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Synthesis and In-vitro Evaluation of N4-Amino Acid Derivatives of Cytarabine for Improving the Oral Delivery of Cytarabine

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ABSTRACT – The present study aimed to investigate the in-vitro characteristics of N4-amino acid derivatives of cytarabine for the oral delivery of cytarabine. After the synthesis of L-Ile-cytarabine, L-Leu-cytarabine and L-Arg-cytarabine, the gastrointestinal stability of each prodrug was examined using artificial gastric juice and intestinal fluids. The cellular uptake characteristics of prodrugs were also examined in Caco-2 cells. While L-Ile-cytarabine and L-Leu-cytarabine appeared to be stable in all the tested biological media during 4-hr incubation, L-Arg-cytarabine was rapidly disappeared within 5 min. Accordingly, the cellular uptake of L-Ile-cytarabine and L-Leu-cytarabine was significantly higher than that of its parent drug, cytarabine in Caco-2 cells but the cellular uptake of L-Arg-cytarabine was similar to that from its parent drug. The cellular uptake of L-Ile-cytarabine and L-Leu-cytarabine appeared to be saturable as drug concentration increased from 0.4 to 4 mM. Collectively, L-Ile-cytarabine and L-Leu-cytarabine could be promising candidates to improve the oral absorption of cytarabine via a saturable transport pathway.

Key words – Cytarabine, Amino acid derivatives, Prodrug, Gastrointestinal stability, Cellular uptake

Cytarabine is one of the most effective drugs used in the treatment of acute myeloid leukaemia, acute lymphoblastic leukaemia and other haematological malignancies.[1,2] In combination with other antitumor agents it is also used against solid tumors.[2] However, its clinical utility is severely limited by a very short plasma half-life and low systemic exposure that are mainly caused by the rapid deamination of cytarabine to the biologically inactive 1-β-D-arabinofuranosyluracil in the liver, spleen and gastrointestinal mucosa.[3,4] Consequently, many prodrug strategies have been explored to avoid the deamination and also to enhance the cellular uptake of cytarabine, but few have led to an approved product.[5,8]

The intestinal peptide transporter (Pept1) plays an important role in transporting dietary peptides as well as pharmacologically active peptidomimetic drugs.[9,10] 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. In previous studies, peptidyl prodrugs targeting the peptide transporter have been successful in improving the bioavailability of poorly absorbable drugs such as α-methyl-dopa, acyclovir, ganciclovir and gemcitabine.[11-14] Furthermore, Cheon et al.[15] have reported that L-valyl derivative of cytarabine was effective to improve the cellular uptake of cytarabine but unfortunately, its metabolic reconversion to the parent drug was not optimal as a potential oral delivery system of cytarabine. Therefore, in order to identify the optimal prodrug of cytarabine, the present study synthesized three N4-amino acid derivatives of cytarabine and evaluated their in-vitro characteristics.

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Materials and Methods

Materials

Cytarabine, small dipeptides, amino acids, acyclovir, 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). Fetal Bovine Serum (FBS), cell culture media, antibiotics and all other reagents used in cell culture studies were purchased from Seolin Science Co. (Seoul, Korea). Caco-2 cells were purchased from ATCC (Rockville, MD, USA). All other chemicals were reagent grade and all solvents were HPLC grade.

Cells

Caco-2 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 1% non-essential amino acids, 1 mM sodium pyruvate, 1% L-glutamine and penicillin (100 U/mL)/streptomycin (100 mg/mL). All cells were maintained in an atmosphere of 5% CO_2 and 90% rel-

