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博士學位論文

약물수송체를 활용한 항암제의 생체이용률 증가에 관한 연구

朝 鮮 大 學 校 大 學 院

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Enhanced oral exposure of anticancer drugs via the utilization of drug transporters

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김명길의 박사학위논문을 인준함

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감사의 글

가장 먼저 저의 대학원 생활에서 부족한 저를 이끌어주시고 많은 가르침을 주시고 또 힘들 때 격려해주시고 실수할 때는 따끔한 충고로 저를 올바르게 인도해주신 지도교수님 한효경 교수님께 무한한 감사와 사랑을 드립니다.

또한 제가 졸업할 때까지 항상 지켜봐주신 최준식교수님, 신상철교수님, 최후균교수님, 홍준희교수님께 감사를 드립니다.

저의 대학원생활에서 같이 실험실에서 공부하며 어려울 때 도와주고 즐거울 때 같이 웃어준 우리 실험실 친구들 부강, 서기수, 임영빈, 고운정, 세진이 그리고 약대대학원에서 처음부터 끝까지 나하고 같이 해준 약제학실의 박영길 선생한테도 깊은 감사를 드립니다

그리고 학교에서의 대학원 생활에서 내가 힘들거나 외로울 때 항상 나에게 기쁨과 자신감을 준 우리 화광축구팀의 선수들한테 진심으로 감사드립니다.

마지막으로 저를 힘들게 키우시는라 수고하신 아버님 어머님한테 저의 영광을 드리고 언제나 항상 나를 믿고 지지해준 아내 원윤홍씨 한테 감사드리고 곧 출생하게 될 나의 아기한테도 이 영광을 같이 나누겠습니다.

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김 명 길

지 도 교 수: 한 효 경

조선대학교 대학원 약학과

본 연구는 주로 항암제의 체내 흡수를 증가시키고 체내 체류시간을 늘여서 항암제의 치료효과를 높이는것을 목표로 수행되었다. 이를 위해 cytarabine과 methotrexate(MTX)의 구조적특성, 생체내에서의 흡수기전 그리고 다른 약물과의 상호작용 등을 이용하여 이들 항암제의 약물동태학적 특성을 개선하고자 하였다.

첫 번째 모델약물인 cytarabine 은 가장 널리 사용되는 골수 백혈병 치료제로서 급성 백혈병, 림프 백혈병 그리고 기타 여러 가지 암에도 많이 쓰이는 암 치료제이다. 그러나 cytarabine 은 체내에서의 분포농도가 낮고 반감기도 짧기 때문에 임상에서 사용할때에 여러가지로 많은 제약이 따른다. 이러한 약동학적 결함은 cytarabine 이 인체 내에서 탈 아미노반응으로 불활성의 1-β-D-arabinofuranosyluracil 을 형성하기 때문이며, 이러한 탈아미노반응은 주요 대사기관인 간뿐만 아니라 위장관내에서도 신속하게 일어나는 것으로 보고되어있다. 따라서 본 연구에서는 이러한 탈아미노반응이 cytarabine의 생체이용률에 미치는 영향을 최소화하기 위하여 cytarabine 의 N_4-amino 그룹에 서로 다른 아미노산을 붙여서 peptidyl prodrugs를 합성하였다. 이러한 prodrug 합성을 통하여 경구투여시 위장관 및 간에서의 약물의 안정성을 증가시키고 궁극적으로는 cytarabine의 혈중농도를 증가시키고자 하였다. 소장내에는 많은 수송체가 존재한다. 그중에 peptide transporter(Pept1) 가 있는데 Pept1은 펩티드 구조를 가진 약물의 수송에 적극적으로 관여한다. 따라서 본 연구에서는 펩티드 구조와 유사한 prodrugs를 합성하였고 이러한 구조적 특성을 이용하여 소장내에서의 prodrugs의 흡수를 개선하고자 하였다.

두 번째 모델약물은 MTX로서 MTX는 급성백혈병, 골육종 등을 포함한 항암치료제로 널리 사용되고 있다. 또한 기타 비악성종양, 건선 등에도 많이 쓰인다. MTX를 다른 약물 혹은 천연식물 추출물 등과 같이 투여했을 경우 MTX의 신 배설이 억제되어 체내 약물농도가 높아진다는것이 보고된바 있다. 최근에는 천연약물에서 추출한 flavonoids가 인체에 유익한 약물로 관심을 끌고 있는데, flavonoids의 하나인 morin은 비교적 독성이 없고 안정한 약물이며 또 임상에서 항알레르기, 항염증, 항암효과 등 다양한 약리작용을 나타내고있다. 뿐만아니라 morin 은 이미 딜티아젬, 파크리탁셀 등의 약물들과 병용투여시 이 약물들의 소장내 흡수를 증가시키는 것으로 보고된바 있다. 그리고 최근에는 morin이 MDCK Cells에서 organic anion transporters(OATs)의 활성을 억제하는 작용이 있음이 본연구진에 의해서 밝혀졌다. 또한 MTX도 신장의 세뇨관에서 OAT에 의해 수송된다는것이 알려져있다. 따라서 MTX와 morin은 모두 신장에 분포하는 OAT와 상호작용이 가능하므로 본 연구에서는 morin에 의한 OATs의 활성억제를 통해 MTX의 신배설을 감소시키고 궁극적으로는 MTX의 혈중농도를 증가시키는 방법을 연구하였다.

Abstract

Enhanced oral exposure of anticacer drugs via the utilization of drug transporters

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This research aimed to improve the pharmacokinetic characteristics of anticancer drugs via the utilization of drug transporters such as (i) prodrug design targeting the peptide transporters and (ii) modulation of organic anion transporters-mediated renal excretion.

(1) Prodrug design targeting the peptide transporters: This 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-Leu-cytarabine, L-Ile-cytarabine and L-Arg-cytarabine, the gastrointestinal stability of each prodrugs was examined using artificial gastric juice and intestinal fluids. The cellular uptake characteristics of prodrugs were also examined in Caco-2 cells. While L-Leu-cytarabine and L-Ile-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-Leu-cytarabine and L-Ile-cytarabine was significantly higher than that of its parent drug, cytarbine in Caco-2 cells but the cellular uptake of L-Arg-ctarabine 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 0.4mM. Collectively, L-Ile-cytarabine and L-Leu-cytarabine could be

promising candidates to improve the oral absorption of cytarabine via a saturable transport pathway. Therefore pharmacokinetic profiles of L-Ile-cytarabine was investigated in rats. The AUC of L-Ile-cytarabine was approximately 1.8-fold higher than that from cytarabine but the appearance of cytarabine was not observed after an oral administration of L-Ile-cytarabine. In conclusion, L-Ile-cytarabine and L-Leu-cytarabine may improve the intestinal absorption of cytarabine via the carrrier-mediated transport pathway but their utility as an oral delivery system of cytarabine could be limited by the low metabolic conversion to cytarabine.

(2) Modulation of organic anion transporter-mediated renal excretion: This study aimed to investigate the effect of morin on the pharmacokinetics of methotrexate (MTX) in rats. Pharmacokinetic parameters of MTX were determined in rats following an intravenous administration of MTX(2mg/kg) in the presence and the absence of morin (25mg/kg, po.). The cellular accumulation of MTX was also examined by using MDCK cells stably overexpressing hOAT1 or hOAT3. Compared with the control given MTX alone, pretreatment with morin 15min prior to MTX administration significantly altered the pharmacokinetics of MTX in rats. Renal clearance and total clearance of MTX were reduced by 42% and 58%, respectively, in the presence of morin. Accordingly, the systemic exposure of MTX in the rats pretreated with morin was significantly higher than that from the control group. The mean residence time (MRT) and terminal plasma half-life of MTX were prolonged by 3.3-and 2.4-fold, respectively, by the concurrent use of morin. The cellular uptake of MTX (20uM) was significantly reduced by the co-incubation with morin (100uM) in MDCK-hOAT1 cells but not in MDCK-hOAT3 cells. Taken together, morin appeared to be effective in altering the pharmacokinetics of MTX in rats, likely by the inhibition of OAT1-mediated renal excretion.

Part1

국문초록

Cytarabine 의 생체이용률 개선을 위한 유도체의 합성 및 특성 평가

본 연구의 목적은 cytarabine의 경구운반시스템으로서 cytarabine의 peptidyl prodrugs인 L-Leu-cytarabine, L-Ile-cytarabine, L-Arg-cytarabine를 합성하고 그 특성을 평가하는것이다. L-Leucine, L-Isoleucine, L-Arginine 을 각각 cytosine ring의 N4-amino group에 삽입하여 prodrugs을 합성한후 다양한 biological media에서 in-vitro 안정성을 측정하였고, Caco-2 cell에서 세포내 흡수 특성에 대해 조사하였다. 또한 실험용 흰쥐에서 cytarabine 과 L-Ile-cytarabine의 약물 동태학적 특성도 평가하였다. 인공위액에서 L-Leu-cytarabine, L-Ile-cytarabine의 반감기는 2-3시간 정도인 반면에 L-Arg-cytarabine는 5분이내에 모든 약물이 신속하게 분해됨을 알수 있었다. 인공장액, cell homogenates, fresh plasma에서는 4시간의 incubation에도 L-Ile-cytarabine와 L-Leu-cytarabine는 거의 분해가 일어나지 않는 안정한 상태를 나타냈다. 그리고 Caco-2 cell에서 세포내 축적은 L-Leu-cytarabine, L-Ile-cytarabine는 cytarabine보다 훨씬 높게 증가하였으나 L-Arg-cytarabine는 parent drug 인 cytarabine과 별로 차이가 없었다. 또한 L-Leu-cytarabine와 L-Ile-cytarabine 는 약물농도가 0.4mM에서 4mM로 증가함에 따라 세포내 유입이 포화되는 경향을 보였으나 L-Arg-cytarabine는 세포내 유입이 약물농도에 비례해서 증가하는 경향을 보여주었다. 이는, L-Ile-cytarabine과 L-Leu-cytarabine은 saturable pathway에 의해 세포내 유입이 이루어지는 반면에 L-Arg-cytarabines는 주로 passive diffusion에 의해 약물흡수가 이루어짐을 암시한다.

