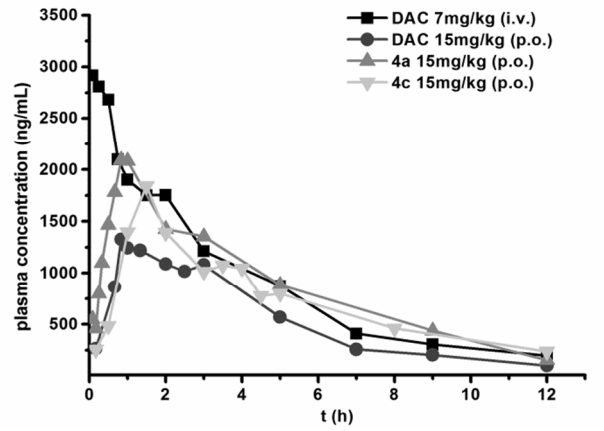


Figure 4. Mean plasma concentration-time profiles of DAC, compound **4a** and **4c** in Sprague-Dawley rats (n = 4): DAC following intravenous (■) at 7 mg/kg and oral administration (●) of DAC at 15 mg/kg; compound **4a** after oral administration of compound **4a** at 15 mg/kg (▲) (calculated as DAC); compound **4c** after oral administration of compound **4c** at 15 mg/kg (▼) (calculated as DAC).

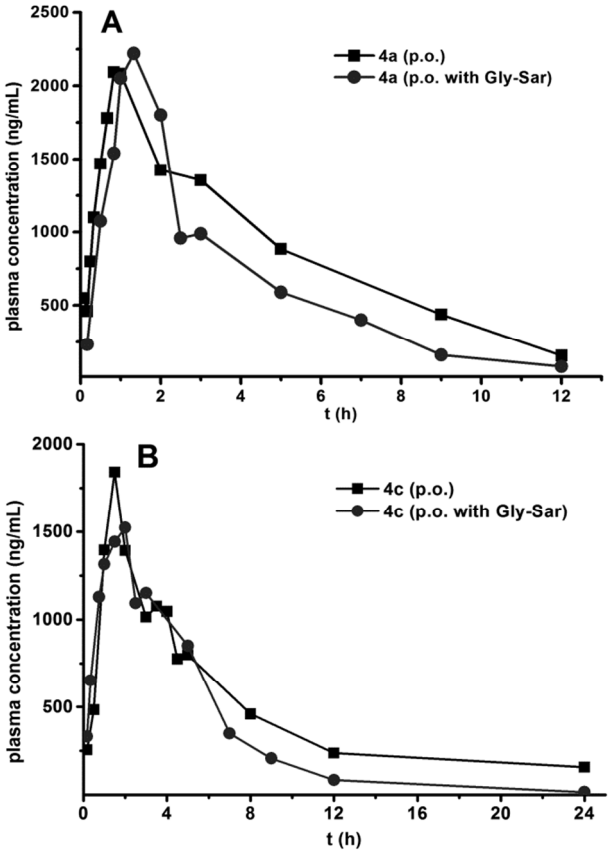


Figure 5. Mean plasma concentration-time profiles of DAC after oral administration of **4a** (A) and **4c** (B) (15 mg/kg, calculated as DAC dose) to Sprague-Dawley rats in the presence and absence of Gly-Sar (n = 4).

Table 3. Pharmacokinetic parameters of DAC, following oral administration of DAC (15 mg/kg), compound **4a** and **4c** (15 mg/kg, calculated as DAC) to Sprague-Dawley rats, respectively (Mean ± SD, n = 4)

	dose			
	4a	**4c**	DAC	
PK params of DAC	15mg/kg(p.o.)	15mg/kg(p.o.)	15mg/kg(p.o.)	7mg/kg (i.v.)
$AUC_{0-\infty}$(μg.h/mL)	11.69±1.21	12.68±4.35	6.82±0.67	11.68±0.77

