

Full-length article

Pharmacokinetic characteristics of *L*-valyl-ara-C and its implication on the oral delivery of ara-C¹Eun-pa CHEON, Hyo-kyung HAN²

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Key wordsara-C; *L*-valyl-ara-C; prodrug; pharmacokinetics; rats

¹ Project supported by the Basic Research Program of Korea Science and Engineering Foundation (KOSEF) (No. R012004000-1001302005).

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Received 2006-05-04

Accepted 2006-08-11

doi: 10.1111/j.1745-7254.2007.00474.x

Abstract

Aim: To evaluate the pharmacokinetic characteristics of *L*-valyl-ara-C, a peptidomimetic prodrug of ara-C. **Methods:** After the synthesis of *L*-valyl-ara-C, the *in vitro* stability of *L*-valyl-ara-C was examined in various biological media. Plasma pharmacokinetic profiles of ara-C and *L*-valyl-ara-C were also evaluated in rats. **Results:** The degradation of *L*-valyl-ara-C was negligible in fresh plasma and also in the presence of plasmin over a 2 h incubation period. 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 chemical hydrolysis of *L*-valyl-ara-C was rather accelerated in acidic pH. 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 prodrug administration. **Conclusion:** 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.

Introduction

The clinical utility of ara-C, one of the most effective drugs against the acute myeloid leukemia, acute lymphoblastic leukemia and other hematological malignancies^[1,2], is severely limited by rapid deamination primarily in the liver, spleen and gastrointestinal mucosa^[3,4]. 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^[3,4]. Therefore, considerable efforts have been directed at an enhancement of the therapeutic efficacy of ara-C, and many prodrug strategies have been explored for the oral delivery of ara-C with varied degrees of success^[5–8]. However, few have led to an approved product and thus, there continues to be a great need for improved methods to deliver pharmacologically active ara-C.

Recently, our group synthesized the *L*-valyl-ara-C by masking the N4-amino group of the cytosine ring in ara-C with *L*-valine in order to enhance the intestinal absorption of ara-C, and evaluated the cellular uptake characteristics of

L-valyl-ara-C in Caco-2 cells^[9]. Compared to ara-C, *L*-valyl-ara-C appeared to be stable in the intestinal lumen and exhibited 5 fold higher cellular uptake via the carrier-mediated transport pathways. Those results indicated that *L*-valyl-ara-C could be a promising candidate for the oral delivery of ara-C. Therefore, the present study aimed to examine the pharmacokinetic characteristics of *L*-valyl-ara-C in rats and evaluate its utility as a potential oral delivery system of ara-C *in vivo*.

Materials and methods

Materials Ara-C, acyclovir and 5-bromo-2'-deoxyuridine were purchased from Sigma Chemical Co (St Louis, MO, USA). Acetonitrile and methanol were obtained from Merck Co (Darmstadt, Germany). All other chemicals were of reagent grade and all solvents were of HPLC grade. AML2 and L1210 cells were purchased from Korean Cell Line Bank (Seoul, Korea).

Cells The L1210 cells were routinely maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and

penicillin (100 U/mL)/streptomycin (50 mg/mL). The AML-2 cells were grown in DMEM supplemented with 10% FBS and penicillin (100 U/mL)/streptomycin (50 mg/mL). All of the cells were maintained in an atmosphere of 5% CO₂ and 90% relative humidity at 37 °C.

Synthesis of *L*-valyl-ara-C Prodrug was synthesized as previously described by Cheon *et al*^[9], and identified by the ¹H-NMR, ¹³C-NMR and EI-MS spectrometers.

***In vitro* stability study** Stabilities of *L*-valyl-ara-C were evaluated at 37 °C by incubating a drug solution (10 μmol/L) 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. Plasmin 30 μL (10 Cu/mL) was added to the thermostated buffer composed of 50 mmol/L Tris-HCl, pH 7.4 and 110 mmol/L NaCl, followed by the addition of the drug solution (10 μmol/L) 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 r/min 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 pH (2.0, 7.4 and 10).

