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Effects of Resveratrol and Licocharcon A on the Bioavailability of Losartan in Rats

조선대학교 대학원

약 학 과

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생체이용률에 미치는 영향

2011 년 8 월 25 일

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이 논문을 약학박사학위신청 논문으로 제출함.

2011 년 4 월

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2011 년 6 월

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

Part I. Effects of Resveratrol on the Bioavailability of Losartan in Rats

Part II. Effects of licocharcon A on the Bioavailability of Losartan in Rats

ABSTRACT

Effects of Resveratrol and Licocharcon A on the Bioavailability of Losartan in Rats

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Resveratrol or licocharcon A and losartan, a calcium channel blocker, could be prescribed as a combination therapy for prevention or treatment of cardiovascular diseases. The purpose of this study was to investigate the possible effects of resveratrol or licocharcon A, on the pharmacokinetics of losartan and its main metabolite, EXP-3174, in rats.

The pharmacokinetic parameters of losartan and EXP-3174 were determined after oral and intravenous administration of losartan (9 or 3 mg/kg) to rats in the presence and absence of resveratrol (0.4, 2 and 8 mg/kg) or licocharcon A (0.4, 2 and 8 mg/kg). The effect of resveratrol or licocharcon A on the P-glycoprotein (P-gp) as well as CYP3A4 and 2C9 activity was also evaluated. Resveratrol or licocharcon A significantly inhibited CYP3A4 and CYP2C9 enzyme. In addition, resveratrol or licocharcon A significantly reduced rhodamine-123 efflux via P-gp in MCF-7/ADR cell overexpressing p-gp. Licocharcon A is more effective than resveratrol in inhibitory effect of P-gp and CYP3A4 activity.

Compared to the control (losartan alone), resveratrol or licocharcon A significantly altered the pharmacokinetic parameters of losartan. The area under the plasma concentration-time curve ($AUC_{0-\infty}$) and the peak plasma concentration (C_{max}) of losartan were significantly increased in the presence of resveratrol or licocharcon A, respectively. The total body clearance (CL/F) was significantly decreased by resveratrol and licocharcon A, respectively. Consequently, the absolute bioavailability of losartan in the presence of resveratrol or licocharcon A were significantly higher than that of the control group, respectively. Pharmacokinetics parameters of intravenous losartan were not affected by resveratrol or licocharcon A.

Resveratrol or licocharcon A increased the $AUC_{0-\infty}$ of its main metabolite, EXP-3174, respectively, but they are not significxant. Moreover, the metabolite-parent AUC ratio (M.R.) in the presence of resveratrol or licocharcon A were significantly decreased compared to those of the control group, respectively.

In conclusion, the enhanced oral bioavailability of losartan by resveratrol or licocharcon A may result from decreased P-gp-mediated efflux in small intestine and inhibition of CYP 3A- and CYP2C9-mediated metabolism in small intestine and/or in the liver and reduction of total body clearace of losartan.

Based on the results, if the drug interactions are confirmed in the patients with cardiovascular diseases, the dosage of losartan should be readjusted when losartan is used concomitantly with resveratrol or licocharcon A.

Key words: Losartan · EXP-3174 · Resveratrol · Licocharcon A · CYP3A · CYP2C9 · P-gp · Pharmacokinetics · Bioavailability · Rats

국 문 초 록

흰쥐에서 레스베라트롤 및 리코찰콘이 로살탄의 생체이용률에 미치는 영향

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레스베라트롤 또는 리코찰콘과 로살탄의 병용투여가 순환기 질환 예방 및 치료를 위해서 처방되는 경우가 있으므로 이에 대한 상호작용을 알아보기 위해서 흰쥐에 로살탄 (9 mg/kg)과 레스베라트롤 (0.4, 2.0 and 8.0 mg/kg) 또는 리코찰콘 (0.4, 2.0 and 8.0 mg/kg)을 병용 경구투여한 후 로살탄 및 그 활성대사체인 EXP-3174 의 약동학적 변수들을 대조군과 비교 검토하였다.

