Enhanced cellular uptake of Ara-C via a peptidomimetic prodrug, L-valyl-ara-C in Caco-2 cells

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Abstract

This study aimed to investigate the gastrointestinal stability and the cellular uptake characteristics of L-valyl-ara-C, a peptidomimetic prodrug of ara-C (cytarabine). After the synthesis of L-valyl-ara-C via the incorporation of L-valine into the N4-amino group of the cytosine ring in ara-C, the gastrointestinal stability of L-valyl-ara-C was examined using artificial gastric juice and artificial intestinal fluids. The cellular uptake characteristics of L-valyl-ara-C were also examined in Caco-2 cells. The disappearance half-life of L-valyl-ara-C was 2.2 h in artificial gastric juice, while the degradation of L-valyl-ara-C was negligible in artificial intestinal fluid and also in the supernatant above the Caco-2 cell monolayer during the 2-h incubation. The cellular accumulation of L-valyl-ara-C was 5-fold higher than that of ara-C in Caco-2 cells. Furthermore, the cellular uptake of L-valyl-ara-C did not increase proportionally to the increase in drug concentration. The cellular accumulation of L-valyl-ara-C was significantly reduced in the presence of uridine, p-aminohippurate, tetraethylammonium and small dipeptides, while it was not changed in the presence of L-valine and benzoic acid, suggesting that L-valyl-ara-C could interact with multiple uptake transporters, including peptide transporters, organic anion and cation transporters and nucleoside transporters, but might not interact with amino acid transporters. In conclusion, L-valyl-ara-C could be effective to improve the oral absorption of ara-C via the carrier-mediated transport pathway.

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Introduction

1-(β-D-Arabinofuranosyl)cytosine (cytarabine, Ara-C), a pyrimidine nucleoside analogue, is one of the most effective drugs used in the treatment of acute myeloid leukaemia, acute lymphoblastic leukaemia and other haematological malignancies (Bryan et al 1974; Grant 1998). In combination with other anti-tumour agents it is also used against solid tumours (Grant 1998). For its antineoplastic activity, ara-C must be converted to cytosine 1-β-D-arabinofuranoside 5'-triphosphate (ara-CTP) via intracellular phosphorylation and subsequently ara-CTP blocks DNA synthesis both by inhibition of DNA polymerase and by incorporation into DNA (Bryan et al 1974; Grant 1998). Recently, ara-C has been reported to induce apoptosis of neoplastic cells (Lickliter et al 2003; Samorapoompichit et al 2003). However, the clinical utility of ara-C is severely limited by rapid deamination to the biologically inactive 1-β-D-arabinofuranosyluracil (ara-U) by cytidine deaminase, primarily in the liver, spleen and gastrointestinal mucosa (Ho & Frei 1971; Rustum & Raymakers 1992). Consequently, ara-C has a very short plasma half-life as well as low systemic exposure and must be administered in continuous infusion or on a complex schedule to provide maximum therapeutic efficacy (Ho and Frei 1971; Rustum and Raymakers 1992). In an attempt to avoid the deamination and also to enhance the cellular uptake of ara-C, many prodrug strategies have been explored with varied degrees of success but few have led to an approved product (Hadfield & Sartorelli 1984; Wipf & Li 1994; Wipf et al 1996; Greenwald et al 2003).

The intestinal peptide transporter Pept1 plays an important role in transporting dietary peptides as well as pharmacologically active peptidomimetic drugs (Smith et al 1993; Leibach & Ganapathy 1996). Due to the broad substrate specificity, the peptide transporter can be a potential target in the prodrug design to improve the intestinal