AUC/dose	0.78±0.08	0.85±0.29	0.45±0.04	1.67±0.01
C_{max}(μg/mL)	2.24±0.41	2.07±0.17	1.88±0.58	2.92±0.08
T_{max}(h)	0.83±0.13	1.5±0.40	1.79±0.93	0
$t_{1/2}$(h)	3.85±1.08	7.26±3.73	2.87±1.04	3.67±0.51
Oral bioavailability	46.7%	50.9%	26.9%	-

The pharmacokinetic studies of DAC, compound **4a** and **4c** after oral administration in rats were carried out to investigate whether PePT1-targeted prodrug strategy could improve the oral absorption of DAC in vivo. Plasma concentration-time profiles of DAC, compound **4a** and **4c** are shown in Figure 4. And the main pharmacokinetic parameters are shown in Table 3. Since compound **4a** and **4c** were rapidly hydrolyzed into the parent drug and their concentration in plasma was very low, the relevant AUC and elimination half-life values could not be accurately determined. Hence, we mainly focused on the pharmacokinetic performances of DAC after oral administration of compound **4a** and **4c**. Following iv injection of DAC to rats (7 mg/kg), the mean AUC was 11.68 μg • h/mL. As shown from Table 3, AUC for DAC after compound **4a**, **4c** (15 mg/kg, calculated as DAC) and DAC (15 mg/kg) oral administration was 11.69, 12.68 and 6.82 μg • h/mL, respectively. Additionally, the absolute bioavailability of DAC following oral administration of compound **4a**, **4c** and DAC was 46.7%, 50.9% and 26.9%, respectively. The oral bioavailability of compound **4a** and **4c** was nearly 1.74-fold and 1.89-fold of the parent drug.

Compound 4a and 4c were found to be the substrates of PepT1 in vitro in the previous study. To further demonstrate the role of PepT1 in the oral absorption of compound **4a** and **4c** in vivo,

inhibition studies were performed by the coadministration with the Gly-Sar, a typical substrate of PepT1. When oral coadministration with Gly-Sar (150 mg/kg), the absolute bioavailability of DAC following oral administration of prodrug at a dose of 15 mg/kg (calculated as DAC dose) decreased from 46.7% to 32.2% for 4a (Fig. 5) and from 50.9% to 32.5% for 4c (Fig. 5), respectively.

■ Discussion

Decitabine, approved by FDA in May 2006, has a low absolute oral bioavailability, which limits its clinical use despite being the most extensively and advanced investigated DNMT inhibitor. To date, little effort has been devoted to design decitabine prodrugs for improving oral absorption.

In this article, a series of amino acid-decitabine conjugates were synthesized for improving the oral bioavailability of decitabine. In Caco-2 permeability experiment, all prodrugs were found to be more permeable than DAC. Consistent with the previous studies, the amino acid ester with the L-configuration was more permeable than the D-counterpart [22, 35].

From Gly-Sar uptake inhibition studies, it was concluded that compound **4a** and **4c** competed with Gly-Sar to interact with PepT1, while decitabine lacked any apparent affinity for the transporter. Additionally, the substitution with amino acid promoieties of decitabine prodrugs did not markedly change the Gly-Sar uptake inhibition of the prodrugs.

The chemical stability analysis showed that the prodrugs were generally more stable at acidic pH than at neutral and alkaline pH. Additionally, $t_{1/2}$ values of compound **4a** and **4c** in the

hepatic/intestinal homogenates and plasma were much shorter than those in the gastrointestinal fluids and buffer solution (Table 2). It suggested the first-pass metabolism may be an essential route for the bioactivation of the two prodrugs to their parent drugs, indicating the amino acid ester prodrugs can maintain enough chemical stability in gastrointestinal tract and was rapidly converted to active parent drug by ester enzymes following transport across the intestinal membrane.

