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조 선 대 학 교 대 학 원

약 학 과

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지도교수 한 효 경

이 논문을 약학 석사학위신청 논문으로 제출함.

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약 학 과

천 은 파

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위원장　조선대학교 교 수　최 준 식　인

위원　조선대학교 교 수　최 후 균　인

위원　조선대학교 교 수　한 효 경　인

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조선대학교 대학원

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천 은 파

지 도 교 수 : 한 효 경

조선대학교 대학원 약학과

이 논문의 목적은 ara-C의 peptide 유사 prodrug인 L-valyl-ara-C를 합성하여 ara-C의 잠재적인 경구 운반 시스템으로써 L-valyl-ara-C를 평가하는 것이다. L-valine을 ara-C의 cytosine ring의 N4-amino group에 삽입하여 L-valyl-ara-C를 합성한 후 다양한 생물학적 용액에서 in-vitro 안정성을 측정하였고, Caco-2 cell에서 세포내 흡수 특성에 대해 조사하였다. 또한, 쥐에서 ara-C와 L-valyl-ara-C의 혈장 약물 동력학적 profile도 평가하였다. L-valyl-ara-C의 인공위액에서의 소실 반감기는 2.2시간인 반면에, 인공장액, fresh plasma, 그리고 serine protease인 plasmin의 존재 시에 2시간 이상의 incubation에도 L-valyl-ara-C의 분해는 거의 일어나지 않았다. 게다가 L-valyl-ara-C는 AML-2와 L1210의 leukemia cell homogenate에서 안정함을 나타내었고, 이어서 동일 cell에서 parent인 ara-C 보다 세포독성을 훨씬 덜 나타냈다. Caco-2 cell에서 L-valyl-ara-C의 세포내 축적은 ara-C와 비교했을 때 5배나 높았다. 게다가 L-valyl-ara-C의 세포내 흡수는 약물 농도의 증가에 비례적으로 증가하지 않았다. L-valyl-ara-C의 세포내 축적은 uridine, PAH, TEA와 작은 dipeptide가 존재할 시에는 매우 감소했으나 L-valine과 benzoic acid가 있을 때는 변화가 없었다. 이는 L-valyl-ara-C가 peptide transporter,

organic anion 및 cation transporter, 그리고 nucleoside transporter을 포함하는 다수의 uptake transporter와 상호작용 할 수 있으나 amino acid transporter와는 작용하지 않음을 나타내었다. 비록 prodrug의 전신적인 분포 용적이 ara-C보다 훨씬 더 높았지만, L-valyl-ara-C의 경구투여 시에 혈장에서 ara-C의 존재가 관찰되었다. Prodrug 투여 시에 ara-C의 생체이용률은 약 4% 정도였다. 결론적으로, L-valyl-ara-C는 carrier-mediated transport pathway를 통해 ara-C의 장관 흡수를 향상시킬 수 있으나 ara-C로의 낮은 대사적 전환으로 인하여 ara-c의 경구 운반 시스템으로써 실제적인 적용은 제한될 수 있다.

Abstract

Evaluation of a peptidomimetic prodrug, L-valyl-ara-C as an oral delivery system of ara-C

Eun Pa Cheon

Advisor: Prof. Hyo-Kyung Han
Department of Pharmacy
Graduate School of Chosun University

The present study aimed to synthesize a peptidomimetic prodrug, L-valyl-ara-C, and evaluate L-valyl-ara-C as a potential oral delivery system of ara-C. After the synthesis of L-valyl-ara-C via the incorporation of L-valine into N4-amino group of the cytosine ring in ara-C, the in-vitro stability of L-valyl-ara-C was examined in the various biological media, and the cellular uptake characteristics of L-valyl-ara-C were examined in Caco-2 cells. Plasma pharmacokinetic profiles of ara-C and L-valyl-ara-C were also evaluated in rats. The disappearance half-life of L-valyl-ara-C was 2.2 hrs in the artificial gastric juice while the degradation of L-val-ara-C was negligible in the intestinal fluid, fresh plasma, and also in the presence of plasmin, a serine protease, over 2-hr incubation. Furthermore, L-valyl-ara-C appeared to be stable in the leukemia cell homogenates and subsequently it was far less cytotoxic than the parent, ara-C in AML2 and L1210 cells. The cellular accumulation of L-valyl-ara-C was 5-fold higher in Caco-2 cells compared to ara-C. Furthermore, the cellular uptake of

L-valyl-ara-C did not increase proportionally to the increase of drug concentration. The cellular accumulation of L-valyl-ara-C was significantly reduced in the presence of uridine, PAH, TEA 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. Following an oral administration of L-valyl-ara-C, the appearance of ara-C was observed in plasma although the systemic exposure of the prodrug was much higher than that of ara-C. The bioavailability of ara-C was about 4 % via the prodrug administration. In conclusion, L-valyl-ara-C may improve the intestinal absorption of ara-C via the carrier-mediated transport pathway but its utility as an oral delivery system of ara-C could be limited by the low metabolic conversion to ara-C.

1. Introduction

Ara-C (1-(β-D-arabinofuranosyl)cytosine, cytarabine), a pyrimidine nucleoside analog, is one of the most effective drugs used in the treatment of acute myeloid leukaemia, acute lymphoblastic leukaemia and other haematological malignancies [5,12]. In combination with other antitumor agents it is also used against solid tumors [12]. For the 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 [5,12]. Recently, ara-C has been reported to also induce apoptosis of neoplastic cells [23,30]. 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 [17, 29]. 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 [17,29]. In attempts 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 [13, 15, 42, 43].

The intestinal peptide transporter (Pept1) plays an important role in transporting dietary peptides as well as pharmacologically active peptidomimetic drugs [22, 33]. 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-methyl-dopa was significantly improved through peptidyl derivatives which were water soluble but well absorbed via a peptide transporter [18]. This approach has been also successful in increasing the bioavailability of the antiviral drugs, acyclovir and ganciclovir [20,39]. The L-valyl ester prodrugs, valacyclovir and valganciclovir, increase the bioavailabiltiy of their parent drugs three to five-fold and 10-fold, respectively, via the peptide transporter-mediated transport pathway [20,39].