transport of low-permeability drugs. For example, the membrane permeability of the polar α-methyldopa is significantly improved through peptidyl derivatives that are water soluble but well absorbed via a peptide transporter (Hu et al 1989). This approach has been also successful in increasing the bioavailability of the antiviral drugs aciclovir and ganciclovir (Weller et al 1993; Jung & Dorr 1999). The L-valyl ester prodrugs, valaciclovir and valganciclovir, increase the bioavailability of their parent drugs 3- to 5-fold and 10-fold, respectively, via the peptide transporter-mediated transport pathway (Weller et al 1993; Jung & Dorr 1999). Recently, Song et al (2005) have reported that amino acid ester prodrugs of the anti-cancer agent gemcitabine could effectively enhance the transport of gemcitabine via the peptide transporter. Furthermore, it has been reported that targeted delivery of drugs to cancer cells would be possible if the peptide transporters exhibit differential expression in cancer cells compared with normal healthy cells. For example, the in-vitro delivery of δ-aminolevulinic acid to rat pancreatic tumour cells via the peptide transporter has been reported, based on the higher expression of the peptide transporter in pancreatic tumours than in normal pancreatic cells (Gonzalez et al 1998; Whitaker et al 2000). Therefore, the peptide transporter would be a good target for prodrug design to improve the intestinal absorption of low-permeability drugs or to potentially target tumour cells with anti-cancer drugs.

To enhance the intestinal absorption of ara-C, this study aimed to design a peptidomimetic prodrug of ara-C targeting the intestinal peptide transporters. Therefore, L-valyl-ara-C was synthesized by masking the N4-amino group of the cytosine ring in ara-C with L-valine, and its

cellular uptake characteristics were examined in Caco-2 cells. The gastrointestinal stability of L-valyl-ara-C was also evaluated by using artificial digestives.

Materials and Methods

Materials

Ara-C, L-valine, benzoic acid, p-aminohippurate (PAH), small dipeptides, uridine, tetraethylammonium (TEA), aciclovir, 5-bromo-2′-deoxyuridine (BDU), 4-dimethyla-minopyridine (DMAP), N,N′-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Pepsin, pancreatin and BCA protein assay kit were also obtained from Sigma. All other chemicals were reagent grade and all solvents were HPLC grade. Caco-2 cells were purchased from ATCC (Rockville, MD, USA).

Cells

Caco-2 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1 mM sodium pyruvate, 1% L-glutamine and penicillin (100 U mL^{-1})/ streptomycin (100 mg mL^{-1}). All cells were maintained in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C.

Synthesis of L-valyl-ara-C

L-valyl-ara-C (**5**) was readily synthesized from ara-C (**1**) (Figure 1). The three hydroxyl groups of the
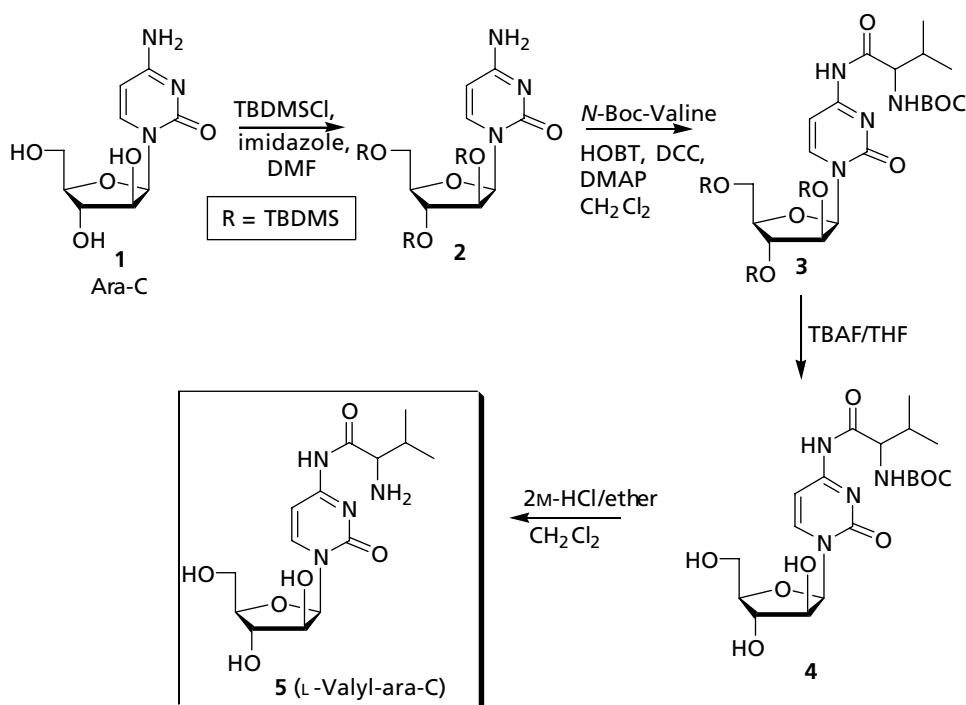