합성한 prodrugs중에서 세포내유입이 가장높은 L-Ile-cytarabine를 실험용 흰쥐에 경구투여하여 혈장내에서의 약물동태학적 특성을 평가하였다. 그 결과

L-Ile-cytarabine 의 혈중농도는 cytarabine보다 훨씬 높게 나타났다. 그러나 prodrug 투여후 혈장에서의 cytarabine의 농도는 정량한계 이하로 나타났다. 결론적으로 L-Ile-cytarabine와 L-Leu-cytarabine는 위장관내에서의 안정성이 높고 소장내 흡수도 cytarabine 보다 우수하지만 체내에서 cytarabine으로의 낮은 대사적 전환으로 인하여 cytarabine의 경구용 약물전달시스템으로써는 적합하지 않음을 알수 있었다. 따라서 cytarabine의 세포내 유입 및 parent drug으로의 reconversion 속도를 최적화할수 있는 새로운 프로드럭의 합성이 필요로 된다.

Abstract

Evaluation of N4-Amino Acid Derivatives of Cytarabine for Improving the Oral Delivery of Cytarabine

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-Leu-cytarabine, L-Ile-cytarabine and L-Arg-cytarabine, the gastrointestinal stability of each prodrugs was examined using artificial gastric juice and intestinal fluids. The cellular uptake characteristics of prodrugs were also examined in Caco-2 cells. While L-Leu-cytarabine and L-Ile-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-Leu-cytarabine and L-Ile-cytarabine was significantly higher than that of its parent drug, cytarbine 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 0.4mM. Collectively, L-Ile-cytarabine and L-Leu-cytarabine could be promising candidates to improve the oral absorption of cytarabine via a saturable transport pathway.

Therefore pharmacokinetic profiles of L-Ile-cytarabine was investigated in rats. The AUC of L-Ile-cytarabine was approximately 1.8-fold higher than cytarabine but the appearance of cytarabine was not observed after an oral administration of L-Ile-cytarabine. In conclusion, L-Ile-cytarabine and L-leu-cytarabine may improve the intestinal absorption of cytarabine via the carrrier-mediated transport pathway but its utility as an oral delivery system of cytarabine could be limited by the low metabolic conversion to cytarabine.

1. Introduction

Cytarabine [(1-β-d-arabinofuranosylcytosine), 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[1-11]. In combination with other antitumor agents it is also used against solid tumors[2]. Recently cytarabine has been reported to also induce apoptosis of neoplastic cells[29,30]. 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.[15,16] 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.[17,20]

Oral absorption of passively absorbed drugs can be predict to some extent in vitro. It is reported that the fraction absorbed of passively absorbed drugs can be predicted from the polar molecular surface area of the drug molecule[24], the permeability through an artificial membrane as well as Caco-2 cell monolayer[25,26]. The intestinal peptide transporter(Pept1) plays an important role in transporting dietary peptides as well as pharmacologically active peptidomimetic drugs.[21,22] 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[23]. 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.[23-26] Furthermore, Cheon et al.[27] 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.

2. Materials and Methods

2-1. Materials

Cytarabine, small depeptides, amino acids, acyclovir, 5-bromo-2′-deoxyuridine (BDU), 4-dimethylaminoptridine(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.

2-2. Cells

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

2-3. 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*** (5.0g, 20.55mmol) were protected with tert-buthyldimethylchlorosilane

(9.2g, 61mmol) in anhydrous DMF (100mL) with imidazole(6.8g, 100mmol) to give compound ***2*** (8.31g, yield 69%), which was purified using column chromatography on silica gel 60 (Hexane:Ethylacetate=1:4). The amino group of compound ***2*** (1.2g, 2.04mmol) was coupled with each N-BOC-amino acid (2.04mmol) using HOBT (283mg, 2.1mmol), DMAP(100mg) and DCC(433mg, 2.1mmol) in anhydrous methylene chloride(20mL) 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***(0.636mmol) with tetra-butylammonium fluoride (3.18mL,1.0M in THF) in THF(10mL) 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 (10mL, 2N HCl solution in ether) in anhydrous methylene chloride (10mL) followed by column chromatography on silica gel 60 (CH_2Cl_2:CH_3OH=5:1), produced the desired compounds (***9-11***).

2-4. In-vitro stability study

Gastrointestinal stability of prodrugs was evaluated at 37℃ by incubating a drug solution (5uM) with artifical digestives. The gastric juice consisted of 320 mg of pepsin, 200mg of NaCl, and 2.4mL of 0.1M HCl in 100mL solution (pH1.2). Artificial intestinal juice contained 2.5 g of pancreatin from porcine and 100mL of 50mM K-phosphate buffer(pH6.8). Stabilities of L-Leucine-cytarabine, L-Isoleucine-cytarabine and L-Aarginine-cytarabine were also evaluated at 37℃ by incubating a drug solution (5uM) in the fresh plasma and cell homogenates. At each time point, 100uL of sample was collected and the metabolic reaction was stopped by adding 200uL of ice-cold acetonitrile followed by vigorous mixing. The mixture was then centrifuged at 3000rpm for 10min at 4℃ and the supernatant was filtered through a membrane filter (0.45um) and analyzed by HPLC. The

chemical stability of prodrugs was also examined in aqueous solutions of different pHs(1.2, 6.8 and 8.0).

2-5. Uptake studies in Caco-2 cells

Cells were seeded in 6-well culture plates at a density of 10^5 cells/mL. At 14 days post-seeding, the cells were washed twice with pH6.0 uptake buffer containing 1mM $CaCl_2$, 1mM $MgCl_2$, 150mM NaCl, 3mM KCl, 1mM NaH_2PO_4, 5mM D-glucose, and 5mM MES. The initial uptake rates of cytarabine, L-Leu-cytarabine, L-Ile-cytarabine, and L-Arg-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 by adding 1mL 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 5min at 3000rpm. After filtration of the supernatant through a membrane filter(0.45uM), 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-Leu-cytarabine, L-Ile-cytarabine and L-Arg-cytarabine above the apical membrane of Caco-2 cell monolayers was also examined during the uptake studies to determine the extent of degradation of L-Leu-cytarabine, L-Ile-cytarabine and L-Arg-cytarabine when in contact with Caco-2 monolayers.

2-6. Pharmacokinetic studies in rats

Male sprague-Dawley rats (270-300g) 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 libtum. All animal studies were performed in accord with the principles for Biomedical Research Involving Animals developed by the Council for International Orgnizations 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 10mg/kg of L-Ile-cytarabine, or cytarabine orally. Drugs were dissolved in saline and the dosing volume was 1mL for each animal. Blood samples were collected from the right femoral artery at 0, 0.25, 0.5, 1, 1.5, 2, 4, 8, 12 and 24hr post-dose and then centrifuged at 13,000 rpm for 10 min to obtain the plasma for the HPLC assay. All samples were stored at -70℃ until analyzed.

2-7. HPLC Assay

Drug concentrations were determined by a HPLC assay described as follows. Acyclovir and 5-bromo-2′-dexyuridine(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 automatic injector (SIL-10A). A UV detector (SPD-10A)(Shimadzu Scienific Instruments, Japan) set at 240nm for L-Leu-cytarabine or 272nm for cytarabine, L-Ile-cytarabine and L-Arg-cytarabine. An octadecylsilane column (Gemini C18, 4.6x250nm, 5um; phenomenex, Torrance,

CA, USA) was eluted with a mobile phase at a flow rate of 1.0 mL/min. The mobile phase was 0.01M ammonium acetate buffer(pH 6.5) containing 9-30% acetonitrile for prodrugs and 0.01M ammonium acetate buffer (pH4.5) containing 1% acetonitrile for cytarabine. The calibration curve from the standard samples was linear over the concentration range of 0.01ug/mL to 5ug/mL. The limit of detection was 0.01ug/mL.

2-8. 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.

3. Results and Discussion

3-1. 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=7.2Hz, 1H), 7.22(d, J=7.2Hz, 1H), 6.21(d, J=2.4Hz, 1H), 5.17(br s, 2H), 4.98(br s,1H), 4.32(d, J=3.3Hz, 1H), 4.21(s, 1H), 4.05 (d, J=3.2Hz, 1H), 3.91(m.1H), 3.70-3.61 (m, 2H), 1.70-1.62 (m, 2H), 1.58-1.53(m, 4H)

L-Leu-cytarabine: yield 24%; ^{1}H NMR (DMSO-d_6, 300 MHz)δ 8.06 (d, J=7.2Hz, 1H), 7.17(d, J=7.2Hz, 1H), 6.02(d, J=7.2Hz, 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).

L-Ile-cytarabine: yield 24%; ^{1}H NMR (DMSO-d_6, 300 MHz)δ 7.70 (d, J=6.0Hz, 1H), 7.52(d, J=6.0Hz, 1H), 5.99(d, J=3.0Hz, 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=6.8Hz, 1H), 3.65 (m,2H), 2.93 (m, 2H), 1.94 (m,1H), 0.97(m, 5H), 0.82(m,3H).

3-2. In-vitro stability study

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

appeared to 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 1hr 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 5min 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.

As shown in Fig.4. L-Ile-cytarabine and L-Leu-cytarabine appeared to be stable in fresh rat plasma and Caco-2 cell homogenates. The degradation of L-Ile-cytarabine and L-Leu-cytarabine was negligible in fresh rat plasma and Caco-2 cell homogenates over the 4 hr-incubation. Results indicated that L-Ile-cytarabine and L-Leu-cytarabine should be resistant to the degradation by plasma. The chemical stability of L-Ile-cytarabine and L-Leu-cytarabine were also examined in aqueous solutions of different pHs(1.2, 6.8, 8.0). While L-Ile-cytarabine and L-Leu-cytarabine were stable in aqueous solutions of pH 6.8 and pH 8.0, the hydrolysis of amide bond appeared to be rather accelerated in acidic pH and the disappearance half-life of L-Ile-cytarabine and L-Leu-cytarabine were approximately 1.2 and 2.9hr in the aqueous solution of pH1.2 (Fig.5). Overall, in-vitro stability studies indicated that L-Ile-cytarabine and L-Leu-cytarabine were metabolically stable in plasma and Caco-2 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[31], chemical hydrolysis of L-Ile-cytarabine and L-Leu-cytarabine may be more favorable in tumor cells.