Recently, it has been reported that amino acid ester prodrugs of the anticancer agent gemcitabine could be effective to enhance the transport of gemcitabine via the peptide transporter.[34] Furthermore, there 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 to normal healthy cells. For example, the in vitro delivery of δ −aminoleuvulinic acid to rat pancreatic tumor cells via the peptide transporter has been reported based on the higher expression of the peptide transporter in pancreatic tumors than normal pancreatic cell [11,40]. Therefore, the peptide transporter would be a good target for the prodrug design to improve the intestinal absorption of low permeability drugs or to potentially target tumor cells with anticancer drugs.

In order to avoid the deamination as well as to enhance an intestinal absorbtion of ara-C, the present study aimed to synthesize a peptidomimetic prodrug, L-valyl-ara-C, and evaluate L-valyl-ara-C as a potential oral delivery system of ara-C.

2. Materials and Methods

Materials : Ara-C, L-valine, benzoic acid, p-aminohippurate(PAH), small dipeptides, uridine, tetraethylammonium(TEA), acyclovir and 5-bromo-2'-deoxyuridine (BDU), 4-dimethylaminopyridine(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 Chemical Co. (St. Louis, MO, USA). All other chemicals were reagent grade and all solvents were HPLC grade. Caco-2 cells were purchased from ATCC (Rockville, MD, USA). AML2 and L1210 cells were purchased from Korean Cell Line Bank (Seoul, Korea).

Cells : Caco-2 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 1% nonessential amino acids, 1 mM sodium pyruvate, 1% L-glutamine and penicillin (100 U/mL)/streptomycin (100 mg/mL). L1210 cells were cultured in RPMI-1640 medium supplementedwith 10% FBS and penicillin (100 U/mL)/streptomycin (50 mg/mL). AML-2 cells were grown in DMEM supplemented with 10% FBS and penicillin (100 U/mL)/streptomycin (50 mg/mL). 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) as depicted in Fig. 1. The three-hydroxyl groups of the starting material 1 (5.0 g, 20.55 mmol) were protected with tert-buthyldimethylchlorosilane (9.2 g, 61 mmol) in anhydrous 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 tetrabutylammonium fluoride (3.18 mL, 1.0 M in 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 BOC group of 4 (400 mg, 0.9 mmol) with etherealhydrochloric acid (10 mL, 2 N 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%).

In-vitro stability study : Gastrointestinal stability of L-valyl-ara-C was evaluated at 37 °C by incubating a 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 pancreatin from porcine and 100 mL of 50 mM K-phosphate buffer (pH 6.8). Stabilities of L-valyl-ara-C were also evaluated at 37 ˚C by incubating a drug solution (10 μM) in the fresh plasma and cell homogenates. Enzymatic cleavage of amide bond between valine and ara-C was also measured in the presence of plasmin. 30 μL plasmin (10 Cu/mL) was added to the thermostated buffer composed of 50 mM Tris-HCl, pH 7.4 and 110 mM NaCl followed by the addition of drug solution (10 μM) to a final volume of 1.0 mL at 37 C. 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 rpm for 10 min at 4 ˚C and the supernatant was filtered through a membrane filter (0.45 μm) and analyzed by HPLC. The chemical stability of L-valyl-ara-C was also examined in aqueous solutions of different pHs (2.0, 7.4 and 10).

Uptake studies in Caco-2 cells : Cells were seeded in 6-well culture plates at a density of 10^5 cells/cm2. At 14 days post-seeding, the cells were washed twice with pH 6.0 uptake buffer containing 1 mM $CaCl_2$, 1mM $MgCl_2$, 150 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 5 mM D-glucose, and 5 mM 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 in 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 rigorously, and centrifuged for 5 min at 3000 rpm. After filtration of the supernatant through a membrane filter (0.45 μm), samples were analyzed 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 the 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/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, it was following the same procedures as mentioned in the uptake studies.

Antiproliferative assays : Cells were resuspended at a density of $1x10^5$cells/mL in growth medium containing serial dilutions of test drugs. Cell viability was determined after 96 hrs at 37 ˚C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method, as previously described by Pauwels et al. (1988)[28]. Cell growth at each drug solution was expressed as percentage of untreated controls and IC_{50} was determined as the concentration resulting in 50% growth inhibition.

Pharmacokinetic studies in rats : Male Sprague-Dawley rats (270-300 g) were purchased from Dae Han Laboratory Animal Research and Co. (Choongbuk, Korea), and given a normal standard chow diet (No.322-7-1) purchased from Superfeed Co. (Gangwon, Korea) and tap water *ad libitum*. All animal studies were performed in accord with the Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences and the experimental protocols were approved by the animal care committee of Chosun University. Animals were kept in these facilities for at least one week before the experiment and fasted for 24 hrs prior to the experiments. At the experiment, rats (n=4 per each treatment) were given a 10 mg/kg of L-valyl-ara-C orally or 2 mg/kg of ara-C intravenously. Drugs were dissolved in saline and the dosing volume was 1 mL for each animal. Blood samples were collected from the right femoral artery at 0, 0.083, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 hr post-dose and then centrifuged at 3,000 rpm for 10 min to obtain the plasma for the HPLC assay. All samples were stored at - 70 ℃ until analyzed.

HPLC Assay : Drug concentrations were determined by a HPLC assay described as follows. Acyclovir and 5-bromo-2'-deoxyuridine were used as the internal standard for the assay of ara-C and L-valyl-ara-C, respectively. The chromatographic system was 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, 5μm; Phenomenex, Torrance, CA, USA) was eluted with a mobile phase at a flow rate of 1.0 mL/min. The mobile phase consisted of 0.01M ammonium acetate buffer (pH 6.5):acetonitrile (93:7, v/v %) for L-valyl-ara-C and 0.01M 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 to 5 μg/mL. The coefficient of variation for the standard curve ranged from 3.5 to 24 %, and the squared correlation coefficient (r^2) was over 0.998. The limit of detection was 0.01 μg/mL.

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

3. 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 the intestinal absorption, N4-amino group of the cytosine ring was masked with L-valine in the present study. Since previous studies have reported that L-valine may have the optimal combination of chain lengthand branching at the beta carbon of the amino acid for the intestinal absorption of peptidomimetic drugs [4,16,35], 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 Fig. 1. L-valyl-ara-C was obtained as white fluffy powders and its purity was = 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) max 275.2 nm ^{1}H NMR (DMSO-*d6*, 300 MHz) 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-*d6*, 75 MHz) 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 $(M+H)^+$

In-vitro metabolic stability studies : The kinetics of L-valyl-ara-C degradation were studied in various conditions. The gastrointestinal stability of L-valy-ara-C was examined by using the artificial digestives. As shown in Fig. 2, L-valyl-ara-C appeared to be more stable in the artificial intestinal fluid than in the gastric juice. The disappearance half-life of L-valyl-ara-C was 2.2 hrs in the artificial gastric juice.