Figure 1 Synthetic scheme of L-valyl-ara-C.

starting material **1** (5.0 g, 20.55 mmol) were protected with *tert*-buthyldimethylchlorosilane (9.2 g, 61 mmol) in anhydrous dimethylformamide (DMF) (100 mL) with imidazole (6.8 g, 100 mmol) to give compound **2** (8.31 g, yield 69%), which was purified using column chromatography on silica gel 60 (hexane–ethylacetate, 1:4). The amino group of compound **2** (1.2 g, 2.04 mmol) was coupled with *N*-BOC-valine (443 mg, 2.04 mmol) using HOBT (283 mg, 2.1 mmol), DMAP (100 mg) and DCC (433 mg, 2.1 mmol) in anhydrous methylene chloride (20 mL) to produce **3** (833 mg, yield 52%), which was purified by column chromatography on silica gel 60 (CH_2Cl_2–CH_3OH, 10:1). Treatment of **3** (500 mg, 0.636 mmol) with tetrabutyl-ammonium fluoride (3.18 mL, 1.0 M in tetrahydrofuran (THF)) in THF (10 mL) provided compound **4** (183 mg, yield 65%), which was purified using column chromatography on silica gel 60 (CH_2Cl_2–CH_3OH, 7:1). Deblocking of the BOC group of **4** (400 mg, 0.9 mmol) with ethereal hydrochloric acid (10 mL, 2 M HCl solution in ether) in anhydrous methylene chloride (10 mL) followed by column chromatography on silica gel 60 (CH_2Cl_2–CH_3OH, 5:1), produced the desired compound, L-valyl-ara-C (**5**) (175 mg, yield 57%).

Uptake studies in Caco-2 cells

Cells were seeded in 6-well culture plates at a density of 10^5 cells/cm^2. At 14 days post-seeding, the cells were washed twice with pH 6.0 uptake buffer containing (in mM): 1 $CaCl_2$, 1 $MgCl_2$, 150 NaCl, 3 KCl, 1 NaH_2PO_4, 5 D-glucose and 5 MES. The initial uptake rates of L-valyl-ara-C and ara-C in Caco-2 cells were determined at 0.4 and 4 mM to examine the concentration dependency of their cellular accumulation. Each drug solution was added to each well and incubated on a plate shaker. At the end of 15 min incubation, drug solution was removed and the cells were washed three times with ice-cold uptake buffer. After cell lysis by adding 1 mL of Milli-Q water, cells were harvested and sonicated for 1–2 min. Acetonitrile was added to the cell lysate, vortexed vigorously and centrifuged for 5 min at 3000 rev min^{-1}. After filtration of the supernatant through a membrane filter (0.45 μm), samples were analysed by HPLC. The protein amount of each sample was determined with BCA protein assay kit following the manufacturer's instruction (Sigma Chemical Co., St Louis, MO, USA). The stability of donor solutions of L-valyl-ara-C above the apical membrane of Caco-2 cell monolayers was also examined during the uptake studies to determine the extent of degradation of L-valyl-ara-C when in contact with Caco-2 monolayers.