In general, the ester bond could be rapidly hydrolyzed by esterases and the amide bond was too stable and difficult to break under the effect of enzymatic hydrolysis. However, there were some notable exceptions and then stability of the amide-based prodrug targeting PepT1 should be analyzed seriously case by case. For instance, amide-based prodrug midodrine could easily be transformed into the active form desglymidodrine in vivo. This carrier-mediated transport strategy improves the bioavailability of desglymidodrine from 50% to 93% [36]. However, amide-based N_4-L-valyl-cytarabine showed very low oral bioavailability of only 4% due to the stable amide linkage in vivo and the absence of active parent drug in the systematic circulation [22]. In our study, the ester-based prodrugs of decitabine could be rapidly bioactivated to parent drug and in vivo bioconversion rate was a lot higher than in vitro, implying that compound **4a** and **4c** possess appropriate chemical stability and in vivo bioactivation behavior.

In the pharmacokinetic studies, no intact prodrugs could be detected, which might result from rapid hydrolysis into the parent drug. This result was consistent with the results of the stability experiment. In addition, the oral bioavailability of compound 4a and 4c was nearly 1.74-fold and 1.89-fold of the parent drug, suggesting that bioconjugation with proper amino acid promoieties is an effective PepT1-targeted prodrug strategy for improving the oral absorption of poorly absorbed drugs.

When oral coadministration with Gly-Sar, the absolute bioavailability of DAC following oral administration of prodrug decreased. Although the inhibitory effect was not so significant, the results did reveal the presence of competitive inhibition of Gly-Sar on the PepT1-mediated transport of compound **4a** and **4c** in the in vivo conditions. Thus, there might exist potential PepT1-mediated drug-drug interactions when such prodrugs are orally coadministrated with some substrate drugs of PepT1, such as angiotensin-converting enzyme inhibitors, β-lactam antibiotics, etc.

In summary, the in vitro and in vivo results have shown that the carrier mediated prodrug strategy developed and described in this work has been very successful in improving the membrane permeability and oral bioavailability of decitabine. Such an approach may allow the reintroduction of DAC through the suitable prodrug form as a standard agent for the treatment of leukemia.

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A carrier-mediated prodrug approach to improve the oral absorption of anti-leukemic drug decitabine

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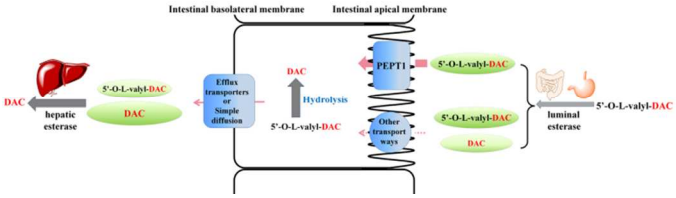