Based on the GI transit study of oral solid preparation in human subjects by gamma-scintigraphy, Weitschies et al. have reported that orally administered solid

preparations were transferred to the small intestine within 1 hr after administration under the fasted condition [38]. 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 hr-incubation. In addition, the degradation of L-valyl-ara-C in donor solution during the 2-hr incubation above the Caco-2 cell monolayer was below 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 reconversion of a prodrug to the parent inside cells appeared to be minimal (less than 15 %). Collectively, the in-vitro stability studies indicated that L-valyl-ara-C could be stable in the intestinal lumen after its oral administration.

As shown in Fig. 3, L-valyl-ara-C appeared to be stable in fresh rat plasma and leukemia cell homogenates. The degradation of L-valyl-ara-C was negligible in fresh rat plasma and leukemia cell homogenates over the 2 hr-incubation. In addition, metabolic stability of L-valyl-ara-C was assessed using pure serine protease plasmin and results indicated that L-valyl-ara-C was resistant to the degradation by plasmin. The chemical stability of L-valyl-ara-C was also examined in aqueous solutions of different pHs (2.0, 7.4 and 10). While L-valyl-ara-C was stable in aqueous solutions of pH 7.4 and pH 10, the hydrolysis of amide bond appeared to be rather accelerated in acidic pH and the disappearance half-life of L-valyl-ara-C was approximately 4 hrs in the aqueous solution of pH 2.0 (Fig. 3).

If the prodrug is stable in plasma and hydrolyzed mainly near the target cells, this should result in a continuous and relatively high concentration of the active agent around target cells. Many types of malignant cells and human tumors display increased concentrations of the protease plasminogen activators that convert plasminogen to the highly active protease, plasmin [1,25,37]. Leukemic cells also secrete these enzymes [41]. Since plasmin rapidly cleaves various low molecular weight compounds coupled to appropriate peptide specifiers, the coupling of such peptide specifiers to anticancer drugs might create "prodrugs" which would be locally activated by tumor-associated plasmin and consequently would be less toxic to

normal cells. For examples, Carl et al. (1980) have reported that the peptide prodrugs of several anticancer agents designed to be specific plasmin substrates showed selective cytotoxicity[7]. Since L-valyl-ara-C is a peptidomimetic prodrug and appeared to be stable in plasma or leukemia cell homogenates, the potential bioconversion of L-valyl-ara-C to ara-C in the surroundings of the leukemia cells was examined using pure plasmin. As shown in Fig. 3, L-valyl-ara-C exhibited high resistance to the degradation by plasmin, implying that L-valyl-ara-C may not be a substrate of plasmin. Overall, in-vitro stability studies indicated that L-valyl-ara-C was metabolically stable in plasma and leukemia cells. However, in the case of chemical stability, the hydrolysis of prodrug appeared to be rather accelerated in acidic pH. Considering that the tumor pH is on average, lower than the pH of normal tissues [10], chemical hydrolysis of L-valyl-ara-C may be more favorable in tumor cells.

Cellular uptake Studies : The cellular uptake characteristics of L-valyl-ara-C as well as ara-C were evaluated in Caco-2 cells. As shown in Fig.4, the cellular uptake of ara-C and L-valyl-ara-C at 0.4 mM was 1.48 ± 0.39 nmol/hr/mg protein and 7.45 ± 1.17 nmol/hr/mg protein, respectively. Therefore, L-valyl-ara-C appeared to be five fold more permeable across the apical membrane of Caco-2 cells compared to ara-C. In addition, to evaluate the potential contribution of a carrier-mediated transport 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 folds 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 of drug concentration (Fig. 4). This result suggests that saturable transport pathways may involve in the cellular uptake of L-valyl-ara-C while the passive diffusion could be predominant for the cellular uptake of ara-C over the tested drug concentrations. Therefore, at the low concentration (0.4 mM), five folds higher cellular uptake of L-valyl-ara-C compared to ara-C may be attributed to the facilitated drug uptake via the carrier-mediated transport pathways. However, at the high 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 high 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 ranged from 0.01 and 0.1 μg/mL [3,24], the carrier-mediated transport pathway for L-valyl-ara-C is unlikely 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, the inhibition studies on the cellular uptake of L-valyl-ara-C was performed in Caco-2 cells. As summarized in Fig. 5, 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. Those results suggest 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. Those results appear to be consistent with the previous reports on the substrate specificities of peptidyl analogues [19,26,32,36]. Sinko et al. (1998) and also Ott et al. (1990) reported that peptide analogs interacted to varying degrees with the organic anion and organic cation transporters as well in addition to peptide transporters[26,32]. Furthermore, the significant overlap in the specificity of the organic anion and organic cation transporters has been demonstrated in the previous reports [19,36]. 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 [6,9,21,27]. Therefore, the contribution of organic anion and organic cation transporters to the intestinal absorption of L-valyl-ara-C may not be significant compared to 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.

Antiproliferative assays : The antiproliferative activity of ara-C and L-valyl-ara-C was evaluated against leukemia cells such as AML-2 and L1210. The IC50 values were 3.89 ± 1.11 and 606 ± 87.3 μM for ara-C and L-valyl-ara-C, respectively in AML-2 cells. In the case of L1210 cells, IC50 values were 0.25 ± 0.03 and 37.5 ± 6.69 μM for ara-C and L-valyl-ara-C, respectively. In both cells, the antiproliferative activity of L-valyl-ara-C was approximately 150-fold less potent than ara-C. Although L-valyl-ara-C appeared to be beneficial to enhance the cellular uptake of ara-C in Caco-2 cells, prodrug itself exhibited far less antiproliferative activity than ara-C in AML-2 and L1210 cells and thus, the rate of conversion of the prodrug to the pharmacologically active parent drug after membrane transport would determine the therapeutic effectiveness of L-valyl-ara-C.