Antiproliferative assays Cells were resuspended at a density of 1 × 10⁵ cells/mL in growth medium containing serial dilutions of test drugs. Cell viability was determined after 96 h 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*^[10]. Cell growth at each drug solution was expressed as percentage of untreated controls, and IC₅₀ 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 (N₀ 322-7-1) purchased from Superfeed Co (Gangwon, Korea) and tap water ad libitum. All animal studies were performed in accordance 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. The animals were kept in these facilities for at least 1 week before the experiment and fasted for 24 h prior to the experiment. At the experiment, the rats (*n*=4 per each treatment) were given 10 mg/kg of *L*-valyl-ara-C orally or 2 mg/kg of ara-C intravenously. The 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 h post-dose and then centrifuged at 3000 r/min for 10 min to obtain the plasma for the HPLC assay. All samples were stored at -70 °C 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, consisting of a pump (LC-10AD), an automatic injector (SIL-10A) and a UV detector (SPD-10A; Shimadzu Scientific Instruments, Japan), was set at 272 nm. An octadecylsilane column (Gemini C18, 4.6 mm × 250 mm, 5 μm; Phenomenex, Torrance, CA, USA) was eluted with a mobile phase at a flow rate of 1.0 mL/min. The mobile phase consisted of 0.01 mol/L ammonium acetate buffer (pH 6.5): acetonitrile (93:7, *v/v*) for *L*-valyl-ara-C and 0.01 mol/L 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 limit of detection was 0.01 μg/mL.

Pharmacokinetic analysis Non-compartmental pharmacokinetic analysis was performed using WinNonlin® (Pharsight Corp, CA, USA). The area under the plasma concentration-time curve (AUC) was calculated using the linear trapezoidal method. The maximum plasma concentration (*C*_{max}) and the time to reach the maximum plasma concentration (*T*_{max}) were observed values from the experimental data. The terminal elimination rate constant (*λ*_z) was estimated from the slope of the terminal phase of the log plasma concentration-time points fitted by the method of least-squares, and then the terminal elimination half-life (*T*_{1/2}) was calculated as 0.693/*λ*_z. Additional estimated parameters using noncompartmental pharmacokinetic analysis were systemic plasma clearance (CL) and the volume of distribution (*V*_{dss}).

Statistical analysis All the means are presented with their standard deviation. An unpaired Student's *t*-test was used to determine the significant difference between treatments. A *P* value <0.05 was considered statistically significant.

Results

***In vitro* metabolic stability studies** The kinetics of *L*-valyl-ara-C degradation were studied in various conditions. As shown in Figure 1, *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 h incubation period. In addition, the metabolic stability of *L*-valyl-ara-C

was assessed using pure serine protease plasmin, and the 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 pH (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 h in the aqueous solution of pH 2.0 (Figure 1).

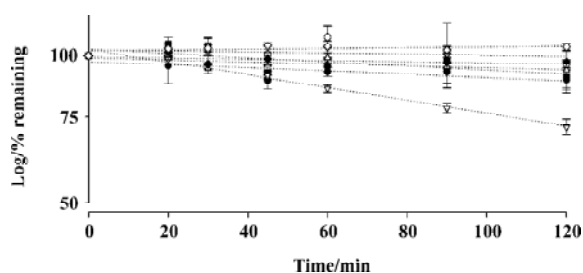


Figure 1. *In vitro* metabolic stability of *L*-valyl-ara-C in various conditions (mean \pm SD, $n=6$). The drug solution (10 μ mol/L) was incubated for 2 h at 37 $^{\circ}$ C in fresh rat plasma (●), cell homogenates [AML2 cells (○), L1210 cells (■)], aqueous solutions [pH 2.0 (▽), pH 7.4 (◆), pH 10 (◇)] and in the presence of plasmin (△).

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 \pm 1.11 and 606.0 \pm 87.3 μ mol/L for ara-C and *L*-valyl-ara-C, respectively in the AML-2 cells. In the case of the L1210 cells, the IC₅₀ values were 0.25 \pm 0.03 and 37.5 \pm 6.69 μ mol/L for ara-C and *L*-valyl-ara-C, respectively. In both types of cells, the antiproliferative activity of *L*-valyl-ara-C was approximately 150 fold less potent than 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 and Figure 2. After an oral administration of *L*-valyl-ara-C, the release of ara-C from the prodrug was observed in the 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 the rats.