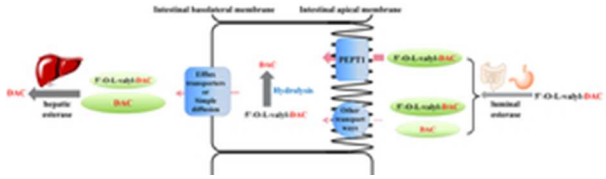

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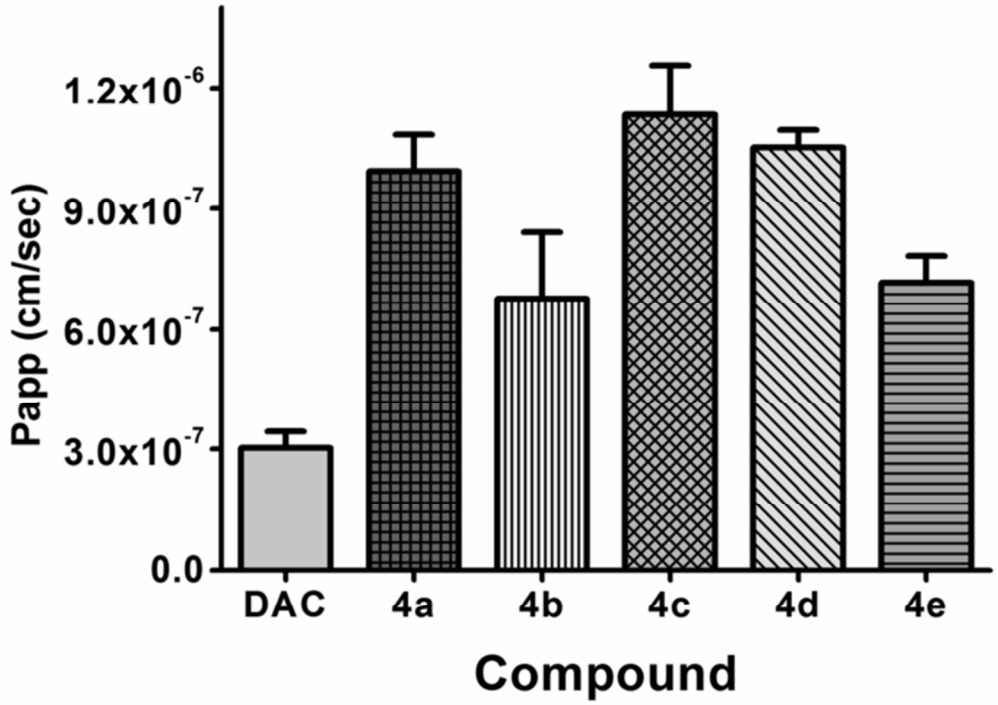

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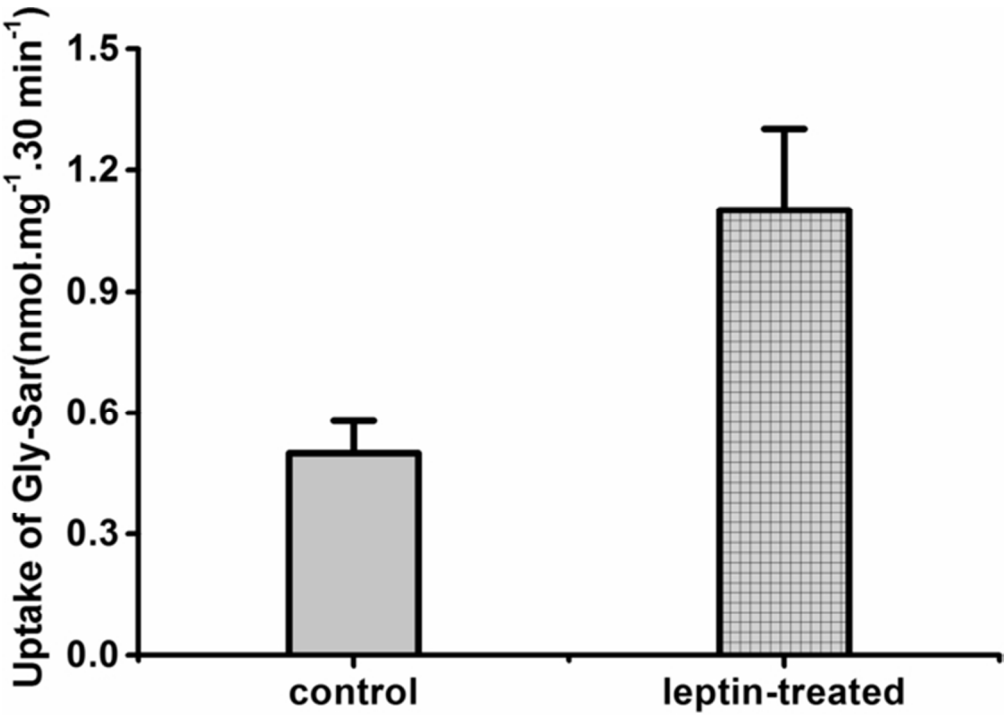