Pharmacokinetic studies : The plasma pharmacokinetic profiles of L-valyl-ara-C and ara-C were determined in rats and summarized in Table 1. After an oral administration of L-valyl-ara-C, the release of ara-C from the prodrug was observed in plasma although the systemic exposure of the prodrug was much higher than that of ara-C. The bioavailability of ara-C was about 4 % via the prodrug administration in rats.

As summarized in Table I, following an intravenous administration of ara-C to rats, ara-C showed a short plasma half-life of 1.5 hr and its volume of distribution was greater than total body water in rats [8]. The pharmacokinetic parameters of ara-C obtained from the present study appeared to be comparable to those from the previous studies [14,31,44]. Following an oral administration of L-valyl-ara-C to rats, the appearance of ara-C was observed in plasma, implying that the prodrug conversion to the parent could happen by intestinal or hepatic metabolism. L-valyl-ara-C is a peptidyl derivative of ara-C and thus during the intestinal absorption it may interact with peptidases which catalyze the hydrolysis of peptide bonds in peptidyl derivatives or the hydrolysis of various simple amides [2]. However, as implicated by the results from the in-vitro stability study of

L-valyl-ara-C, the metabolic conversion of L-valyl-ara-C appeared to be minimal in rats. Consequently, the systemic exposure (AUC) of the prodrug was much higher than that of ara-C and the bioavailability of ara-C was low (about 4 %) via the prodrug administration. Therefore, the metabolic conversion of the prodrug to the parent did not appear to be sufficient to ensure the therapeutic effectiveness in the treatment of tumors, although L-valyl-ara-C could prevent the rapid deamination of ara-C by masking the N4-amino group of the cytosine ring. Further studies should be required for the considerable tuning of the metabolic stability of prodrugs by varying its amino acid component.

4. Conclusions

The present study has demonstrated that L-valyl-ara-C could be stable in the intestinal lumen, L-valyl-ara-C was five-fold more permeable across the apical membrane of Caco-2 cells compared to ara-C, and 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 be effective to improve the oral absorption of ara-C via the carrier-mediated transport pathways.

However, the amide bond of L-valyl-ara-C was stable against the enzymatic hydrolysis and the utility of L-valyl-ara-C as an oral delivery system of ara-C appeared to be limited by its low metabolic conversion to ara-C in rats.

References

1. Axelrod JH, Reich R and Miskin R. (1989) Expression of human recombinant plasminogen activators enhances invasion and experimental metastasis of H-ras-transformed NIH 3T3 cells. *Mol. Cell Biol*. **9**:2133-2141.
2. Bai JPF and Amidon GL. (1992) Structural specificity of mucosal cell transport and metabolism of peptide drugs: Implication for oral peptide drug delivery. *Pharm. Res*. **9**:969-978.
3. Balis FM, Holcenberg JS and Bleyer WA (1983). Clinical pharmacokinetics of commonly used anticancer drugs. *Clin. Pharmacokinet.* **8**:202-232**.**
4. Beauchamp LM, Orr GF, de Miranda P, Burnette T and Krenitsky TA (1992). Amino acid ester prodrugs of acyclovir. *Antiviral. Chem. Chemother.* **3**:157-164.
5. Bryan JH, Henderson ES and Leventhal BG. (1974) Cytosine arabinoside and 6-thioguanine in refractory acute lymphocytic leukemia. *Cancer* **33**:539-544.
6. Burckhardt BC and Burckhardt G. (2003). Transport of organic anions across the basolateral membrane of proximal tubule cells. *Rev. Physiol. Biochem. Pharmacol.* **146**:95-158.
7. Carl PL, Chakravarty PK, Katzenellenbogen JA and Weber MJ. (1980) Protease-activated "prodrugs" for cancer chemotherapy. *Proc Natl Acad Sci USA*. **77**:2224-2228.
8. Davies B and Morris T. (1993)Physiological parameters in laboratory animals and humans. *Pharm. Res*. **10**:1093-1095.
9. Dresser MJ, Leabman MK and Giacomini KM (2001). Transporters involved in the elimination of drugs in the kidney: organic aniontransporters and organic cation transporters. *J. Pharm. Sci.* **90**:397-421.
10. Gerweck LE and Seetharaman K. (1996) Cellular pH gradient in tumor versus

normal tissue: potential exploitation for the treatment of cancer. *Cancer Res*. **56**:1194-1198.

11. Gonzalez DE, Covitz KM, Sadee W and Mrsny RJ (1998). An oligopeptide transporter is expressed at high levels in the pancreatic carcinoma cell lines AsPc-1 and Capan-2. Cancer Res. **58**:519-25.
12. Grant S. (1998) Ara-C: cellular and molecular pharmacology. *Adv. Cancer Res*. **72**:197-233.
13. Greenwald RB, Choe YH, McGuire J and Conover CD. (2003) Effective drug delivery by PEGylated drug conjugates. *Adv. Drug Deliv. Rev*. **55**:217-250.
14. Groothuis DR, Benalcazar H, Allen CV, Wise RM, Dills C, Dobrescu C, Rothholtz V, Levy RM. (2000) Comparison of cytosine arabinoside delivery to rat brain by intravenous, intrathecal, intraventricular and intraparenchymal routes of administration. *Brain Res*. **856**:281-90.
15. Hadfield AF and Sartorelli AC. (1984) The pharmacology of prodrugs of 5-fluorouracil and 1-beta-D-arabinofuranosylcytosine. *Adv. Pharmacol.Chem*other. **20**:21-67.
16. Han H-K, de Vrueh RLA, Rhie JK, Covitz K-MY, Smith PL, Lee C-P, Oh D-M, Sadee W and Amidon G.L (1998). 5'-amino acid esters of antiviral nucleosides, acyclovir and AZT are absorbed by the intestinal PEPT1 peptide transporter. *Pharm. Res.* **15**: 1154-1159.
17. Ho DH and Frei E 3rd. (1971) Clinical pharmacology of 1-beta-d-arabinofuranosyl cytosine. *Clin. Pharmacol. Ther*. **12**:944-954.
18. Hu M, Subramanian P, Mosberg HI and Amidon G.L (1989). Use of the peptide carrier system to improve the intestinal absorption of L-α-methyldopa: carrier kinetics, intestinal permeabilities, and in vitro hydrolysis of dipeptidyl derivatives of L-α-methyldopa. *Pharm. Res.* **6**:66-70.
19. Izzedine H, Launay-Vacher V and Deray G (2005). Renal tubular transporters and

antiviral drugs: an update. *AIDS.* **19**:455-62.