레스베라트롤 또는 리코찰콘과 병용 투여시 로살탄의 약물동태학적 변수는 유의성 있게 변화하였다. 대조군에 비해 레스베라트롤 (2.0 and 8.0 mg/kg) 또는 리코찰콘 (2.0 and 8.0 mg/kg)과 병용투여군에서 로살탄의 혈장농도곡선하면적 ($AUC_{0-\infty}$)과 최고혈중농도 (C_{max})는 각각 유의성있게 증가되었으며, 전신클리어런스는 유의성 있게 각각 감소되었다. 절대적 생체이용률은 대조군에 비해 각각 유의성 있게 증가되었으며 상대적 생체이용률은 1.25-1.63 배 증가되었다.

정맥투여군에서는 레스베라트롤 또는 리코찰콘은 로살탄의 약동학적 변수에는 거의 영향을 주지못하였다.

레스베라트롤 (8.0 mg/kg) 또는 리코찰콘 (8.0 mg/kg)과 로살탄을

병용투여한군에서 대조군에 비해 활성대사체인 EXP-3174 의 혈장농도곡선하면적 ($AUC_{0-\infty}$)이 증가되었으나 유의성은 없었다. 그리고 레스베라트롤 또는 리코찰콘은 로살탄의 대사율 (M.R.)을 각각 유의성 있게 감소시켰다. 레스베라트롤 및 리코찰콘에 의해서 P-gp 와 CYP3A 및 CYP2C9 활성을 억제시켰으나 리코찰콘이 레스베라트롤 보다 CYP3A4 와 P-gp 억제효과가 더 컸다.

본 연구에서 항산화작용 및 심혈관 개선작용이 있는 레스베라트롤 또는 리코찰콘을 각각 고혈압치료제인 로살탄과 병용투여 하였을 때 경구투여된 로살탄의 생체이용률이 유의성 있게 증가된 것은 레스베라트롤 및 리코찰콘에 의해서 신배설 감소 보다는 주로 소장에 존재하는 P-gp 억제에 의한 흡수증가와, 주로 소장과 간장에 존재하는 CYP3A 와 CYP2C9 억제에 의한 로살탄의 초회통과효과(대사) 감소와 전신클리어런스의 감소에 기인한 것으로 사료된다.

Part I. Effects of Resveratrol on the Bioavailability of Losartan in Rats

Abstract

The present study was to investigate the effect of resveratrol, an antioxidant, on the bioavailability and pharmacokinetics of losartan and its active metabolite, EXP-3174, in rats. Pharmacokinetic parameters of losartan and EXP-3174 in rats were determined after an oral and intravenous administration of losartan (9 mg/kg) in the presence or absence of resveratrol (0.4, 2 and 8 mg/kg).

The effects of resveratrol on the P-glycoprotein (P-gp) as well as CYP3A4 and 2C9 activity were also evaluated. Resveratrol inhibited CYP3A4 and CYP2C9 enzyme activity with 50% inhibition concentration (IC_{50}) of 1.0 and 8.4 μM, respectively. In addition, resveratrol significantly enhanced the cellular accumulation of rhodamine-123 in MCF-7/ADR cells overexpressing P-gp in a concentration-dependent manner.

The pharmacokinetic parameters of losartan were significantly altered by the presence of resveratrol compared with the control group (given losartan alone). Presence of resveratrol significantly (2 mg/kg, $p < 0.05$; 8 mg/kg, $p < 0.01$) increased the area under the plasma concentration–time curve ($AUC_{0-\infty}$) of losartan by 24.9–52.3% and peak plasma concentration (C_{max}) of losartan by 25.0–50.0%. The total body clearance (CL/F) was significantly decreased (2 mg/kg, $p < 0.05$; 8

mg/kg, $p < 0.01$) by resveratrol. Consequently, the absolute bioavailability (A.B.) of losartan in the presence of resveratrol was 31.7–38.7%, which was enhanced significantly ($p < 0.05$) compared with the oral control group (25.4%). The relative bioavailability (R.B.) of losartan was increased by 1.10- to 1.52-fold than that of the control group. However, there was no significant change in the peak plasma concentration (T_{max}) and terminal half-life ($t_{1/2}$) of losartan in the presence of resveratrol. Presence of resveratrol increased the $AUC_{0-\infty}$ of EXP-3174 but it is not significant. Metabolite-parent AUC ratio in the presence of resveratrol (8 mg/kg) significantly ($p < 0.05$) decreased by 21.0% compared to the control group, implying that coadministration of resveratrol could be effective to inhibit the CYP3A4-mediated metabolism of losartan.