3-3. 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.5, 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 dug concentration, while the permeability of L-Ile-cytarabine and L-Leu-cytarabine decreased significantly as drug concentration increased from 0.4 to 4mM. 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.

3-4. Pharmacokinetic studies

The plasma pharmacokinetic profiles of L-Ile-cytarabine and cytarabine were determined in rats and summarized in Table 1. The AUC and terminal plasma

half-life of L-Ile-cytarabine were prolonged by 2.7 and 2-fold, respectively, but the appearance of cytarabine was did not observed after the oral administration oral of L-Ile-cytarabine.

The metabolic conversion of the prodrug to the parent drug did not appear to be sufficient to ensure the therapeutic effectiveness in the treatment of tumors, although L-Ile-cytarabine could prevent the rapid deamination of cytarabine 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 compnent.

4. Conclusions

The present study has demonstrated that L-Ile-cytarabine and L-Leu-cytarabine were more permeble across the apical membrane of Caco-2 cells than cytarabine. These results also suggest that L-Ile-cytarabine and L-Leu-cytarabine could be effective to improve the oral absorption of cytarabine via the carrier-mediated transport pathways. However, due to the low metabolic conversion to the parent drug, the utility of prodrugs could be limited..

5. References

1. Bryan, J. H.; Henderson E. S.; Leventhal, B. G. Cytosine arabinoside and 6-thioguanine in refractory acute lymphocytic leukemia, *Cancer*. **33**, 539-544 (1974).
2. Grant, S. Ara-C: cellular and molecular pharmacology, Adv. *Cancer Res*. **72**, 197-233 (1998).
3. Sancho, J. M.; Ribera, J. M.; Romero, M. J.; Martin-Reina, V.; Giraldo, P.; Ruiz, E. Compassionate use of intrathecal depot liposomal cytarabine as treatment of central nervous system involvement in acute leukemia: report of 6 cases. *Haematologica*. **91**, ECR02 (2006).
4. Omura, G. A.; Vogler, W. R.; Lefante, J.; Silberman, H.; Knospe. W.; Gordon, D. Treatment of acute myelogenous leukemia: influence of three induction regimens and maintenance with chemotherapy of BCG immunotherapy. *Cancer*. **49**, 1530-1536 (1982).
5. Glantz, M. J.; LaFollette. S.; Jaeckle, K. A.; Shapiro, W.; Swinnen, L.; Rozental, J. R. Randomized trial of a slow-release versus a standard formulation of cytarabine for the intrathecal treatment of lymphomatous meningitis. *Clin Oncol* **17**, 3110-31116 (1999).
6. Goldstone, A. H.; Burnett, A. K.; Wheatly, K,.; Smith, A. G.; Hutchinson, R. M.; Clarke, R. E.; Attempts to improve the treatment outcomes in acute myelogenous leukemia(AML) in older patients: the results of the United Kingdom Medical Reseach Council AML11 trial. *Blood*. **98**, 1302-11 (2001).
7. Kims; Chatelut, E.; Kim, J. C.; Howell, S. B.; Cates, C.; Kormanik, P. A. Extended CSF cytarabine exposure following intrathecal administration of DTC 101. *J Clin Oncol*. **11**, 2186-2193 (1993).
8. Fenaux, P.; Chomienne, C.; Degos, L. Treatment of acute promyelocytic leukaemia. *Best Pract Res Clin Haematol*. **14**, 153-174 (2001).

9. McClune, B.; Buadi, F.; Aslam, N. Intrathecal liposomal cytarabine for prevention of meningeal disease in patients with acute lymphocytic leukemia and high-grade lymphoma. *Leuk Lymphoma*. **48,** 1849-1851 (2007).
10. Weiss, M. A.; Drulinsky, P.; Masalk P.; Scheinberq, D.; Golde, D. W. A phase I trial of a single high dose of idarubicin combined with high-dose cytarabine as induction therapy in relapsed or refractory adult patients with acute lymphoblastic leukemia. *Leukemia* **12**, 865-868 (1998).
11. Weiss, M. A.; Aliff, T. B,; Tallman, M. S.; Frankel, S. R.; Kalaycio, M. E.; Maslak, P. G. A single, high dose of idarubicin combined with cytarabine as induction therapy for adult patients with recurrent of refractory acute lymphoblastcd leukemia. *Cancer*. **95**, 581-587 (2002).
12. Palm, K.; Luthman, K.; Ungell, A.; Strandlund, G.; Beigi, F.; Lundahl, P.; Artursson, P. Evaluation of dynamic polar molecular surface area as predictor of drug absorption: comparison with other computational and exprimental predictors. *J Med Chem*. **41**, 5382-5392 (1998).
13. Chong, S.; Dando, S. A.; Souck, K. M.; Morrison, A. *In vitro* permeability through Caco-2 cells is not quantitatively predictive of *in vivo* absorption for peptide-like drugs absorbed via the dipeptide transport system. *Pharm.Res*. **13**, 120-123 (1996).
14. Gres, M. C.; Julian, B.; Bourrie, M.; Meunier, V.; Roques, C.; Berger, M.; Boulenc, X.; Berger, Y.; Fabre, G. Correlation between oral drug absorption in humans, and apparent drug permeability in TC-7 cells, a human epithelial intestinal cell line: comparison with the parental Caco-2 cell line. *Pharm Res*. **15**, 726-733 (1998)
15. Ho, D. H.; Frei, E. Clinical pharmacology of 1-beta-d-arabinofuranosyl cytosine. *Clin Pharmacol Ther*. **12**, 944-54 (1971).

16. Rustum, Y. M.; Raymakers, R. A. 1-Beta-arabinofuranosylcytosine in therapy of leukemia: preclinical and clinical overview. *Pharmacol Ther*. **56**, 307-21 (1992)

17. Greenwald, R. B.; Choe, Y. H.; McGuire, J.; Conover, C. D. Effective drug delivery by PEGylated drug conjugates. *Adv Drug Deliv Rev*. **55**, 217-50 (2003)

18. Hadfield, A. F.; Sartorell, A. C. The pharmacology of prodrugs of 5-fluorouracil and 1-beta-D-arabinofuranofuranosylcytosine. *Adv Pharmacol. Chemother*. **20**, 21-67 (1984)

19. Wipf, P.; Li, w. prodrugs of ara-c, *Drugs RFuture*. **1**9, 49-54 (1994)

20. Wipf, P.; Li, W.; Adeyeye, C. M.; Rusnak, J. M.; Lazo, J. S. Synthesis of chemoreversible prodrugs of ara-C with variable time-release profiles. Biological evaluation of their apoptotic activity. *Bioorg Med Chem*. **4**, 1584-1596 (1996)

21. Leibach, F. H. Ganapathy, v. Peptide transporters in the intestine and the kidney. *Annu Rev Nutr*. **16**, 99-119 (1996)

22. Smith, P. L.; Eddy, E. p.; Lee, C. P.; Wilson, G. Expoloitation of the intestinalk oligopeptide transporter to enhance drug absorption. *Drug Del*. **1**, 103-111 (1993)

23. Hu, M.; Subramanian, P.; Mosberg, H. I.; Amidon, G. L.; Use of the peptide carrier system to improve the intestinal absorption of L-a'-methyldopa: carrier kinetics, intestinal permebilities, and *in vitro* hydrolysis of depeptidyl derivatives of L-a'-methyldopa. *pharm Res*. **6**, 66-70 (1989)

24. Jung, D.; Dorr, A. Single-dose pharmacokinetics of valganciclovir in HIV-and CMV-seropositive subjects. *J Cin Pharmacol*. **39**, 800-804 (1999).

25. Weller, S.; Blum, M. R.; Doucette, M.; Burnette, T.; Cederberg, D. M.; de Miranda, P.; Smiley, M. L. Pharacokinetics of the acyclovir pro-drug valaciclovir after escalating single-and multiple-dose administration to normal volunteers. *Clin Pharmacol Ther*. **54**, 595-605 (1993)

26. Song, X.; Lorenzi, P. L.; Landowski, C. P.; Vig, B. S.; Hilfenger, J. M.; Amidon, G. L. Amino acid ester prodrugs of the anticancer agent gemcitabine: synthesis, bioconversion, metabolic bioevasion, and hPEPT1-mediated transport. *Mol Pharm.* **2**, 157-167 (2005).
27. Cheon, E. P.; Hong, J. H.; Han, H. K. Enhanced Cellular Uptake of Ara-C via a Peptidomimetic Prodrug, L-Valyl-ara-C in Caco-2 cells. *J Pharm Pharmacol.* **58**, 927-932 (2006).
28. Weitschies, W.; Cardini, D.; Karaus, M.; Trahms, L.; Semmler, W. Magnetic marker monitoring of esophageal, gastric and duodenal transit of non-disntegrating capsules. *Pharmazie*. **54**, 426-430 (1999)
29. Lickliter, J. D.; Wood N. J.; johnson, L.; McHugh, G.; Tan, J.; Wood, F.; Cox, J.; Wickham, N. W. Ha14-1 selectively induces apoptosis in Bcl-2-overexpressing leukemia/lymphoma cells, and enhances cytarabine-induced cell death. *Leukemia*. **17**, 2074-2080 (2003).
30. Samorapoompichit, P.; Steiner, M.; Lucas, T.; Wachtler, F.; Schedled, A.; Sperr, W. R.; Valent, P. Induction of apoptosis in the human mast cell leukemia cell line HMC-1 by various antineooplastic drugs. *Leuk Lymphoma.* **44**, 509-15 (2003).
31. Gerweck LE and Seetharaman K. Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer. *Cancer Res.* **56**, 1194-1198 (1996)