20. Jung D and Dorr A (1999). Single-dose pharmacokinetics of valganciclovir in HIV- and CMV-seropositive subjects. *J. Clin. Pharmacol*. **39**:800-4.
21. Katsura T and Inui K (2003). Intestinal absorption of drugs mediated by drug transporters: mechanisms and regulation. *Drug Metab. Pharmacokinet*. **18**:1-15.
22. Leibach FH and Ganapathy V (1996). Peptide transporters in the intestine and the kidney. *Annu. Rev. Nutr.* **16**:99-119.
23. Lickliter JD, Wood NJ, Johnson L, McHugh G., Tan J, Wood F, Cox J and Wickham NW (2003). HA14-1 selectively induces apoptosis in Bcl-2-overexpressing leukemia/lymphoma cells, and enhances cytarabine-induced cell death. *Leukemia*. **17**:2074-80.
24. Mellett LB (1974). Pharmacodynamic and pharmacokinetic measurements of antitumor agents. *Clin. Pharmacol. Ther.* **16**:230-242.
25. Nagy B, Ban J and Brdar B. (1977) Fibrinolysis associated with human neoplasia: production of plasminogen activator by human tumours. *Int. J. Cancer*. **19**:614-620.
26. Ott RJ, Hai AC and Giacomini KM (1990). Mechanisms of interactions between organic anions and the organic cation transporter in renal brush border membrane vesicles. *Biochem. Pharmacol.* **40**:659-61.
27. Pastor-Anglada M, Cano-Soldado P, Molina-Arcas M, Lostao MP, Larrayoz I, Martinez-Picado J and Casado FJ (2005). Cell entry and export of nucleoside analogues. *Virus Res.* **107**:151-64.
28. Pauwels R, Balzarini J, Baba M, Snoeck R, Schols D, Herdewijn P, Desmyter J, De Clercq E. (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J. Virol. Methods*. **20**:309-321.
29. Rustum YM and Raymakers RA. (1992) 1-Beta-arabinofuranosylcytosine in therapy of leukemia: preclinical and clinical overview. *Pharmacol. Ther*.

56:307-321.

30. Samorapoompichit P, Steiner M, Lucas T, Wachtler F, Schedled A, Sperr WR and Valent P (2003). Induction of apoptosis in the human mast cell leukemia cell line HMC-1 by various antineoplastic drugs. *Leuk. Lymphoma.* **44**:509-15.
31. Scott-Moncrieff JC, Chan TC, Samuels ML, Cook JR, Coppoc GL, DeNicola DB and Richardson RC. (1991) Plasma and cerebrospinal fluid pharmacokinetics of cytosine arabinoside in dogs. *Canc. Chemother. Pharmacol.* **29**:13–18.
32. Sinko PJ and Balimane PV (1998). Carrier-mediated intestinal absorption of valacyclovir, the L-valyl ester prodrug of acyclovir: 1. Interactions with peptides, organic anions and organic cations in rats. *Biopharm. Drug Dispos.* **19**:209-17.
33. Smith PL, Eddy EP, Lee CP and Wilson G. (1993). Exploitation of the intestinal oligopeptide transporter to enhance drug absorption. *Drug. Del.* **1**:103-111.
34. Song X, Lorenzi PL, Landowski CP, Vig BS, Hilfinger JM and Amidon GL (2005). Amino acid ester prodrugs of the anticancer agent gemcitabine: synthesis, bioconversion, metabolic bioevasion, and hPEPT1-mediated transport. *Mol. Pharm.* **2**:157-67.
35. Sugawara M, Huang W, Fei YJ, Leibach FH, Ganapathy V and Ganapathy ME (2000). Transport of valganciclovir, a ganciclovir prodrug, via peptide transporters PEPT1 and PEPT2, *J. Pharm. Sci.* **89**:781–789.
36. Takeda M, Khamdang S, Narikawa S, Kimura H, Kobayashi Y, Yamamoto T, Cha SH, Sekine T and Endou H (2002). Human organic anion transporters and human organic cation transporters mediate renal antiviral transport. *J.Pharmacol. Exp. Ther.* **300**:918-24.
37. Tucker WS, Kirsch WM, Martinez-Hernandez A and Fink LM. (1978) In vitro plasminogen activator activity in human brain tumors. *Cancer Res*. **38**:297-302.
38. Weitschies W, Cardini D, Karaus M,Trahms L and Semmler W (1999). Magnetic marker monitoring of esophageal, gastric and duodenal transit of

non-disintegrating capsules. *Pharmazie.* **54**: 426-30.

39. Weller S, Blum MR, Doucette M, Burnette T, Cederberg DM, de Miranda P and Smiley ML (1993). Pharmacokinetics of the acyclovir pro-drug valaciclovir after escalating single- and multiple-dose administration to normal volunteers. *Clin. Pharmacol. Ther.* **54**:595-605.
40. Whitaker CJ, Battah SH, Forsyth MJ, Edwards C, Boyle RW and Matthews EK (2000). Photosensitization of pancreatic tumour cells by delta-aminolaevulinic acid esters. *Anticancer Drug Des.* **15**:161-70.
41. Wilson EL, Jacobs P and Dowdle EB. (1983) The secretion of plasminogen activators by human myeloid leukemic cells in vitro. *Blood*. **61**:568-574.
42. Wipf P and Li W (1994). Prodrugs of ara-C. *Drugs Future* **19**:49-54.
43. Wipf P, Li W, Adeyeye CM, Rusnak JM and Lazo JS (1996). Synthesis of chemoreversible prodrugs of ara-C with variable time-release profiles. Biological evaluation of their apoptotic activity. *Bioorg. Med. Chem*. **4**:1585-96.
44. Zimmerman CL. (1990) The disposition of cytosine arabinoside and its metabolite after single doses to rabbits. *Biopharm. Drug Dispos*. **11**:121-9.