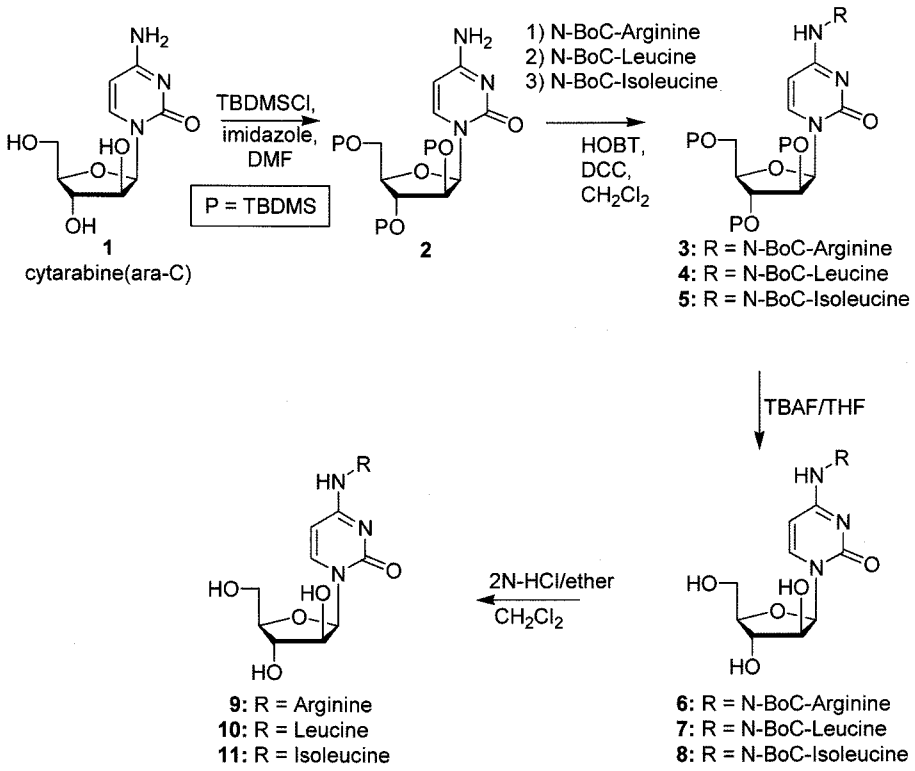

Figure 1—Synthetic scheme for N4-derivatives of cytarabine.

ative humidity at 37°C.

Synthesis of N4-prodrugs of cytarabine

N-amino acid prodrugs of cytarabine (**9-11**) were readily synthesized from cytarabine (**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 each *N*-BOC-amino acid (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 **3-5**, which were purified by column chromatography on silica gel 60 (CH_2Cl_2 : CH_3OH = 10 : 1). Treatment of **3-5** (0.636 mmol) with tetrabutylammonium fluoride (3.18 mL, 1.0 M in THF) in THF (10 mL) provided compound **6-8**, which were purified using column chromatography on silica gel 60 (CH_2Cl_2 : CH_3OH = 7 : 1). Deblocking of BOC group of **6-8** (0.9 mmol) with ethereal hydrochloric 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 compounds (**9-11**).

Gastrointestinal stability study

Gastrointestinal stability of prodrugs 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). At each time point, 100 μL of sample was collected and the metabolic reaction was stopped by adding 200 μL of 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.

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 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 5 mM D-glucose and 5 mM MES. The initial uptake rates of prodrugs and cytarabine 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, 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 prodrugs in donor solutions above the apical membrane of Caco-2 cell monolayer was also examined during the uptake studies to determine the extent of degradation of prodrugs when in contact with Caco-2 monolayer.

HPLC Assay

Drug concentrations were determined by a HPLC assay described as follows. Acyclovir and 5-bromo-2'-deoxyuridine (BDU) were used as the internal standard for the assay of cytarabine and prodrugs, respectively. The chromatographic system consisted of a pump (LC-10AD) and an automatic injector (SIL-10A). A UV detector (SPD-10A) (Shimadzu Scientific Instruments, Japan) set at 240 nm for L-leu-cytarabine or 272 nm for cytarabine, L-Ile-cytarabine and L-Arg-cytarabine. 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. The mobile phase was 0.01 M ammonium acetate buffer (pH 6.5) containing 9~30% acetonitrile for prodrugs and 0.01 M ammonium acetate buffer (pH 4.5) containing 1% acetonitrile for cytarabine. 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.

Statistical analysis

All the means were presented with their standard deviation. Statistical analysis was performed using Student's t-test or 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.

Results and Discussion

Synthesis of N4-prodrugs of cytarabine

To reduce the rapid deamination of cytarabine, N4-prodrugs of cytarabine were synthesized by masking the N4-amino group of a cytosine ring with amino acids (L-isoleucine, L-leucine or L-arginine) as illustrated in Fig. 1. N4-prodrugs were obtained as white fluffy powders with the purity greater than 98% as determined by HPLC. The identities of prodrugs were confirmed by H^1-NMR as follows.

L-Arg-cytarabine

yield 10%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.67 (d, J= 7.2 Hz, 1H), 7.22 (d, J=7.2 Hz, 1H), 6.21 (d, J=2.4 Hz, 1H), 5.17 (br s, 2H), 4.98 (br s, 1H), 4.32 (d, J=3.3 Hz, 1H), 4.21 (s, 1H), 4.05 (d, J=3.2 Hz, 1H), 3.91 (m, 1H), 3.70-3.61 (m, 2H), 1.70-1.62 (m, 2H), 1.58-1.53 (m, 4H).