Scheme 1: N4-cytarabine 전구체의 합성

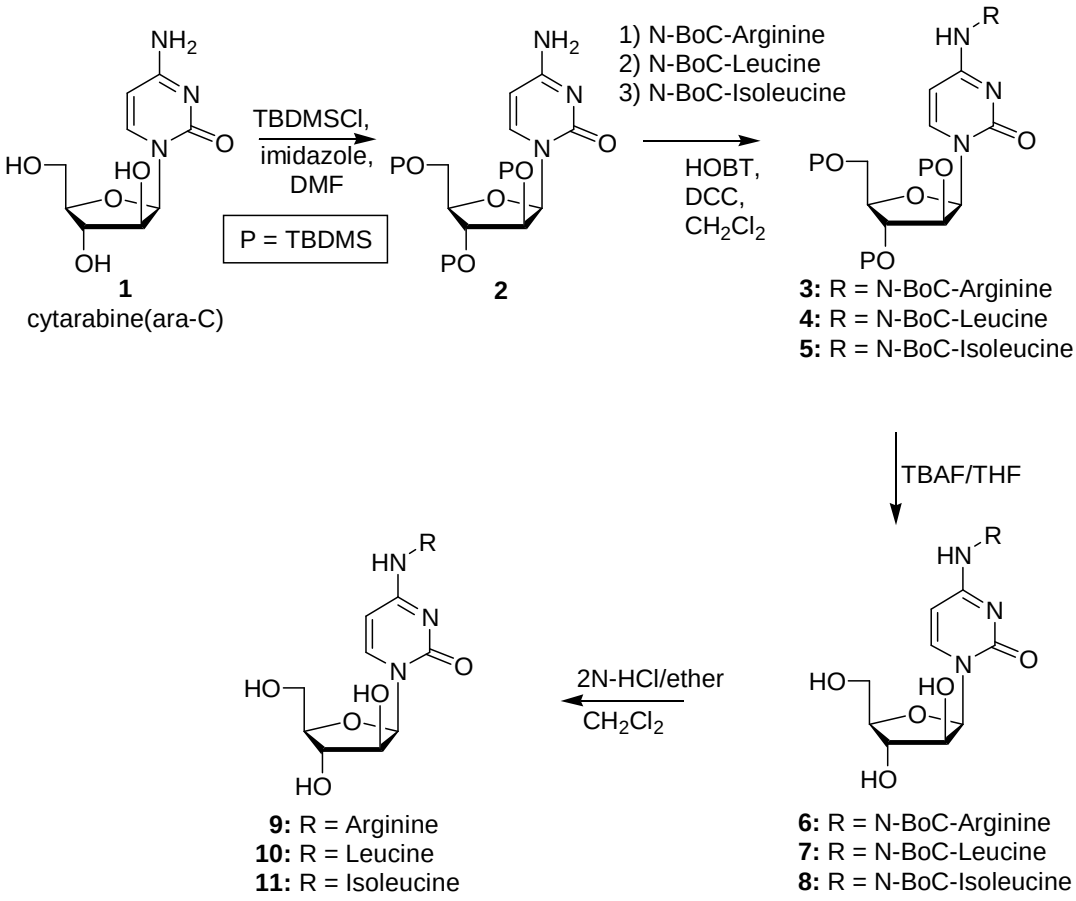


Fig. 1: Synthetic scheme for N4-derivatives of cytarabine

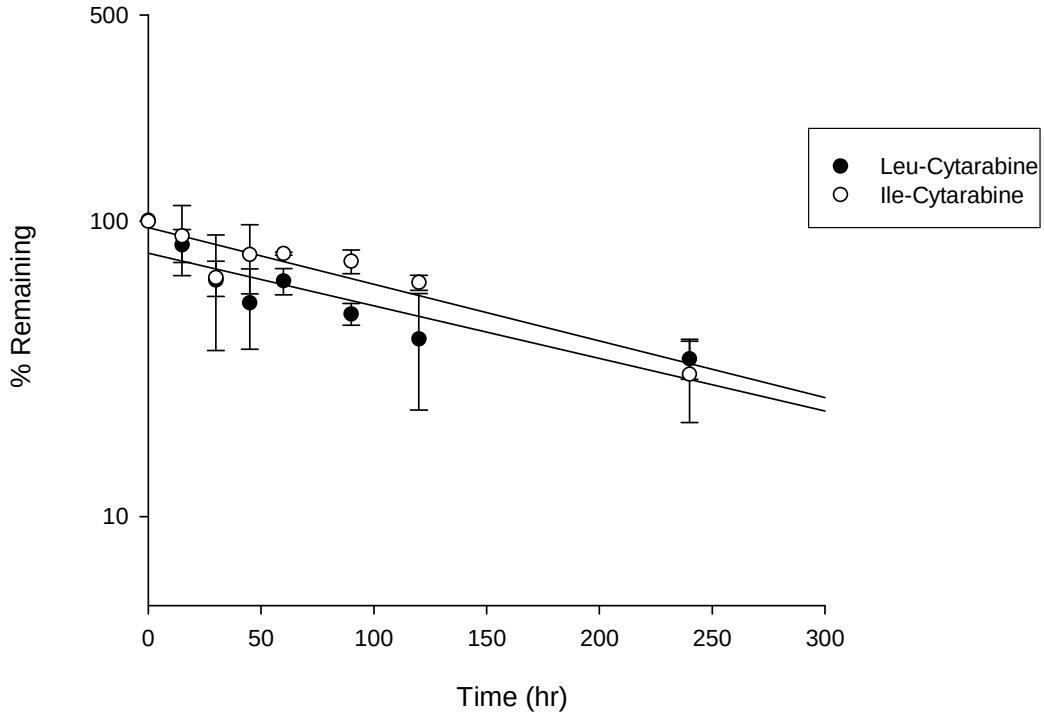


Fig. 2: In-vitro stability of cytarabine prodrugs in the artificial gastric juice (Mean±SD, n=3)

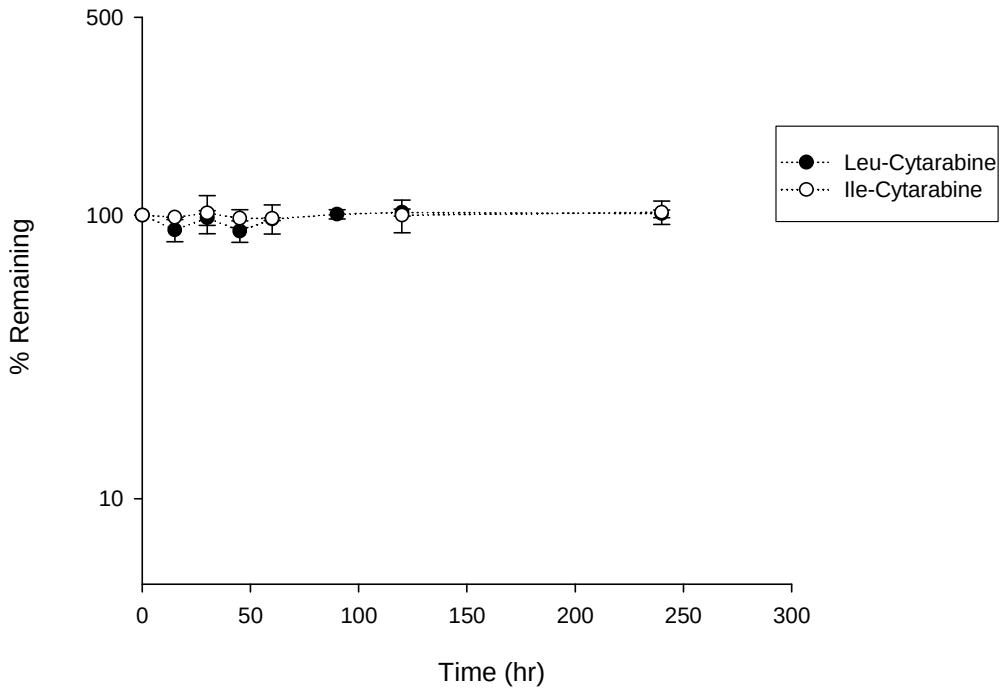


Fig. 3: In-vitro stability of peptidyl prodrugs in the artificial intestinal fluids(Mean±SD, n=3).

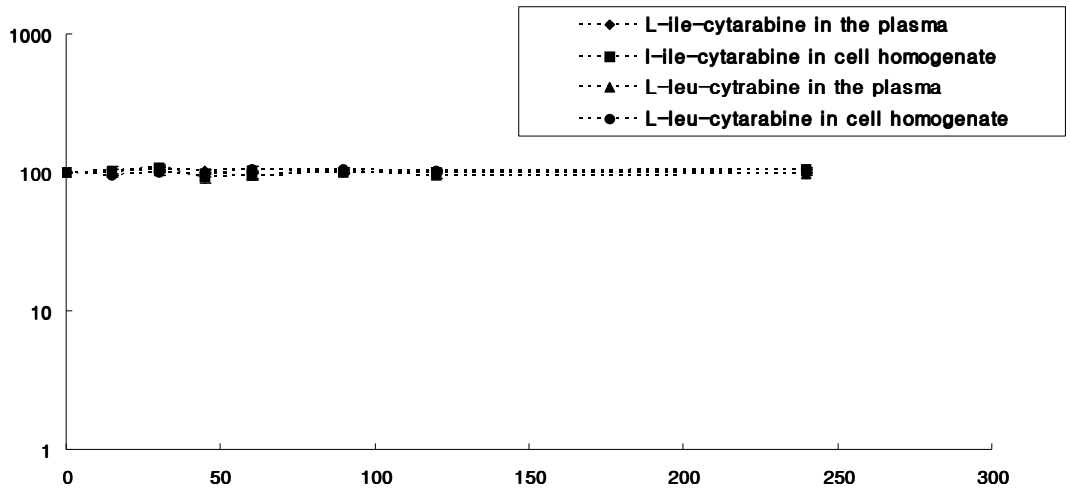


Fig. 4: In-vitro stability of cytarabine prodrugs in cell homogenate and plasma. (Mean±SD, n=3).