Inhibition studies on L-valyl-ara-C uptake in Caco-2 cells

At 14 days post-seeding, the medium was removed and cells were washed twice with pH 6.0 uptake buffer. L-Valyl-ara-C solution (0.4 mM) was prepared with or without an inhibitor such as 1 mM uridine (for nucleoside transporters), 4 mM L-valine (for amino acid transporters), 4 mM benzoic acid (for monocarboxylic acid transporters), 4 mM Gly-Sar and 4 mM Phe-Pro (for peptide transporters), 4 mM PAH (for organic anion transporters), or 4 mM TEA (for organic cation transporters). Each drug solution was added to the wells and incubated for 15 min. After 15 min incubation, the same procedures were followed as in the uptake studies.

Gastrointestinal stability study

Gastrointestinal stability of L-valyl-ara-C was evaluated at 37°C by incubating drug solution (100 μM) with artificial digestives. The gastric juice consisted of 320 mg of pepsin, 200 mg of NaCl and 2.4 mL of 0.1 M HCl in 100 mL solution (pH 1.2). Artificial intestinal juice contained 2.5 g of porcine pancreatin and 100 mL of 50 mM K-phosphate buffer (pH 6.8). At each time point, the metabolic reaction was stopped by adding ice-cold acetonitrile followed by vigorous mixing. The mixture was then centrifuged at 3000 rev min^{-1} for 10 min at 4°C and the supernatant was filtered through a membrane filter (0.45 μm) and analysed by HPLC.

HPLC assay

Drug concentrations were determined by an HPLC assay as follows. Aciclovir and 5-bromo-2′-deoxyuri-dine were used as the internal standard for the assay of ara-C and L-valyl-ara-C, respectively. The chromatographic system consisted of a pump (LC-10AD), an automatic injector (SIL-10A) and a UV detector (SPD-10A) (Shimadzu Scientific Instruments, Japan) set at 272 nm. An octadecylsilane column (Gemini C18, 4.6 × 250 mm, 5 μm; Phenomenex, Torrance, CA, USA) was eluted with a mobile phase at a flow rate of 1.0 mL min^{-1}. The mobile phase consisted of 0.01 M ammonium acetate buffer (pH 6.5)–acetonitrile (93:7, v/v%) for L-valyl-ara-C and 0.01 M ammonium acetate buffer (pH 4.5)–acetonitrile (99:1, v/v%) for ara-C. The calibration curve from the standard samples was linear over the concentration range of 0.01 μg mL^{-1} to 5 μg mL^{-1}. The coefficient of variation for the standard curve ranged from 3.5–24%, and the squared correlation coefficient (r^2) was over 0.998. The limit of detection was 0.01 μg mL^{-1}.

Statistical analysis

All the means are presented with their standard deviation. Statistical analysis was performed using a one-way analysis of variance, followed by *a posteriori* testing with the use of the Dunnett correction. $P < 0.05$ was considered statistically significant.