In conclusion, the enhanced oral bioavailability of losartan by resveratrol may result from decreased P-gp-mediated efflux transporter in small intestine and inhibition of CYP 3A- and CYP2C9-mediated metabolism in small intestine and in the liver and/or reduction of total body clearace of losartan.

Based on the results, if the drug interactions are confirmed in the patients with cardiovascular diseases, the dosage of losartan should be readjusted when losartan is used concomitantly with resveratrol.

Key words: Losartan · EXP-3174 · Resveratrol · CYP3A · CYP2C9 · P-gp · Pharmacokinetics · Bioavailability · Rats

Introduction

Losartan potassium (DuP 753 or MK-954), an angiotensin II receptor antagonist, is the first of a new class of agents that was introduced for the treatment of hypertension [1, 2]. Two angiotensin receptor subtypes, angiotensin receptor-1 (AT1) and angiotensin receptor-2 (AT2), have been proposed base on ligand-binding studies [3]. Studies confirmed that losartan is an orally active, long-lasting selective antagonist of AT receptors. Losartan is nearly completely absorbed and extensively metabolized to the active metabolite, EXP-3174 [4]. After oral losartan, about 5% of the dose is excreted unchanged form in the urine and about 8% of the dose is excreted in the urine as EXP-3174. The remainder of the drug is excreted in urine and feces as inactive metabolites (oxidative metabolites or glucuronide conjugates) [5]. Soldner *et al*. suggested that losartan should be a substrate of both cytochrome P450 (CYP) 3A and P-glycoprotein (P-gp) [6]. *In vitro* and *in vivo* studies demonstrated that losartan is metabolized by the CYP3A4 [7-12]. Considering that P-gp is co-localized with CYP3A4 in small intestine, P-gp and CYP3A4 may act synergistically for the presystemic drug metabolism and lead to the prolonged exposure of P-gp substrates to CYP3A4, resulting in the limited absorption of drugs [13-17].

Resveratrol (*trans*-3′,4′,5-trihydroxy-stilbene), a phytoalexin is present in mulberries, peanuts, and grapes, is regularly consumed in the human diet [18]. It is synthesized by grapes in response to fungal infections and is found, therefore, in

red wine at concentrations between 1 and 10 μM [19]. Resveratrol inhibits events associated with tumor initiation, promotion, and progression [20]. Resveratrol is a mechanism-based inactivator of CYP3A4 [21]. Resveratrol inhibits CYP3A subfamily and 1A subfamily in rat and human liver microsomes [22]. Resveratrol inhibits the metabolizing activity of phase-I enzymes (CYPs), and have an effect on P-gp in KB-2 cells [23], but there is no report about relationship between lostartan, P-gp substrate and resveratrol. Resveratrol is metabolized to piceatannol by human CYP1A1 and 1A2 [24]. Piceatannol has potential anticancer activity by the inhibition of an enzyme in human tumor cells [25]. Piceatannol inhibits rat CYP1A1 and 1A2 [26].

It is summarized that the bioavailability of losartan is mainly affected by CYP3A4 and P-gp via the first-pass metabolism. Resveratrol, as a dual inhibitor of CYP3A4 and P-gp, could be expected a therapeutic benefit to improve the bioavailabilty of losartan. Resveratrol and losartan could be prescribed for the prevention or treatment of cardiovascular diseases as a combination therapy. Therefore, the aim of this study is to investigate the effects of resveratrol on the bioavailability or pharmacokinetics of losartan and an active metabolite, EXP-3174, in rats

Materials and Methods

Materials

Losartan, EXP-3174 (a metabolite of losartan) and L-158.809 (internal standard) were obtained from the Merck Co. (NJ, U.S.A.). Resveratrol was purchased from the Sigma-Aldrich Co. (St. Louis, MO, USA). Acetonitrile, methanol, and tert-butylmethylether were purchased from Merck Co. (Darmstadt, Germany). All other chemicals were reagent grade and all solvents were HPLC grade.