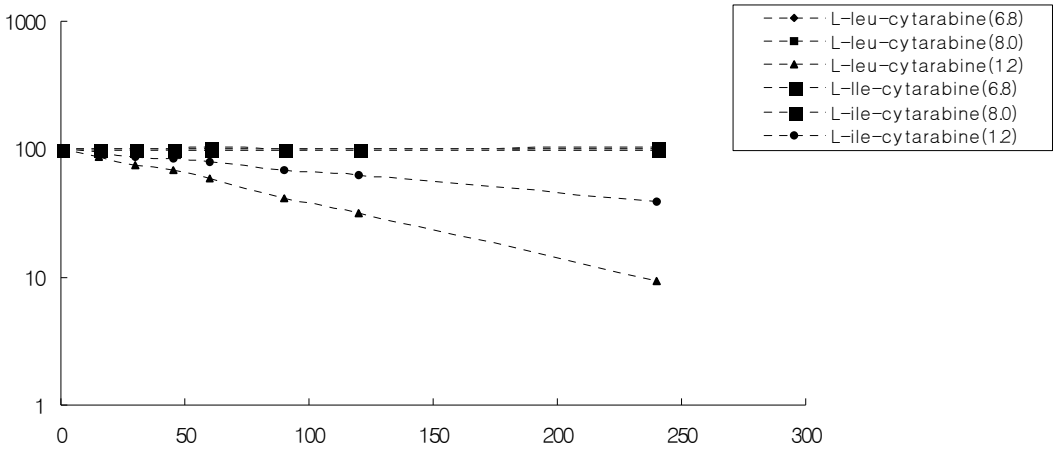


Fig. 5: In-vitro stability of cytarabine prodrugs in aqueous solutions of different pHs(1.2, 6.8, 8.0). (Mean±SD, n=3).

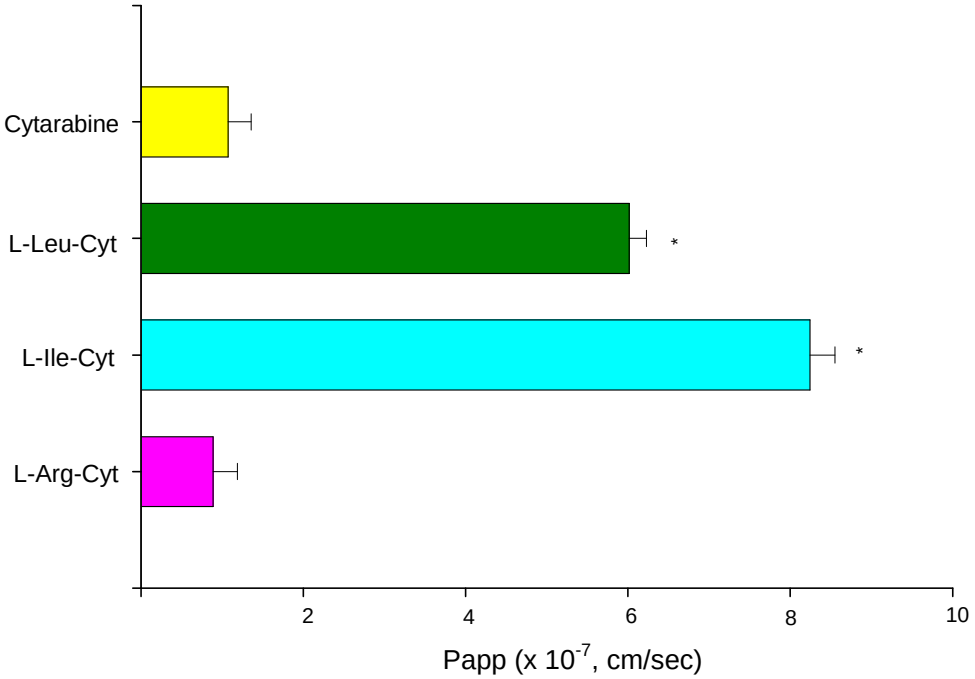


Fig. 6: Cellular uptake of cytarabine and its prodrugs in Caco-2 cells (Mean±SD, n=5-6). *:$p<0.05$, compared to the parent drug.

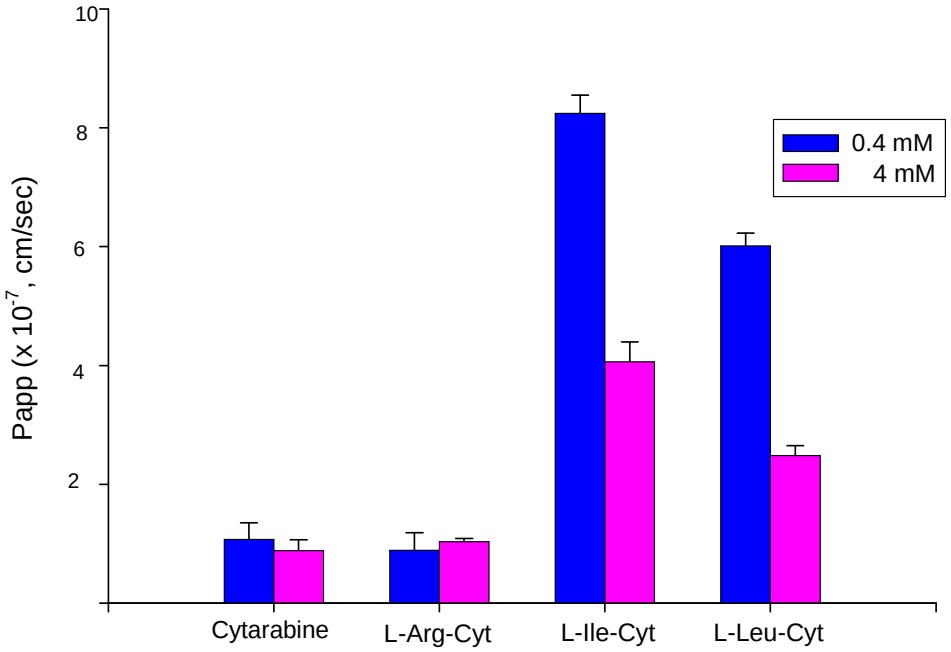


Fig. 7: Concentration dependency in membrane permeabilities of cytarabine and its peptidyl prodrugs in Caco-2 cells (Mean±SD, n=5-6)

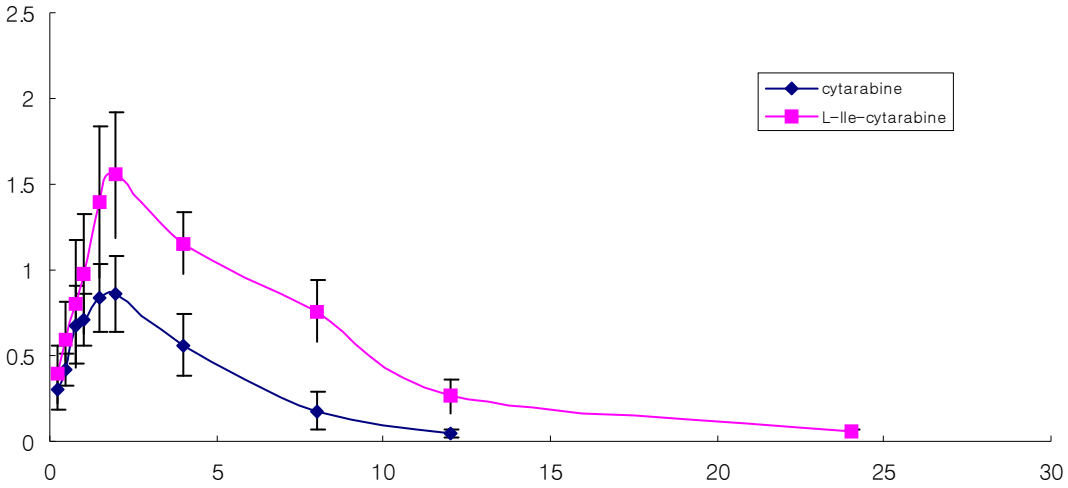


Fig. 8: Mean plasma concentration-time curves of cytarabine and L-Ile-cytarabine after an oral administration to rats (Mean±SD, n=5). ◆;Cytarabine 10mg/kg, ■;L-Ile-cytarabine 10mg/kg

Table 1. Mean pharmacokinetic parameters of L-Ile-cytarabine and cytarabine after an oral administration to rats (Mean±SD, n=4)

Drugs	*Cytarabine*	*L-Ile-cytarabine*	
Analytes	Cytarabine	Cytarabine	L-Ile-cytarabine
Cmax(ng/mL)	876±193	—	1570±385*
Tmax(hr)	1.9±0.3	—	1.9±0.3
AUC(ug*hr/mL)	4600±1070	—	12400±1730*
$T_{1/2}$ (h)	2.3±0.4	—	4.5±0.7*

*$p<0.05$, compared to the control

Part2

국문초록

흰쥐에서 Methotrexate의 신배설 감소를 통한 생체이용률의 증가

본 연구는 흰쥐에서 morin 이 Methotrexate(MTX)의 약물동력학적특성에 미치는 영향을 연구하였다. 동물실험에서 대조군은 MTX(2mg/kg)만 정맥투여했고 병용투여군은 Morin(25mg/kg) 과 MTX(2mg/kg)을 같이 투여하였다. 그 결과 병용투여군은 대조군에 비해 MTX 의 신장클리어런스와 총 클리어런스는 각각 42%, 58% 감소하였다. 또한 morin 과 병용투여했을 경우 MTX의 혈장내 농도는 대조군보다 유의성있게 증가했고 체내 평균체류시간과 혈장 반감기도 대조군에 비해 각각 3.3배와 2.4배 높았다.