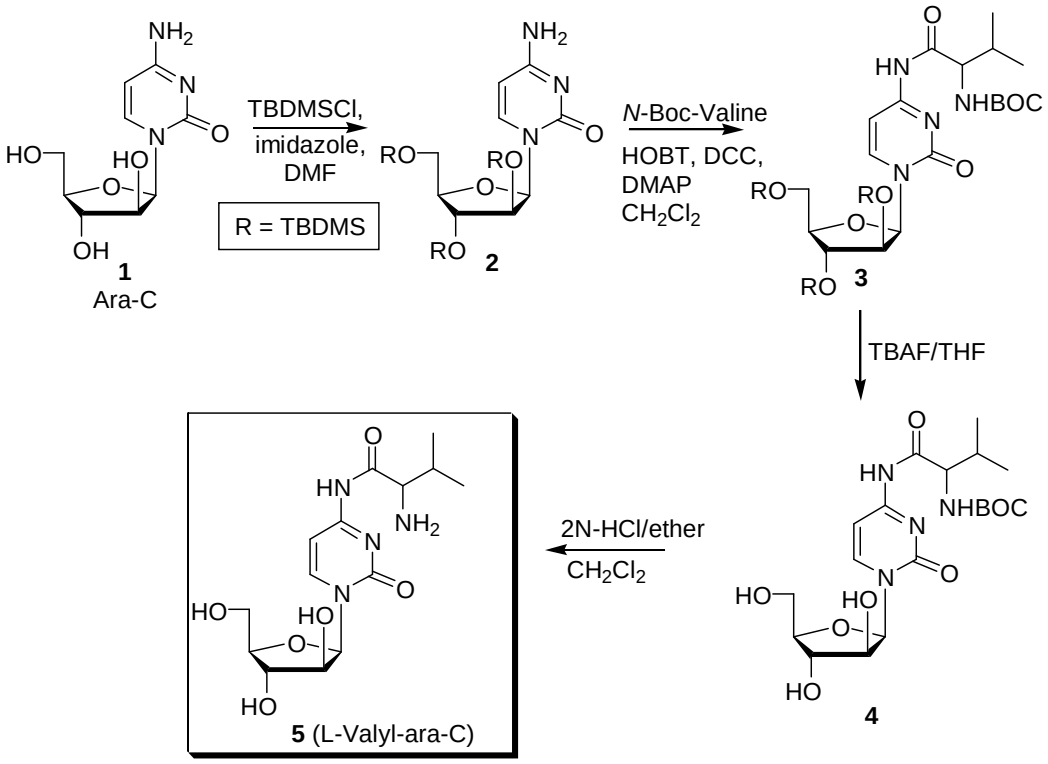

Fig. 1: Synthetic scheme of L-valyl-ara-C

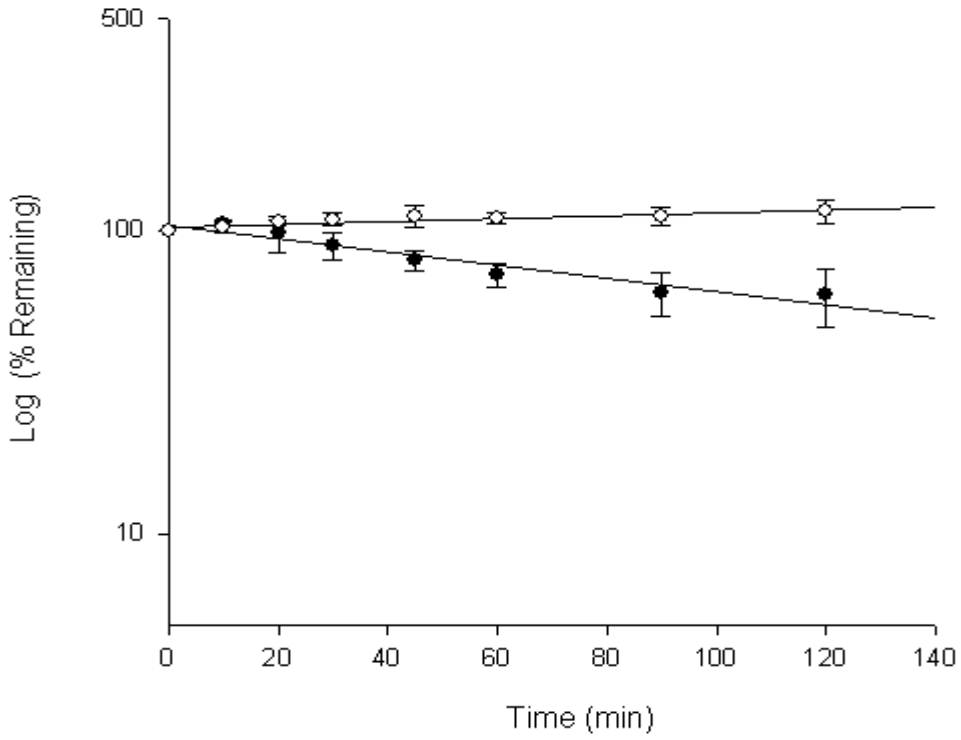

Fig. 2: In-vitro stability of L-valyl-ara-C in the artificial gastric juice and intestinal fluids (Mean±SD, n=6). Drug solution (100 µM) was incubated for 2 hrs at 37 °C. ● % remaining of L-valyl-ara-C in the artificial gastric juice, ○ % remaining of L-valyl-ara-C in the artificial intestinal fluids. The compositions of the artificial gastric juice and intestinal fluids were described under *Materials and Methods*.