Animal studies

The experimental protocols were approved by the Animal Care Committee of Chosun University. Male Sprague-Dawley rats (280-300 g) were purchased from Dae Han Laboratory Animal Research and Co. (Eumsung, Republic of Korea), and had free access to normal standard chow diet (Superfeed Company, Wonju, Republic of Korea) and tap water during the experiment. The animals were housed, four or five per cage, in laminar flow cages maintained at 22 ± 2^{o}C, 50-60% relative humidity, under a 12 h light-dark cycle. The animals were kept in these facilities for at least one week before the experiment. All animal studies were performed in accordance with the "Guiding Principles in the Use of Animals in Toxicology" adopted by the Society of Toxicology (USA) in July 1989 and revised in March 1999.

Drug administration

The rats were divided into the following five groups (n = 6, each group): an oral control group (9 mg/kg of losartan dissolved in distilled water) with or without (control) oral administration of resveratrol at a dose of 0.4, 2 or 8 mg/kg (mixed in distilled water) and 3 mg/kg of losartan dissolved in 0.9% NaCl solution for intravenous administration. The rats were fasted for at least 24 h prior to beginning of the experiments. Each animal was anesthetized with ether and the right femoral artery (for blood sampling) was cannulated with a polyethylene tube (SP45, I.D. 0.58 mm, O.D. 0.96 mm; Natsume Seisakusho Co. Ltd, Tokyo, Japan). Oral losartan was administered intragastrically using a feeding tube, and resveratrol was administered intragastrically 30 min before the oral administration of losartan. Losartan for intravenous (i.v.) administration was injected through the femoral vein within 0.5 min. A 0.45-ml of blood was collected into heparinized tubes from the femoral artery at 0 (to serve as control), 0.017 (only for i.v. group), 0.25, 0.5, 1, 2, 3 (only for oral group), 4, 6, 8, 12, 24 and 36 h (only for oral group) after the administering of losartan. The blood samples were centrifuged at 13,000 rpm for 5 min, and the plasma samples (0.2 ml) were stored at –20℃ until HPLC assay of losartan and EXP-3174.

HPLC assay

The plasma concentrations of losartan were determined by the HPLC assay modified from Zarghi *et al* [27]. Briefly, a 50 μl aliquot of L-158.809 (0.2 μg/ml

dissolved in methanol; an internal standard) and a 0.5 ml aliquot of acetonitril were added to a 0.2 ml aliquot of the plasma sample in a 2.0 ml polypropylene microtube. The mixture was then stirred for 2 min and centrifuged (13,000 rpm, 10 min). A 0.4 ml aliquot of the organic layer was transferred to a clean test tube and evaporated under a gentle stream of nitrogen gas at 35°C. The residue was reconstituted with a 150 μl aliquot of the mobile phase and centrifuged (13,000 rpm, 5 min). A 50 μl aliquot of the water layer was injected into the HPLC system. The HPLC system consisted of two solvent delivery pumps (Model LC-10AD, Shimadzu Co., Japan), a UV-Vis detector (Model SPD-10A), a system controller (Model SCL-10A), degasser (Model DGU-12A) and an autoinjector (SIL-10AD). The UV detector was set to a wavelength of 254 nm. The stationary phase was a Kromasil KR 100-5C_8 column (5 μm, 4.6 × 250 mm, EKA chemicals, Sweden) and the mobile phase was acetonitrile : 0.01 M phosphate buffer (70 : 30 v/v, pH 3, adjusted with phosphoric acid). The retention times at a flow rate of 2 ml/min were as follows: internal standard at 4.2 min, losartan at 11.0 min and EXP-3174 at 21.0 min. The lower limit of quantification for losartan and EXP-3174 in the rat plasma were all 5 ng/ml. The variation coefficients of losartan and EXP-3174 were <13.9% and <15.9%, respectively.

CYP inhibition assay

The assays of inhibition on human CYP3A4 and 2C9 enzyme activities were performed in a multiwell plate using the CYP inhibition assay kit (GENTEST,

Woburn, MA) as described previously [28]. Briefly, human CYP enzymes were obtained from baculovirus-infected insect cells. CYP substrates [7-benzyloxy-4-trifluoromethylcoumarin (7-BFC) and 7-methoxy-4-(trifluoromethyl) coumarin (7-MFC) for CYP3A4 and 2C9, respectively] were incubated with or without test compounds in the reaction mix containing 1 pmol of P450 enzyme and NADPH generating system (1.3 mM NADP, 3.54mM glucose 6-phosphate, 0.4 U/ml glucose 6-phosphate dehydrogenase and 3.3 mM $MgCl_2$) in potassium phosphate buffer (pH 7.4). Reactions were terminated by adding stop solution after 45 min. Metabolite concentrations were measured with a spectrofluorometer (Molecular Device, Sunnyvale, CA) to set at an excitation wavelength of 409 nm and an emission wavelength of 530 nm. Positive controls (1 μM ketoconazole and 2 μM sulfaphenazole for CYP3A4 and 2C9, respectively) were run on the same plate and produced 99% of inhibition. All experiments were performed in duplicate, and results were expressed as the percent of inhibition.