MTX의 세포내 축적 실험에서는 hOAT1 또는 hAOT3가 과다발현된 MDCK 세포를 사용하였다. 동물실험과 마찬가지로 대조군은 MTX(20uM)만 사용했고 병용투여군은 MTX 와 Morin(100uM)을 같이 사용하였다. 세포실험의 결과를 보면 hOAT1이 발현된 MDCK 세포에서 병용투여군이 대조군에 비하여 MTX 의 세포내 축적이 유의성있게 감소하였으나 hOAT3 가 발현된 MDCK 세포에서는 유의성있는 차이가 나타나지 않았다.

결과적으로 동물실험과 세포실험 모두에서 Morin 은 MTX 의 약물동력학적 특성에 현저한 영향을 미침을 알수있는데, 이는 주로 Morin이 MTX의 신장배설을 억제시키기때문으로 Morin의 MTX에 대한 신배설억제에는 신장에 분포하는 OAT1이 관여한다는것을 알수있다.

Abstract

Enhanced Systemic Availability of Methotrexate in the Presence of Morin in Rats

The present study aimed to investigate the effect of morin on the pharmacokinetics of methotrexate (MTX) in rats. Pharmacokinetic parameters of MTX were determined in rats following an intravenous administration of MTX(2mg/kg) in the presence and absence of morin (25mg/kg, po.). The cellular accumulation of MTX was also examined by using MDCK cells stably overexpressing hOAT1 or hOAT3. Compared with the control given MTX alone, pretreatment with morin 15min prior to MTX administration significantly altered the pharmacokinetics of MTX in rats. Renal clearance and total clearance of MTX were reduced by 42% and 58%, respectively, in the presence of morin. Accordingly, the systemic exposure of MTX in the rats pretreated with morin was significantly higher than that from the control group. The mean residence time (MRT) and terminal plasma half-life of MTX were prolonged by 3.3-and 2.4-fold, respectively, by the concurrent use of morin. The cellular uptake of MTX (20uM) was significantly reduced by the co-incubation with morin (100uM) in MDCK-hOAT1 cells but not in MDCK-hOAT3 cells. Taken together, morin appeared to be effective in altering the pharmacokinetics of MTX in rats, likely by the inhibition of OAT1-mediated renal excretion.

1. Introduction

Methotrexate (MTX, 2,4-diamino-N^{10}-methylpteroyl-L-glutamic acid), a classical antifolate, has been used in the treatment of various human neoplastic disorders including childhood acute leukemia, head and neck cancer and osteosarcoma[1-3]. MTX is also used in other non-malignant conditions including psoriasis and rheumatic disease[4,5]. As the side effects of MTX include acute renal failure, bone marrow depression and hepatitis through an increase in blood MTX levels when it was coadministered with other drugs[6,7], the prediction of potential drug interactions with MTX should be important in combination therapy. Some previous studies have reported life threatening interactions of MTX with conventional drugs or plant extracts[8-10]. For example, the coadministration of Pueraria lobata root decoction decreased the elimination of MTX and resulted in markedly increased systemic exposure and toxicity of MTX in rats[10]. Probenecid inhibited the tubular secretion of MTX and thus increased the plasma MTX concentration by 2-to 3-fold[8,9].

Recently flavonoids have gained much attention as complementary and alternative medicines due to their beneficial effects on human health[11,12]. In particular, morin (3,5,7,2′,4′-pentahy-droxy-flavone)appeared to be non-toxic [13] and displayed a variety of biological actions such as anti-allergic, anti-inflammatory, antimutagenic and anticarcinogenic effects [14,15]. Moreover they are effective as modulators of pharmacokinetics or cellular uptake of certain therapeutic drugs including diltiazem, paclitaxel and gamma-hydroxybutyrate[16-18]. Recently it was also demonstrated that morin could modulate the transport activity of human organic anion transporter 1 (hOAT1) by using transfected MDCK cells[19]. Given that MTX can be taken up by organic anion transporters on the basolateral side of the proximal tubule in kidney (20), there could be a potential drug interaction

between MTX and morin by the modulation of renal excretion. Therefore, this study aimed to investigate the effect of morin on the pharmacokinetics of MTX in rats.

2. Materials and Methods

2-1. Materials

MTX (4-amino-10-methylfolic acid), morin and 5-bromo-2′-deoxyuridine were obtained from Sigma Chemicals (St Louis, MO, USA). Acetonitrile and methanol were purchased from Merck Co. (Darmstadt, Germany). 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). Madin-Darby canine kidney cells stably transfected with hOAT1(MDCK-hOAT1 cells) were kindly provided by Dr John B. Pritchard(NIEHS, North Carolina, USA). MDCK cells overexpressing hOAT3 (MDCK-hOAT3 cells) were generated as described in a previous report[21] and the functional expression of hOAT3 was confirmed by the uptake of [H^3]-estrone sulfate. All other chemicals were reagent grade and all the solvents used were HPLC grade.

2-2. Cell culture

MDCK cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 1% nonessential amino acids, 1mM sodium pyruvate, 1% L-glutamine and penicillin (100U/mL) streptomcin (100mg/mL). MDCK-hOAT1 and MDCK-hOAT3 cells were maintained in the same media added with G-418(200ug/mL) or blasticidin S(2ug/mL), respectively. All cells were maintained in an atmosphere of 5% CO_2 and 90% relative humidity at 37℃.

2-3. Cellular uptake study of MTX

Cells were seeded in 6-well culture plates at a density of 10^5 cells/cm^2. At 5 days post-seeding, the cells were washed twice with pH 7.4 uptake buffer and then were incubated with a drug solution containing 20uM MTX in the absence and the presence of 100uM morin. At the end of 15min incubation, the drug solution was removed and the cells were washed three times with ice-cold phosphate-buffered saline. After the cell lysis, the cells were harvested and sonicated for 1-2min. Acetonitrile (1mL) was added to the cell lysate, vortexed rigorously and centrifuged for 5min at 3000rpm. After filtration of the supernatant through a membrane filter(0.45uM), the samples were analysed by HPLC. The protein amount of each sample was determined with a BCA protein assay kit following the manufacturer's instruction (Sigma Chemical Co, St Louis, MO, USA).

2-4. Pharmacokinetic studies in rats

Male Sprague-Dawley rats (270-300g) were purchased from Dae Han Laboratory Animal Research and Co. (Choongbuk, Korea), and had free access to normal standard chow (Jae chow, korea) and tap water. The animals were kept in these facilities for at least 1 week before the experiment and fasted for 24h prior to the experiments. On the day of experiments, MTX(2mg/kg) was administered intravenously to rats with and without pretreatment with rats with morin (25mg/kg) orally 15min prior to MTX administration. Blood samples were collected from the femoral artery at 0, 0.083, 0.25, 0.5, 1, 2, 4, 8, 12 and 24h postdose. Urine was also collected for 24h from the same group of rats. Blood samples were centrifuged to obtain plasma. Urine samples were centrifuged for 10min at 3000rpm and then passed through a membrane filter (0.45uM). All samples were stored at

-70℃ until analysed.

2-5. HPLC Assay

The concentrations of MTX were determined by a HPLC assay as described by Ueda et al[22]. In brief. 20ul (10ug/ml) of aminopterin as an intemal standard was to 200ul plasma samples and the samples were deproteinized by adding 400ul acetonitrile. After centrifugation of the samples at 13000rpm for 10min, the supematant was completely evaporated for dryness. The residue was reconstituted with 200ul of the mobile phase, and then 50ul aliquots were injected directly into the HPLC system(Shimadzu Scientific Instruments, Japan). 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 320nm. An octadecylsilane column (Gemini C18, 4.6×250mm, 5um; Phenomenex, Torrance, CA, USA) was eluted with a mobile phase consisted of 0.01M ammonium acetate buffer: acetonitrile (94:6, V/V %) for plasma samples and 0.01M KH_2PO_4(PH 6.3): acetonitrile : methanol(92.5 : 6 : 1.5, V/V/V%) for urine samples. The standard curves covered the drug concentrations of 0.01～5ug/mL(Fig.10).

2-6. Pharmacokinetic analysis

The pharmacokinetic parameters of MTX were obtained by noncompartmental analysis using WinNonlin version 2.1 (Pharsight, Mountain View, CA). The area under the plasma concentration-time curve(AUC) and the area under the first moment-time curve from time zero to infinity(AUMC) were calculated using the linear trapezoidal method. And the mean residence time(MRT) was calculated by AUMC/AUC. 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 $0.693/\lambda_z$. The total clearance (CL) was estimated by dividing dose by AUC and the apparent volume of distribution at steady state (V_{dss}) was calculated as CL multiplied by MRT. Renal clearance (CL_r) was determined as CL_r=Ae/AUC, where Ae (amount of unchanged drug eliminated in urine) and AUC were measured over the same time interval.

2-7. Statistical analysis

All data are expressed as the mean±SD. The statistical significance of differences between treatments was evaluated using the unpaired Student's t-test, and a value of $p<0.05$ was considered to be statistically significant.

3. Results and Discussion

The mean plasma concentration-time profiles of MTX in the presence and the absence of morin are illustrated in Figure 11. The mean pharmacokinetic parameters of MTX are also summarized in Table 2. Kuroda et al[23]. demonstrated that the intravenous pharmacokinetics of MTX was linear over the dose range 0.5-2.5mg/kg in rats. Therefore, considering the assay sensitivity as well as the dose range for the linear pharmacokinetics, MTX was dosed at 2mg/kg in the present study and achieved a plasma level comparable to the therapeutic concentrations in humans[9]. As shown in Table 2, pretreatment with morin 15min prior to MTX administration significantly ($p<0.05$) altered the pharmacokinetics of MTX in rats, compared with the control given MTX alone. Renal clearance and total clearance of MTX decreased by 42% and 58%, respectively, in the presence of morin. Consequently, the systemic exposure (AUC) of MTX was significantly($p<0.05$) higher than the control given MTX alone. Furthermore, the mean residence time (MRT) and terminal plasma half-life of MTX were prolonged by 3.3-and 2.4-fold,respectively, by the concurrent use of morin. Taken together, pretreatment with morin prior to MTX administration significantly altered the renal excretion and plasma pharmacokinetics of MTX in rats.