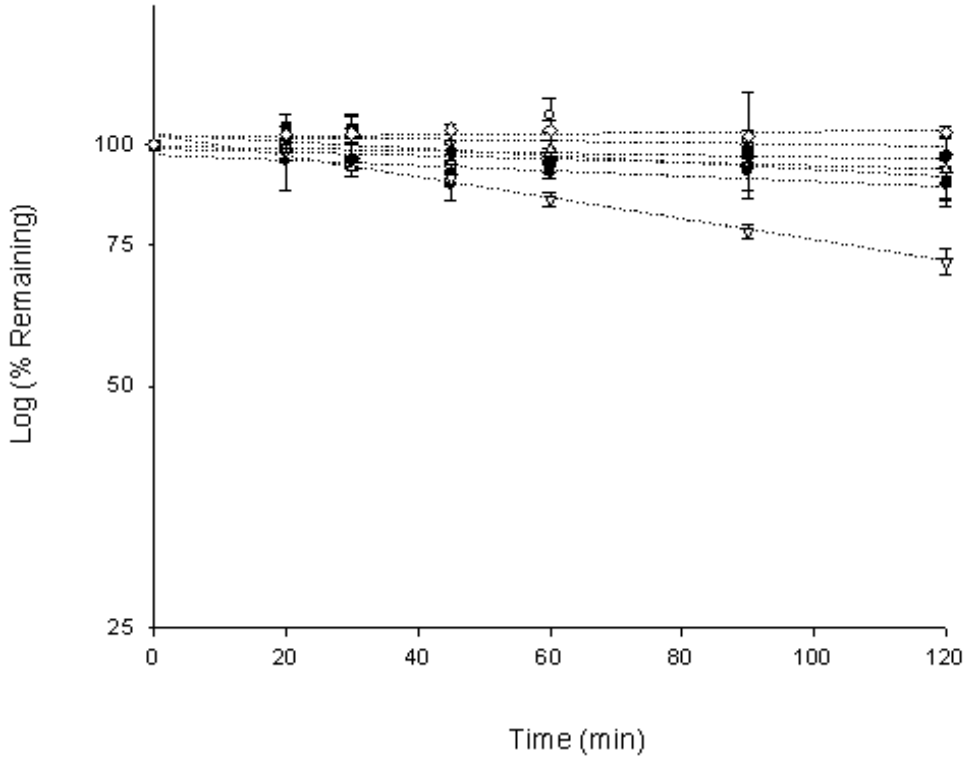

Fig. 3: In-vitro metabolic stability of L-valyl-ara-C in various conditions (Mean±SD, n=6). Drug solution (10 μM) was incubated for 2 hrs at 37 °C in fresh rat plasma (●), cell homogenates (AML2 cells (○), L1210 cells (■)), aqueous solutions (pH 2.0 (▽), pH 7.4 (◆), pH 10 (◇)) and in the presence of plasmin (△).

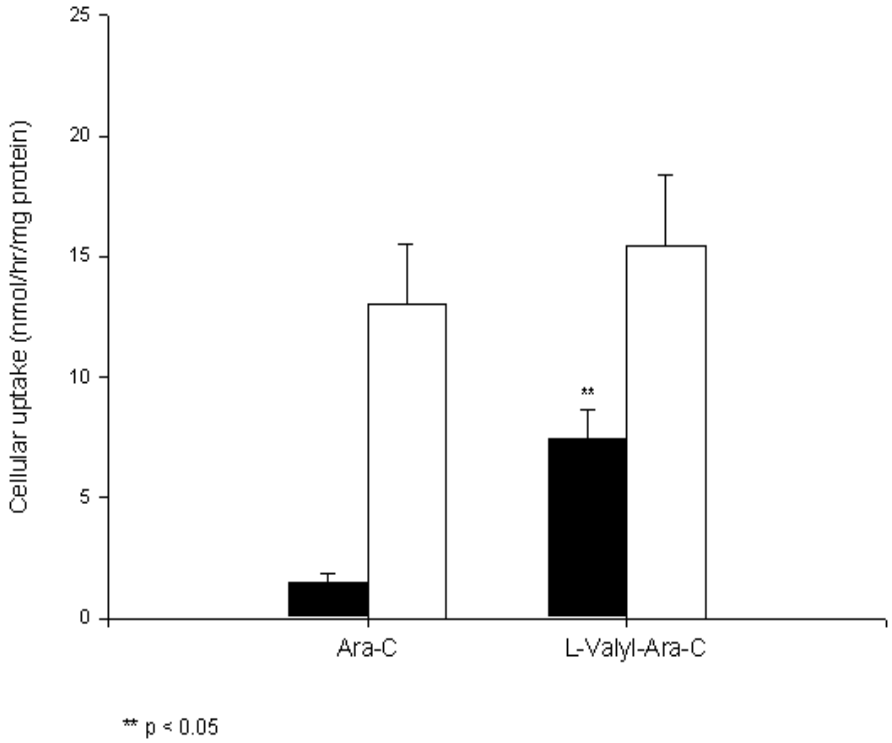

Fig. 4: Cellular uptake of ara-C and L-valyl-ara-C in Caco-2 cells (Mean±SD, n=6). ■: drug uptake at 0.4 mM, □ drug uptake at 4 mM. A *P* value was calculated to determine the statistically significant difference in the drug uptake between L-valyl-ara-C and ara-C.