Rhodamine-123 retention assay

The procedures used for the Rho-123 retention assay were similar to a previously reported method [29]. The P-gp overexpressed MCF-7/ADR cells were seeded in 24-well plates. At 80% confluence, the cells were incubated in FBS-free DMEM for 18 h. The culture medium was changed to Hanks' balanced salt solution and the cells were incubated at 37 °C for 30 min. After incubation of the cells with 20 μM rhodamine-123 in the presence and absence of resveratrol (10 or 100 μM) or

verapamil (100 μM) for 90 min, the medium was completely removed. The cells were then washed three times with an ice-cold phosphate buffer (pH 7.0) and lysed in EBC lysis buffer. The rhodamine-123 fluorescence in the cell lysates was measured using excitation and emission wavelengths of 480 and 540 nm, respectively. Fluorescence values were normalized to the total protein content of each sample and presented as the percentage ratio to control.

Pharmacokinetic analysis

The plasma concentration data were analyzed using a noncompartmental method on WinNonlin software version 4.1 (Pharsight Co., Mountain View, CA, USA). The elimination rate constant (K_{el}) was calculated by log-linear regression of losartan concentration data during the elimination phase. The terminal half-life ($t_{1/2}$) was calculated by $0.693/K_{el}$. The peak plasma concentration (C_{max}) and time to reach peak plasma concentration (T_{max}) of losartan in plasma were obtained by visual inspection of the data from the concentration–time curve. The area under the plasma concentration-time curve (AUC_{0-t}) from time zero to the time of last measured concentration (C_{last}) was calculated by the linear trapezoidal rule. The AUC zero to infinite ($AUC_{0-\infty}$) was obtained by the addition of AUC_{0-t} and the extrapolated area determined by C_{last}/K_{el}. The absolute bioavailability (A.B.) of losartan was calculated by $AUC_{oral}/AUC_{i.v.} \times Dose_{i.v.}/Dose_{oral} \times 100$, and the relative bioavailability (R.B.) of losartan was estimated by $AUC_{with\ resveratrol}/AUC_{control} \times 100$. The metabolite-parent ratio (M.R.) was estimated by

($AUC_{EXP-3174}/AUC_{losartan}$).

Statistical analysis

All data were expressed as the mean ± SD. The pharmacokinetic parameters were compared with 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

Inhibition of CYP3A4 and CYP2C9

The inhibitory effect of resveratrol on CYP3A4 and CYP2C9 activity is shown in Fig. 4. Resveratrol inhibited CYP3A4 and CYP2C9 enzyme activity and the 50% inhibition concentration (IC_{50}) values of resveratrol on CYP3A4 and CYP2C9 activity were 1.0 and 8.4 μM, respectively.

Rhodamine-123 retention assay

The cell-based P-gp activity test using rhodamine-123 showed that resveratrol (100 μM, $p < 0.01$) significantly inhibited P-gp activity (Fig. 5).

Effect of resveratrol on the pharmacokinetics of oral losartan

The mean plasma concentration-time profiles of losartan in the presence or absence of resveratrol were characterized in Fig.6. The mean pharmacokinetic parameters of losartan were also summarized in Table 1.

As shown in Table 1, resveratrol (2 or 8 mg/kg) significantly altered the pharmacokinetic parameters of losartan compared to the control (given losartan alone). Resveratrol significantly (2 mg/kg, $p < 0.05$; 8 mg/kg, $p < 0.01$) increased the area under the plasma concentration–time curve ($AUC_{0-\infty}$) of losartan by 24.9–52.3% and peak plasma concentration (C_{max}) of losartan by 25.0–50.0%. The total body clearance (CL/F) was significantly decreased by resveratrol (2 mg/kg, $p <$

0.05; 8 mg/kg, $p < 0.01$). Consequently, the absolute bioavailability (A.B.) of losartan in the presence of resveratrol was 31.7–38.7%, which was enhanced significantly ($p < 0.05$) compared with the oral control group (25.4%). The relative bioavailability (R.B.) of losartan was increased by 1.10- to 1.52-fold than that of the control group. However, there was no significant change in the peak plasma concentration (T_{max}) and terminal half-life ($t_{1/2}$) of losartan in the presence of resveratrol.