The altered renal excretion of MTX in the presence of morin may be caused by competition between MTX and morin for the transport system such as organic anion transporters (OATS) expressed in the renal proximal tubules, considering that MTX can be taken up by OAT1 and OAT3 across the basolateral membrane of proximal tubules [20,24,25] and morin can also interact with those transporters [19]. Therefore, the cellular uptake of MTX was determined in the presence and absence of morin by using transfected MDCK cells overexpressing hOAT1 or hOAT3. As shown in Figure 12, the cellular accumulation of MTX in MDCK-hOAT1 significantly decreased in the presence of morin while there was no change in

MDCK-hOAT3 cells. This result suggests that morin might be more effective in inhibiting the hOAT1-mediated cellular uptake of MTX than the hOAT3-mediated uptake of MTX. Therefore, the altered pharmacokinetics of MTX via the concurrent use of morin might be explained, at least in part, by the inhibition effect of morin on the OAT1-medated renal excretion of MTX. Some previous studies also reported that probenecid and piperacillin significantly reduced the renal elimination of MTX, probably by blocking the organic anion transport system in kidney[9,26]. However, since MTX and morin could be also recognized by other transporters including P-gp and MRPs[27-29], additional mechanisms for pharmacokinetic interaction between MTX and morin should not be excluded at this moment.

Considering that the urinary clearance of MTX accounts for approximately 70% to 90% of the total clearance in humans [9], the interaction involving urinary excretion between MTX and morin may have more profound impact on the pharmacokinetics of MTX in humans than that observed in rats. Therefore, the clinical importance of this finding should be further investigated in clinical studies.

4. Conclusion

Pretreatment with morin significantly enhanced the systemic exposure of intravenously administered MTX. Therefore, the concomitant use of morin or morin-containing dietary supplements with MTX may require close monitoring for potential drug interactions in clinics.

5. References

1. Djerassi, I. Methotrexate infusions and intensive supportivecare in the management of children with acutelymphocytic leukemia: follow-up report. *Cancer Res.* **27**, 2561-2564 (1967).
2. Levitt, M.; Mosher M. B.; DeConti R. C. Improved therapeutic index of methotrexate with 'eucovorin rescue. *Cancer Res.* **33**, 1729-1734 (1973)
3. Frei, E. 3rd,; Jaffe, N.; Tattersall, M. H.; Pitman, S.; Parker, L.; New approaches to cancer chemotherapy with methotrexate. *N Engl J Med.* **292**, 846 - 851 (1975).
4. Chladek, J.; Grim, J.; Martinkova, J. Pharmacokinetics and pharmacodynamics of low-dose methotrexate in the treatment of psoriasis. *Br J Clin Pharmacol.* **54**, 147 - 156 (2002).
5. Burchini, G.; Orsi, C. Rheumatoid arthritis. *N Engl J Med.* **351**, 1360 - 1361 (2004).
6. Maiche, A. G. Acute renal failure due to concomitant action of methotrexate and indomethacin. *Lancet.* **1**, 1390 (1986).
7. Thyss, A.; Milano, G.; Kubar, J.; Namer, M.; Schneider, M. Clinical and pharmacokinetic evidence of a life-threatening interaction between methotrexate and ketoprofen. *Lancet.* **1**, 256 - 258 (1986).
8. Bourke, R. S,; Chheda, G.; Bremer, A.; Watanabe, O.; Tower, D. B. Inhibition of renal tubular transport of methotrexate by probenecid. *Cancer Res.* **35**, 110 - 116 (1975).
9. Aherne, G. W.; Piall, E.; Marks, V.; Mould, G.; White, W. F. Prolongation and enhancement of serum methotrexate concentrations by probenecid. Br Med J. **1**, 1097 - 1099 (1978).
10. Chiang, H. M,; Fang, S. H.; Wen, K. C. Life-threatening interaction between the root extract of Pueraria lobata and methotrexate in rats.

Toxicol Appl Pharmacol. **209**, 263 - 268 (2005).

11. Cassileth, B. R.; Deng, G. Complementary and alternative therapies for cancer. *Oncologist*. **9**, 80 - 89 (2004).
12. Richardson, M. A.; Straus, S. E. Complementary and alternative medicine: opportunities and challenges for cancer management and research. *Semin Oncol*. **29**, 531 - 545 (2002).
13. Wu, T. W,; Zeng, L. H.; Wu, J.; Fung, K. P. Morin: a wood pigment that protects three types of human cells in the cardiovascular system against oxyradical damage. *Biochem Pharmacol*. **47**, 1099 - 1103 (1994).
14. Robak, J.; Gryglewski, R. J. Flavonoids are scavengers of superoxide anion. *Biochem Pharmacol*. **37**, 83 - 88(1988).
15. Stavric, B. Role of chemopreventers in human diet. *Clin Biochem*. **27**, 319 - 332 (1994).
16. Choi, B. C.; Choi, J. S.; Han, H. K. Altered pharmacokinetics of paclitaxel by the concomitant use of morin in rats. *Int J Pharm*. **323**, 81 - 85 (2006).
17. Choi, J. S.; Han, H. K. Pharmacokinetic interaction between diltiazem and morin, a flavonoid, in rats. *Pharmacol Res*. **52**, 386 - 391 (2005).
18. Wang, Q.; Morris, M. E. Flavonoids modulate monocarboxylate transporter-1-mediated transport of gamma-hydroxybutyrate *in vitro* and in vivo. *Drug Metab Dispos*. **35**, 201 - 208 (2007).
19. Hong, S. S.; Seo, K.; Lim, S. C.; Han, H. K. Interaction characteristics of flavonoids with human organic anion transporter 1 (hOAT1) and 3 (hOAT3). *Pharmacol Res*. **56**, 468-473 (2007).
20. Takeda, M.; Khamdang, S.; Narikawa, S. Characterization of methotrexate transport and its drug interactions with human organic anion transporters. *J Pharmacol Exp Ther*. **302**, 666 - 671 (2002).
21. Aslamkhan, A. G.; Han, Y. H.; Yang, X. P.; Zalups, R. K.; Pritchard, J. B. Human renal organic anion transporter 1-dependent uptake and toxicity of mercuric-thiol conjugates in Madin-Darby canine kidney cells.

Mol Pharmacol. **63**, 590 - 596 (2003).

22. Ueda, K.; Kato, Y.; Komatsu, K.; Sugiyama, Y. Inhibition of biliary excretion of methotrexate by probenecid in rats: Quantitative prediction of interaction from in vitro data. *J Pharmacol Exp Ther*. **297**, 1036 - 1043 (2001).
23. Kuroda, T.; Namba, K.; Torimaru, T.; Kawashima, K.; Hayashi, M. Species differences in oral bioavailability of methotrexate between rats and monkeys. *Biol Pharm Bull*. **23**, 334 - 338 (2000).
24. Uwai, Y.; Taniquchi, R.; Motohashi, H.; Saito, H.; Okuda, M.; Inui, K. I. Methotrexate - oxoprofen interaction: involvement of human organic anion transporters hOAT1 and hOAT3. *Drug Metab Pharmacokinet*. **19**, 369 - 374 (2004).
25. Kwak, J. O.; Kim, H. W.; Oh, K. J.; Kim, D. S.; Han, K. O.; Cha, S. H. Colocalization and interaction of organic anion transporter 1 with caveolin-2 in rat kidney. *Exp Mol Med*. **37**, 204 - 212 (2005).
26. Iven, H.; Brasch, H. Influence of the antibiotics piperacillin, doxycycline, and tobramycin on the pharmacokinetics of methotrexate in rabbits. Cancer Chemother Pharmacol. 17, 218 - 222 (1986).
27. Zeng, H.; Chen, Z. S.; Belinsky, M. G.; Rea, P. A.; Kruh, G. D. Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. *Cancer Res*. **61**, 7225 - 7232 (2001).
28. Gifford, A. J.; Kavallaris, M.; Madafiglio, J. P-glycoprotein- mediated methotrexate resistance in CCRF-CEM sublines deficient in methotrexate accumulation due to a point mutation in the reduced folate carrier gene. *Int J Cancer*. **78**, 176 - 181 (1998).
29. Morris, M. E.; Zhang, S. Flavonoid - rug interactions: effects of flavonoids on ABC transporters. *Life Sci*. **78**, 2116 - 2130 (2006).

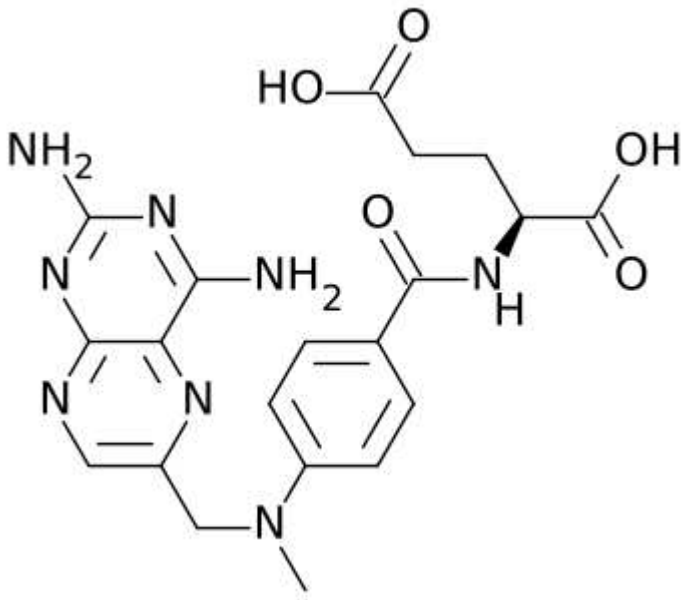
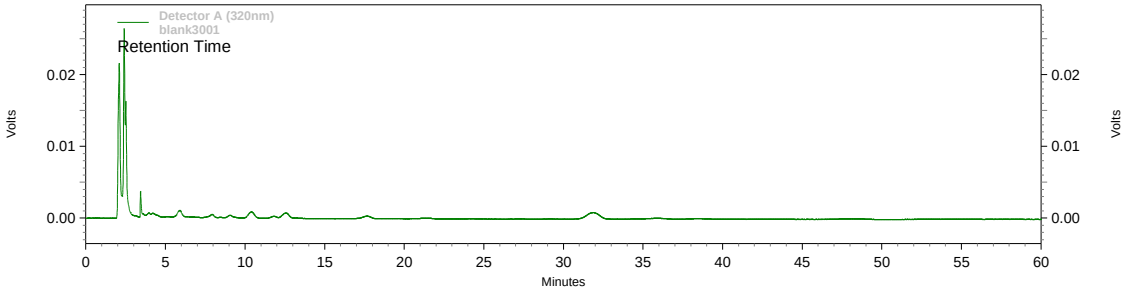