L-Ile-cytarabine

yield 24%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.70 (d, J= 6.0 Hz, 1H), 7.52 (d, J=6.0 Hz, 1H), 5.99 (d, J=3.0 Hz, 1H), 5.52 (s, 2H), 5.28 (s, 1H), 4.54 (s, 1H), 4.37 (s, 1H), 4.12 (s, 1H), 3.98 (s, d, J=6.8 Hz, 1H), 3.65 (m, 2H), 2.93 (m, 2H), 1.94 (m, 1H), 0.97 (m, 5H), 0.82 (m, 3H).

L-Leu-cytarabine

yield 22%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.06 (d, J= 7.2 Hz, 1H), 7.17 (d, J=7.2 Hz, 1H), 6.02 (d, J=7.2 Hz, 1H), 5.66 (s, 1H), 5.56 (s, 2H), 5.28 (s, 1H), 4.22 (s, 1H), 4.05 (s, 1H), 3.90 (m, 1H), 3.66-3.57 (m, 2H), 2.90 (m, 2H), 1.97 (m, 1H), 0.95 (m, 6H).

Gastrointestinal stability of N4-prodrugs

The gastrointestinal stability of prodrugs was examined by using the artificial digestives. As shown in Fig. 2 and 3, L-Ile-

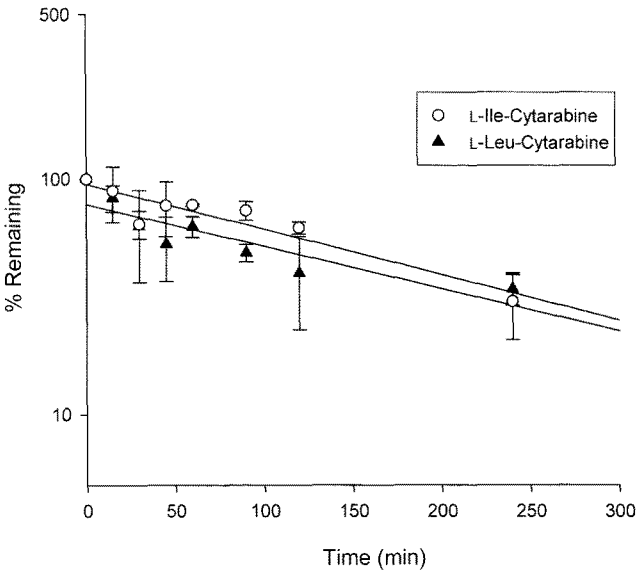

Figure 2—In-vitro stability of cytarabine prodrugs in the artificial gastric juice (Mean±S.D., n=3).

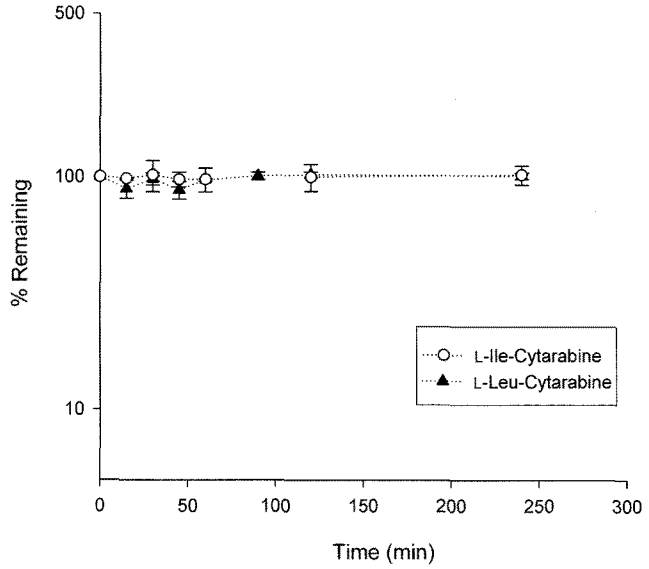

Figure 3—In-vitro stability of cytarabine prodrugs in the artificial intestinal fluids (Mean ± S.D., n=3).