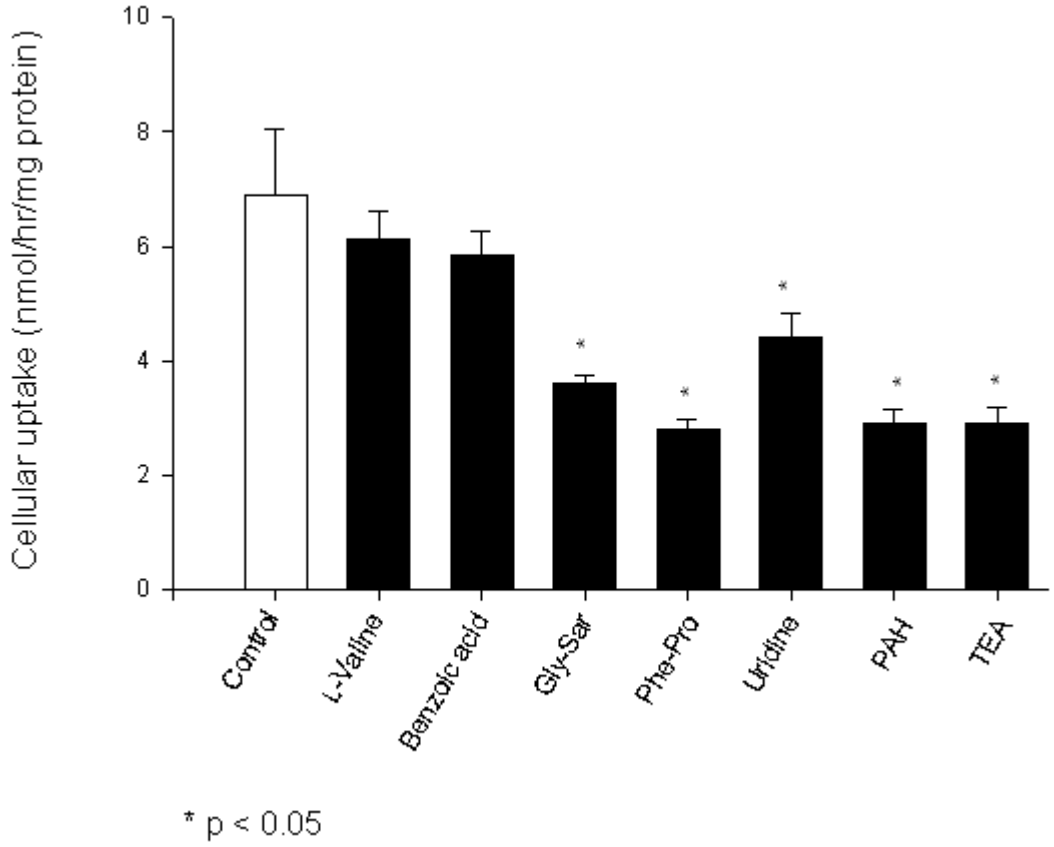

Fig. 5: Inhibition studies on the cellular uptake of L-valyl-ara-C (0.4 mM) in Caco-2 cells (Mean±SD, n = 6). □ drug uptake in the absence of inhibitors (Control), ■: drug uptake in the presence of each inhibitor. The concentration of each inhibitor was described under *Materials and Methods*. * p <0.05: compared to the control.

Table 1. Mean pharmacokinetic parameters of L-valyl-ara-C and ara-C in rats (Mean± SD, n = 4)

Drugs	Ara-C [a]	L-valyl-ara-C [b]	
Analytes	Ara-C	Ara-C	L-valyl-ara-C
C_{max} (μg/mL)	-	0.14±0.11	1.58±0.36
T_{max} (hr)	-	6.7±2.3	5.0±2.0
AUC_{inf} (μg*hr/mL)	7.29±2.84	1.01±0.92	16.1±3.81
CL ($L \cdot hr^{-1} \cdot kg^{-1}$)	0.34±0.03	-	-
V_{dss} ($L \cdot kg^{-1}$)	0.61±0.07	-	-
$T_{1/2}$ (hr)	1.5±0.5	-	11.3±2.76

[a]: IV, 2 mg/kg

[b]: PO, 10 mg/kg

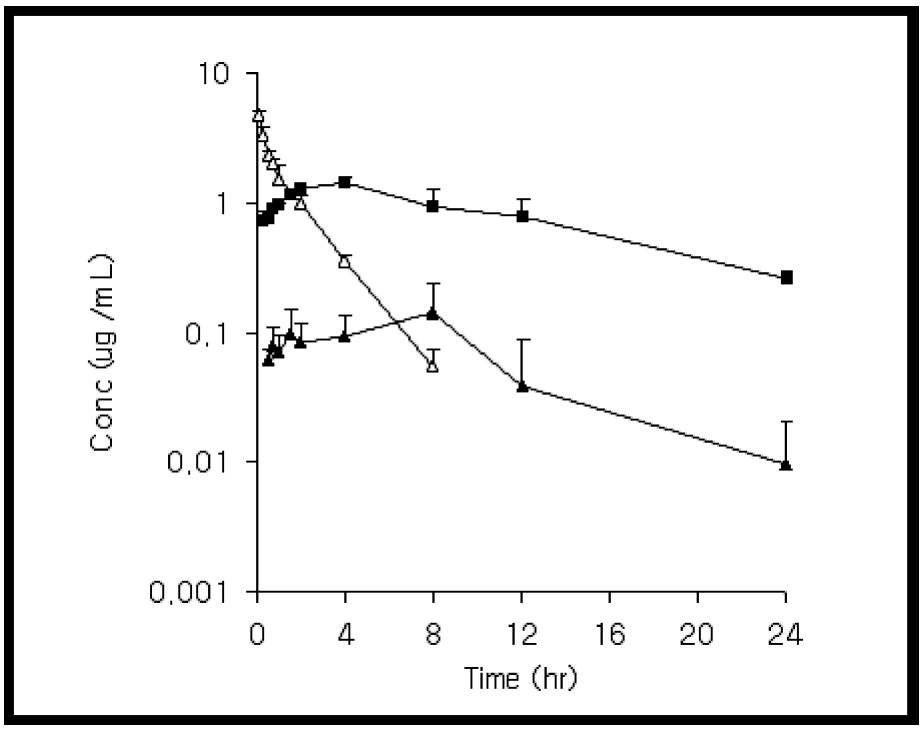

Fig. 6: Mean plasma concentration-time profiles of L-valyl-ara-C and ara-C in rats (Mean + SD, *n* = 4). (Δ; ara-C (i.v., 2 mg/kg), (■) L-valyl-ara-C (p.o., 10 mg/kg), (▲; ara-C via the oral administration of L-valyl-ara-C (10 mg/kg).

감사의 글

가장 먼저 하나님께 영광을 돌립니다.

2년간의 대학원 생활에서 부족한 저를 이끌어주시고, 힘들 때마다 격려해 주시며 때론, 따끔한 충고를 아끼지 않으신 저의 지도 교수님, 한효경 교수님께 무한한 감사와 사랑을 드립니다.

또한, 항상 뒤에서 저를 지켜봐 주신 최준식 교수님과 최후균 교수님께 감사드립니다.

저의 대학원 생활을 보다 더 즐겁고 행복하게 만들어 준 나의 선배, 오정현, 오요한, 김명길, 그리고 내 후배 서기수에게도 깊은 감사를 드립니다. 특히 동물실험에서 김선생님의 도움엔 더욱더 깊은 감사를 드립니다.

그리고 실험실에만 있는 재미없는 친구를 늘 응원해 주는, 사랑하는 나의 친구들, 정아, 시은, 성지, 라나, 희정, 민선, 그리고 지혜, 너무 고맙습니다.

마지막으로 사랑하는 나의 가족들, 부모님과 여동생 은비에게, 힘들 때 끝까지 내 편이 되어주는 그 분들께 진실로 무한한 감사와 사랑을 드립니다.

저작물 이용 허락서

학 과	약 학 과	학 번	20057077	과 정	석사
성 명	한글: 천 은 파		영문 : Cheon Eun Pa		
주 소	경남 거제시 옥포 2동 영진한솔빌라 802호				
연락처	E-MAIL :llovemyself82@hotmail.net				
논문제목	한글 :Ara-C의 경구 운반시스템으로서, peptide 유사 prodrug인 L-valyl-ara-C의 평가				
	영문 :Evaluation of a peptidomimetic prodrug, L-valyl-ara-C as an oral delivery system of ara-C				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(0) 반대( )

2007년 2월 23일

저작자: 천 은 파 (서명 또는 인)

조선대학교 총장 귀하