Results and Discussion

Synthesis of L-valyl-ara-C

Ara-C is given parenterally not orally because on absorption it would be rapidly deaminated to its inactive metabolite ara-U by the intestinal mucosa. Therefore, to reduce the rapid deamination during intestinal absorption, the N4-amino group of the cytosine ring was masked with L-valine in this study. Since previous studies have reported that L-valine may have the optimal combination of chain length and branching at the beta carbon of the amino acid for the intestinal absorption of peptidomimetic drugs (Beauchamp et al 1992; Han et al 1998; Sugawara et al 2000), L-valine was selected to mask the N4-amino group of the cytosine ring in ara-C and subsequently a peptidomimetic prodrug, L-valyl-ara-C was synthesized as illustrated in Figure 1. L-Valyl-ara-C was obtained as white fluffy powder and its purity was $\geq 98\%$ as determined by HPLC. The identities of L-valyl-ara-C were confirmed by EI-MS, H^1 NMR and C^{13} NMR as follows: mp 163–165°C; UV (H_2O) λ_{max} 275.2 nm; ^1H NMR (DMSO-d_6, 300 MHz) δ 7.58 (d, $J = 9.0$ Hz, 1H), 7.49 (d, $J = 7.2$ Hz, 1H), 7.44 (s, 1H), 6.98 (s, 1H), 7.58 (d, $J = 9.0$ Hz, 1H), 7.49 (d, $J = 7.2$ Hz, 1H), 7.44 (s, 1H), 6.98 (s, 1H), 5.96 (d, $J = 3.6$ Hz, 1H), 5.89 (d, $J = 7.2$ Hz, 1H), 5.36 (d, $J = 4.8$ Hz, 2H), 4.99 (t, $J = 5.4$ Hz, 1H), 4.41 (dd, $J = 8.7$, 6.6 Hz, 1H), 3.89–3.81 (m, 2H), 3.67 (dd, $J = 8.1$, 2.1 Hz, 1H), 3.53 (d, $J = 5.1$ Hz, 2H), 1.94 (m, 1H), 0.83 (dd, $J = 6.6$, 2.7 Hz, 6H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 173.03, 163.67, 155.06, 141.80, 93.34, 85.94, 84.95, 76.34, 74.77, 61.18, 57.83, 30.56, 19.27, 18.16. ; EI-MS: 343 $(M+H)^+$.

Gastrointestinal stability of L-valyl-ara-C

The gastrointestinal stability of L-valy-ara-C was examined by using the artificial digestives. As shown in Figure 2, L-valyl-ara-C appeared to be more stable in artificial intestinal fluid than in gastric juice. The disappearance half-life of L-valyl-ara-C was 2.2 h in the artificial gastric juice. Based on the gastrointestinal transit study of oral solid preparations in man, by gamma-scintigraphy, Weitschies et al (1999) have reported that orally administered solid preparations were transferred to the small intestine within 1 h of administration under the fasted condition. Therefore, the stability of L-valyl-ara-C in gastric juice appeared to be appropriate for the oral delivery of L-valyl-ara-C. The degradation of L-valyl-ara-C was negligible in the artificial intestinal fluid over the 2-h incubation. In addition, the degradation of L-valyl-ara-C in the donor solution during the 2-h incubation above the Caco-2 cell monolayer was < 10%, implying that L-valyl-ara-C was stable against the hydrolysis at the apical membrane of Caco-2 cells. During the cellular uptake studies in caco-2 cells, the re-conversion of a prodrug to the parent inside cells appeared to be minimal (< 15%).
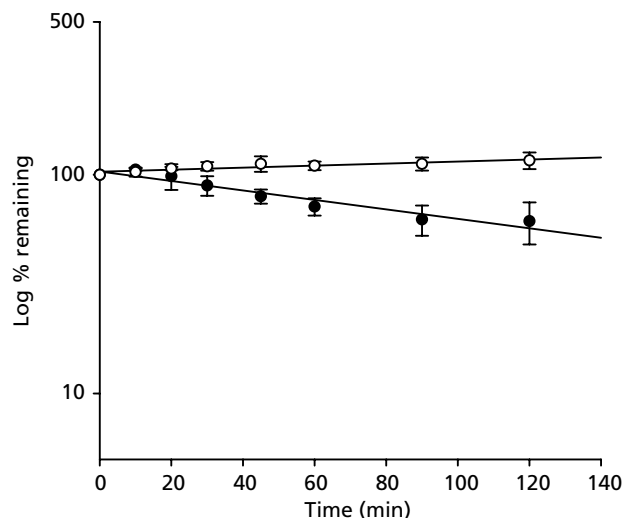

Figure 2 In-vitro stability of L-valyl-ara-C in artificial gastric juice (closed circles) and intestinal fluids (open circles) (mean $\pm$ s.d., n = 6). Drug solution (100 μM) was incubated for 2 h at 37°C. The compositions of the artificial gastric juice and intestinal fluids are described under Materials and Methods.