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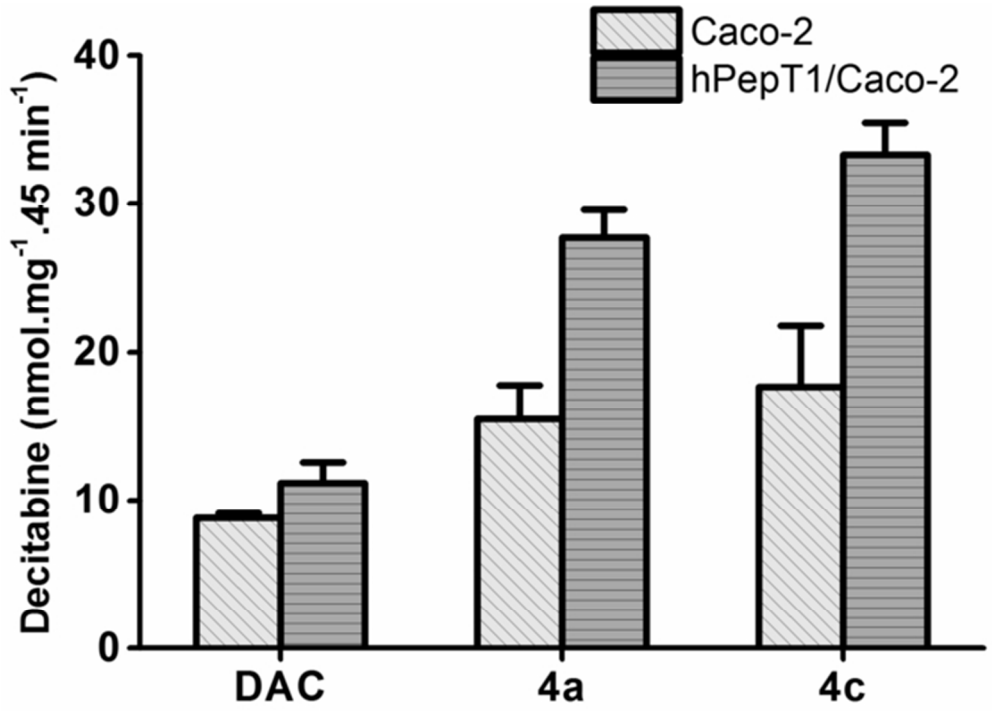

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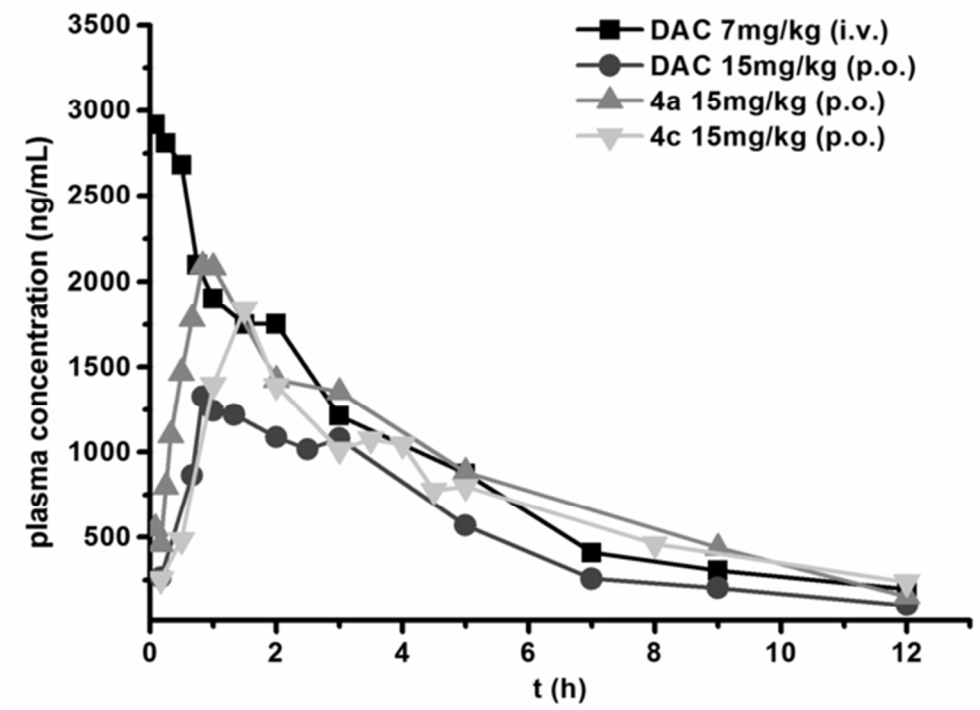