Discussion

The development of oral alternatives to intravenous administration of ara-C would not only avert the high costs dictated by hospital treatments, but also be more patient friendly. In addition, it may also be possible to incorporate prodrug strategies that could reduce undesirable side effects such as toxicity, and improve therapeutic action into

Table 1. Mean pharmacokinetic parameters of *L*-valyl-ara-C and ara-C in rats (mean \pm SD, $n=4$).

Drugs Analytes	Ara-C ^a	<i>L</i> -valyl-ara-C ^b	
	Ara-C	Ara-C	<i>L</i> -valyl-ara-C
C_{max} (μ g/mL)	–	0.14 \pm 0.11	1.58 \pm 0.36
T_{max} (h)	–	6.7 \pm 2.3	5.0 \pm 2.0
AUC _{inf} (μ g·h/mL)	7.29 \pm 2.84	1.01 \pm 0.92	16.10 \pm 3.81
CL (L·h ⁻¹ ·kg ⁻¹)	0.34 \pm 0.03	–	–
V_{dss} (L·kg ⁻¹)	0.61 \pm 0.07	–	–
$T_{1/2}$ (h)	1.5 \pm 0.5	–	11.30 \pm 2.76

^aiv, 2 mg/kg; ^bpo, 10 mg/kg.

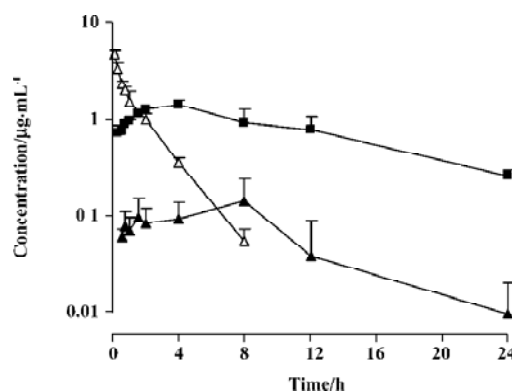


Figure 2. Mean plasma concentration-time profiles of *L*-valyl-ara-C and ara-C in rats (mean \pm SD, $n=4$). (△) ara-C (iv, 2 mg/kg); (■) *L*-valyl-ara-C (po, 10 mg/kg); (▲) ara-C via the oral administration of *L*-valyl-ara-C (10 mg/kg).

such oral alternatives. In our previous studies, *L*-valyl-ara-C appeared to be beneficial in enhancing the cellular uptake of ara-C in Caco-2 cells^[9]. However, in the present study, 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.

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^[11–13]. Leukemic cells also secrete these enzymes^[14]. Since plasmin rapidly cleaves various low molecular weight compounds coupled to appropriate peptide specifiers, the coupling of such peptide specifiers to anti-

cancer drugs might create 'prodrugs' which would be locally activated by tumor-associated plasmin and consequently would be less toxic to normal cells. For example, Carl *et al*^[15] reported that the peptide prodrugs of several anti-cancer agents designed to be specific plasmin substrates showed selective cytotoxicity. Since *L*-valyl-ara-C is a peptidomimetic prodrug and appears 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 Figure 1, *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 have indicated that *L*-valyl-ara-C is 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^[16], chemical hydrolysis of *L*-valyl-ara-C may be more favorable in tumor cells.

As summarized in Table 1, following an intravenous administration of ara-C to rats, ara-C showed a short plasma half-life of 1.5 h, and its volume of distribution was greater than total body water in the rats^[17]. The pharmacokinetic parameters of ara-C obtained from the present study appeared to be comparable to those from previous studies^[18–20]. Following an oral administration of *L*-valyl-ara-C to the rats, the appearance of ara-C was observed in the plasma, implying that the prodrug conversion to the parent could occur by intestinal or hepatic metabolism (Figure 2). *L*-valyl-ara-C is a peptidyl derivative of ara-C and thus, during intestinal absorption, it may interact with peptidases which catalyze the hydrolysis of peptide bonds in peptidyl derivatives or the hydrolysis of various simple amides^[21]. 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 the 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 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.

In conclusion, 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 the rats.

Acknowledgement

The authors wish to express their appreciation to Mr Ming-ji JIN for his help with the animal experiment.

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The 7th Chinese Symposium on Calcium Signalling (The 7th CSCS Meeting)

October/November 2008, Wuhan, China

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