Fig. 9: structure of MTX

A



B

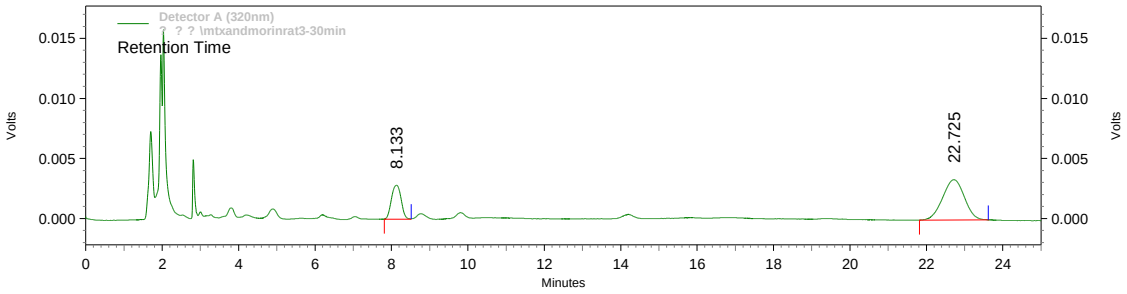


Fig. 10: Chromatograms of blank plasma (A) and plasma spiked (B) with internal standard (8.113 min) and MTX (22.725 min)

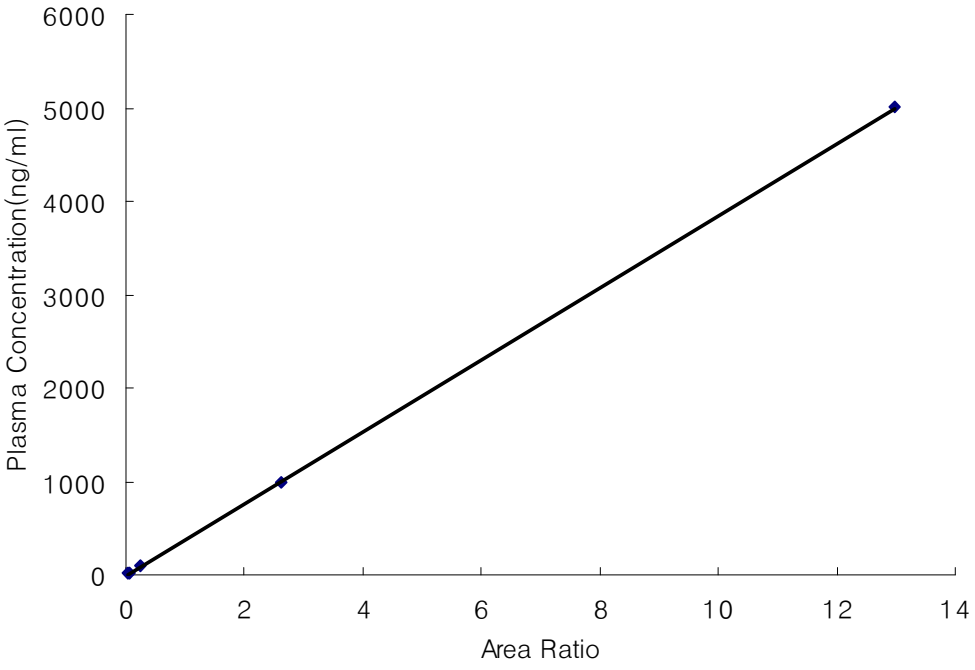


Fig. 11: Calibration curve of Plasma Concentration
(y=385.33x-2.7993, r=1)

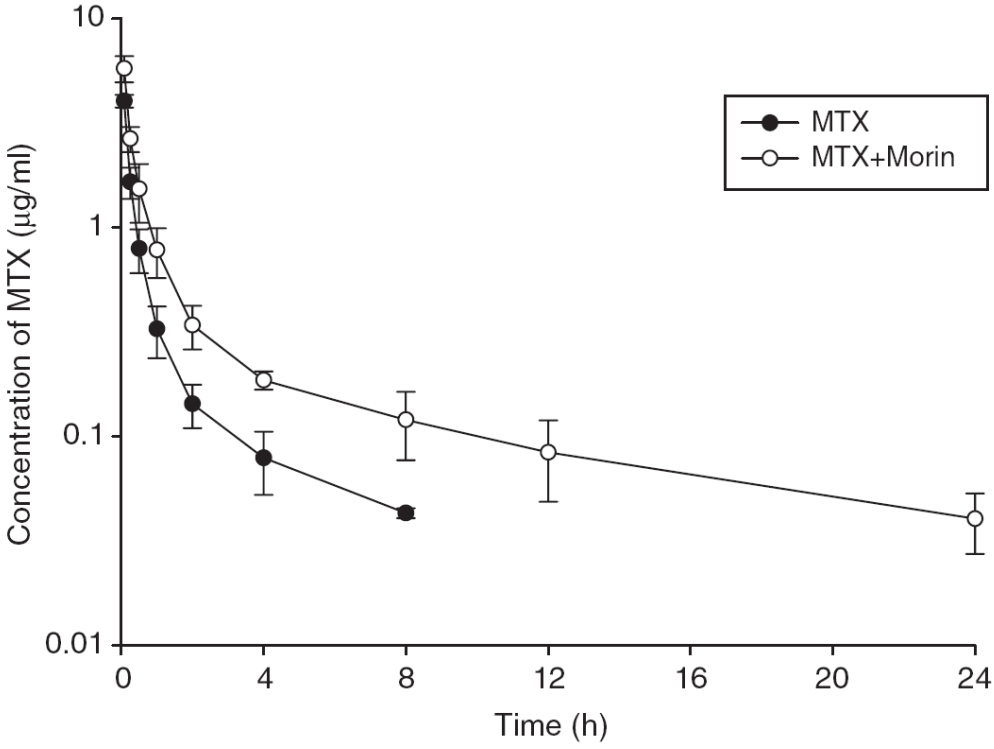


Fig. 12: Mean plasma concentration-time profiles of MTX after an intravenous administration of MTX (2mg/kg) to rats in the presence and absence of morin (Mean±SD, n=4).

Table 2: Pharmacokinetic parameters of MTX following an intravenous administration of MTX (2mg/kg) to rats in the presence and the absence of morin (Mean±SD, n=4).

Parameter	*MTX*	*MTX + Morin*
$AUC_{0-\infty}$ (ng.h/mL)	2340 ± 329	5550±664*
CL(1/h/kg)	0.876±0.118	0.364±0.046*
CL_r(1/h/kg)	0.156±0.092	0.091±0.022*
V_{dss}(1/kg)	1.71±0.684	2.37±0.918
$T_{1/2}$ (h)	3.1±1.4	7.5±3.4*
MRT(h)	2.0±0.78	6.5±2.6*

*$p<0.05$, compared with the control group given MTX alone.

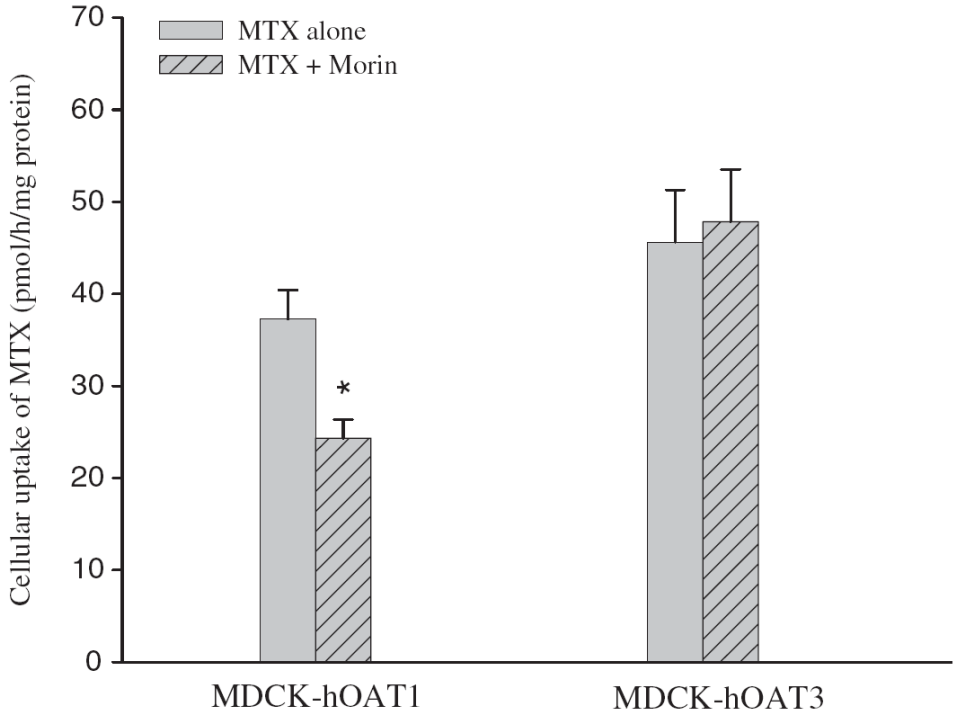


Fig. 13: Celluar uptake of MTX in MDCK-hOAT1 and MDCK-hOAT3 cells(Mean±SD, n=3). Cells were incubated with MTX(20uM) for 15min in the presence and absence of morin(100uM). *$p<0.05$, compared with the control group given MTX alone.