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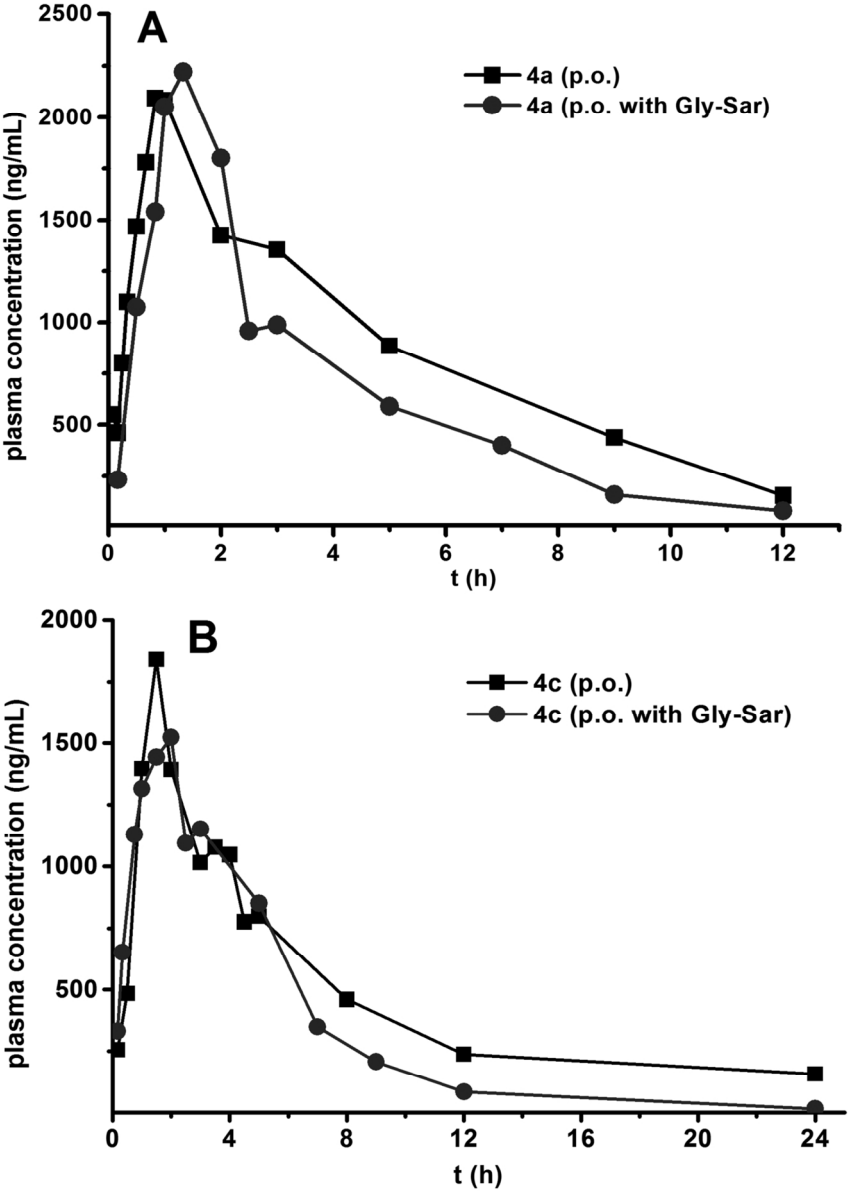

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