Collectively, the in-vitro stability studies indicated that L-valyl-ara-C could be stable in the intestinal lumen after its oral administration.

Cellular uptake studies

The cellular uptake characteristics of L-valyl-ara-C, as well as ara-C, were evaluated in Caco-2 cells. The cellular uptake of ara-C and L-valyl-ara-C at 0.4 mM was 1.48 ± 0.39 nmol h^{-1} (mg protein)$^{-1}$ and 7.45 ± 1.17 nmol h^{-1} (mg protein)$^{-1}$, respectively (Figure 3). Therefore, L-valyl-ara-C appeared to be 5-fold more permeable across the apical membrane of Caco-2 cells compared with ara-C. In addition, to evaluate the potential contribution of a carrier-mediated transport
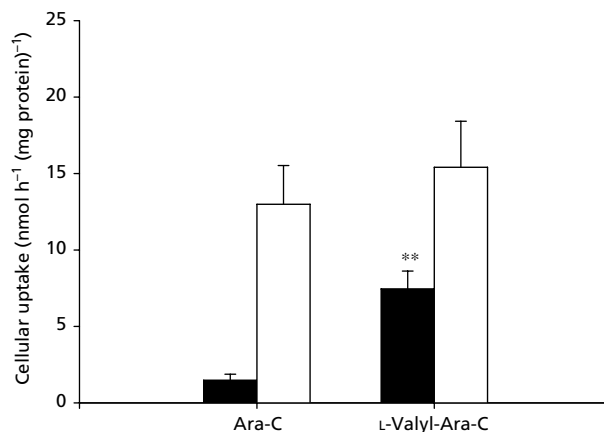

Figure 3 Cellular uptake of Ara-C and L-valyl-ara-C in Caco-2 cells (mean $\pm$ s.d., n = 6) at 0.4 mM (shaded columns) and 4 mM (open columns). **P < 0.05, uptake of L-valyl-ara-C vs ara-C.

mechanism to the cellular uptake of drugs, the concentration dependency in the membrane transport of L-valyl-ara-C was examined in Caco-2 cells. While the cellular uptake of ara-C increased approximately 10 fold as drug concentration increased from 0.4 to 4 mM, the uptake rate of L-valyl-ara-C did not increase proportionally to the increase in drug concentration (Figure 3). This result suggests that saturable transport pathways may be involved in the cellular uptake of L-valyl-ara-C, while passive diffusion could predominate for the cellular uptake of ara-C over the tested drug concentrations. Therefore, at the lower concentration (0.4 mM), the 5-fold higher cellular uptake of L-valyl-ara-C compared with ara-C may be attributed to the facilitated drug uptake via carrier-mediated transport pathways. However, at the higher concentration (4 mM), the carrier-mediated transport pathway could be saturated and, thus, the passive diffusion became predominant for the cellular uptake of L-valyl-ara-C. Consequently, at the higher concentration, the cellular uptake of L-valyl-ara-C was similar to that of ara-C. Considering that the therapeutic concentration of ara-C is in the range 0.01–0.1 $\mu g\,mL^{-1}$ (Mellett 1974; Balis et al 1983), the carrier-mediated transport pathway for L-valyl-ara-C is unlikely to be saturated at the therapeutic dose level, although further studies should be performed in-vivo for more clarification.