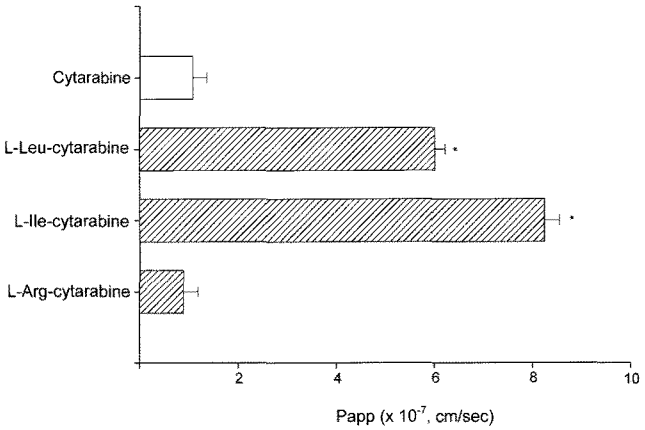

Figure 4—Cellular uptake of cytarabine and its prodrugs in Caco-2 cells (Mean ± S.D., n=5~6). *: $p<0.05$, compared to the parent drug.

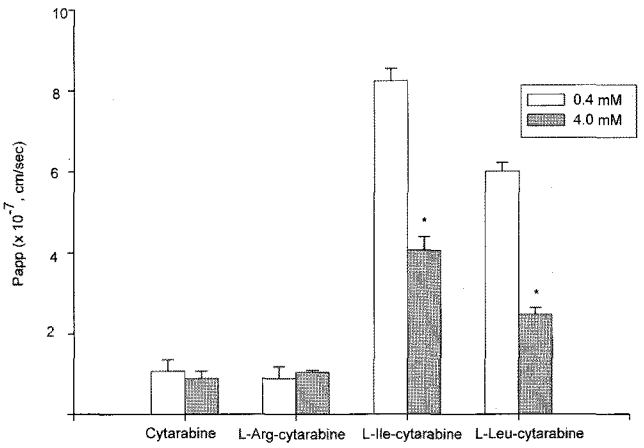

Figure 5—Concentration dependency in membrane permeabilities of cytarabine and its prodrugs in Caco-2 cells (Mean ± S.D., n=5~6). *: $p<0.05$ for the comparison between 0.4 mM and 4 mM.

cytarabine and L-Leu-cytarabine appeared to be more stable in the artificial intestinal fluid than in the gastric juice. The disappearance half-life of L-Ile-cytarabine and L-Leu-cytarabine was about 2.5~3.0 hrs in the artificial gastric juice. Considering that orally administered solid preparations were transferred to the small intestine within 1 hr after administration under the fasted condition,[16] the stability of L-Ile-cytarabine and L-Leu-cytarabine in gastric juice appeared to be appropriate for the oral delivery of cytarabine. The degradation of L-Ile-cytarabine and L-Leu-cytarabine was negligible in the artificial intestinal fluid over the 4 hr-incubation. In contrast to L-Ile-cytarabine and L-Leu-cytarabine, L-Arg-cytarabine was rapidly disappeared within 5 min in all the tested biological media. Therefore, the utility of L-Arg-cytarabine as a prodrug for the oral delivery of cytarabine should be minimal. Collectively, the in-vitro stability studies indicated that L-Ile-cytarabine and L-Leu-cytarabine could be stable in the intestinal lumen after the oral administration.

Cellular uptake studies

The cellular uptake characteristics of prodrugs as well as parent drug were evaluated in Caco-2 cells. As expected from the metabolic instability in artificial digestives, L-Arg-cytarabine was rapidly converted to its parent drug before the permeation across the apical membrane of Caco-2 cells, so that the cellular uptake profile of L-Arg-cytarabine should be similar to that of its parent drug. Indeed, as shown in Fig. 4, the cellular uptake of L-Arg-cytarabine was similar to that from its parent drug while L-Ile-cytarabine and L-Leu-cytarabine appeared to be 6~8 folds more permeable across the apical membrane of Caco-2 cells compared to cytarabine. In addition, the membrane permeability of L-Arg-cytarabine and cytarabine was not changed regardless of the increase of drug concentration, while the permeability of L-Ile-cytarabine and L-Leu-cytarabine decreased significantly as drug concentration increased from 0.4 to 4 mM. This result suggests that saturable transport pathways may involve in the cellular uptake of L-Ile-cytarabine and L-Leu-cytarabine while the passive diffusion could be predominant during the incubation of L-Arg-cytarabine and cytarabine. Taken all together, L-Ile-cytarabine and L-Leu-cytarabine exhibited the appropriate gastrointestinal stability and much greater cellular accumulation compared to the parent drug.

Conclusion

L-Ile-cytarabine and L-Leu-cytarabine could be promising candidates to improve the oral absorption of cytarabine via saturable transport pathways.

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