To identify the membrane transporters responsible for the intestinal transport of L-valyl-ara-C, inhibition studies on the cellular uptake of L-valyl-ara-C were performed in Caco-2 cells. The transport of L-valyl-ara-C across the apical membrane of the Caco-2 cell monolayers was markedly inhibited in the presence of small dipeptides, PAH, TEA and uridine, while L-valine and benzoic acid had no effect (Figure 4). This suggests that L-valyl-ara-C could

interact with multiple transporters, such as peptide transporters, organic anion and cation transporters and nucleoside transporters, but might not interact with amino acid transporters. These results appear to be consistent with previous reports on the substrate specificities of peptidyl analogues (Ott et al 1990; Sinko & Balimane 1998; Takeda et al 2002; Izzedine et al 2005). Sinko & Balimane (1998) and also Ott et al (1990) reported that peptide analogues interacted to varying degrees with the organic anion and organic cation transporters in addition to peptide transporters. Furthermore, the significant overlap in the specificity of the organic anion and organic cation transporters has been demonstrated in the previous reports (Takeda et al 2002; Izzedine et al 2005). Therefore, it is not surprising that L-valyl-ara-C, a peptidyl derivative, could interact with organic anion and organic cation transporters in addition to peptide transporters. However, the tissue distribution of organic anion and organic cation transporters seems to be restricted to a few cell types and they do not appear to significantly contribute to drug absorption in the intestine (Dresser et al 2001; Burckhardt & Burckhardt 2003; Katsura & Inui 2003; Pastor-Anglada et al 2005). Therefore, the contribution of organic anion and organic cation transporters to the intestinal absorption of L-valyl-ara-C may not be significant compared with the intestinal peptide transporters. The quantitative contribution of each transporter to the overall transport of L-valyl-ara-C should be clarified further in the future studies.

Conclusions

This study has demonstrated that L-valyl-ara-C could be stable in the intestinal lumen, that L-valyl-ara-C was
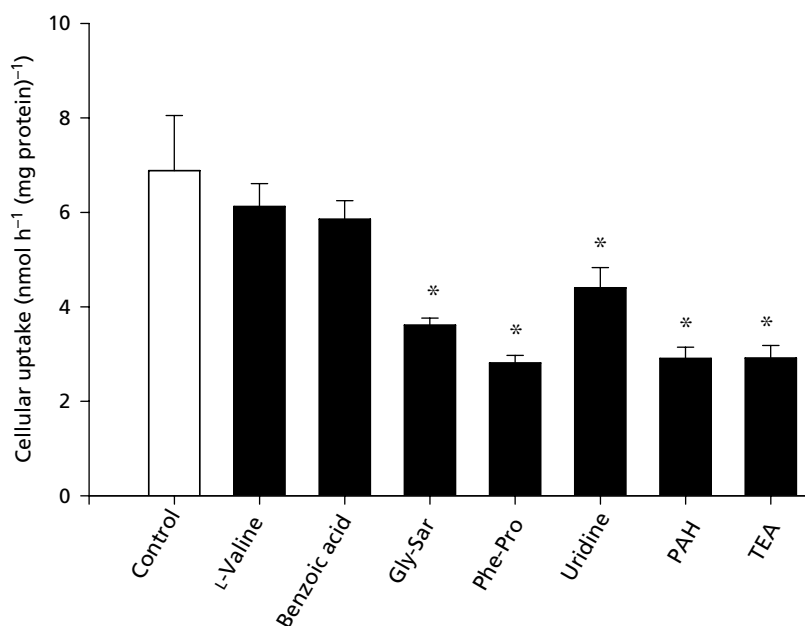

Figure 4   Inhibition studies on the cellular uptake of L-valyl-ara-C (0.4 mM) in Caco-2 cells (mean ± s.d., n = 6) in the absence of inhibitors (open column, control) and in the presence of each inhibitor (shaded columns). The concentration of each inhibitor is described under Materials and Methods. *$P < 0.05$, compared with control.

5-fold more permeable across the apical membrane of caco-2 cells compared with ara-C, and that L-valyl-ara-C could interact with multiple uptake transporters in the apical membrane of Caco-2 cells. These results suggest that L-valyl-ara-C could effectively improve the oral absorption of ara-C via the carrier-mediated transport pathways.

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