Effect of resveratrol on the pharmacokinetics of EXP-3174

The mean plasma concentration-time profiles of EXP-3174 in the presence or absence of resveratrol were characterized in Fig.7. The mean pharmacokinetic parameters of EXP-3174 were also summarized in Table 2.

As summarized in Table 2, resveratrol (8 mg/kg) significantly increased $AUC_{0-\infty}$ of EXP-3174 but it is not significant. Metabolite-parent AUC ratio in the presence of resveratrol (8 mg/kg) significantly decreased by 21.0 % ($p < 0.05$) compared to the control group, suggesting that the presence of resveratrol could be effective to inhibit the CYP3A mediated metabolism of losartan.

Effect of resveratrol on the pharmacokinetics of intravenous losartan

Mean arterial plasma concentration-time profiles of losartan after an intravenous administration of losartan (3 mg/kg) to rats in the presence or absence of resveratrol (0.4, 2 and 8 mg/kg) are shown in Figure 8, the corresponding

pharmacokinetic parameters are shown in Table 3.

The $AUC_{0-\infty}$ of losartan was increased, but was not statistically significant compared to that in the control. The $t_{1/2}$ of losartan was also prolonged, but this increase was not significant. The pharmacokinetics of intravenous losartan was not affected by the concurrent use of resveratrol in contrast to those of oral losartan. Therefore, the enhanced oral bioavailability in the presence of resveratrol, while there was no significant change in the pharmacokinetics of intravenous losartan, may be mainly due to inhibition of the CYP3A-mediated metabolism of losartan in the small intestine and/or in the liver rather than to reduction of renal elimination of losartan by resveratrol.

Discussion

With the great interest in herbal products as alternative medicines, much effort is currently being expanded toward identifying natural compounds from plant origins that modulate P-gp as well as metabolic enzymes. However, there is far less information on the pharmacokinetic interactions between herbal products and medicines. Therefore, more preclinical and clinical investigations on the herbal constituents-drug interaction should be performed to prevent potential adverse reactions or to utilize those interactions for a therapeutic benefit.

Based on the broad overlap in the substrate specificities as well as co-localization in the small intestine, the primary site of absorption for orally administered drugs, CYP3A4 and P-glycoprotein have been recognized as a concerted barrier to the drug absorption [30, 31]. Therefore, dual inhibitors against both CYP3A4 and P-gp should have a great impact on the bioavailability of many drugs where CYP3A4 metabolism as well as P-gp mediated efflux is the major barrier to the systemic availability. Besides the extensive metabolism by CYP3A4, losartan appeared to be the substrate of P-glycoprotein, suggesting that P-glycoprotein and CYP3A4 should act synergistically to limit the oral bioavailability of losartan [14, 32]. Therefore, the present study evaluated the effect of resveratrol, an antioxidant, on the bioavailabilty and pharmacokinetics of losartan in rats to examine a potential drug interaction between resveratrol and losartan via the dual inhibition of CYP3A4 and P-gp.

As shown in Table 1, the presence of resveratrol (2 and 8 mg/kg) significantly enhanced AUC of losartan. Losartan is a substrate of both CYP3A4 and P-gp [6], and resveratrol was reported to modulate CYP3A4-mediated metabolism as well as P-gp efflux pump [25-26]. Therefore, resveratrol might be due to the inhibition of P-gp and CYP3A4 increased absorption of losartan in the intestine. These results were consistent with previous study, resveratrol inhibited both CYP3A4 and P-gp and increased bioavailability of diltiazem and nicardipine in rats, respectively [33, 34].

The pharmacokinetic profiles of EXP-3174 were also evaluated in the presence or absence of resveratrol (Table 2). The metabolite-parent ratio in the presence of 8 mg/kg of resveratrol decreased significantly ($p < 0.05$) compared to the control, suggesting that the presence of resveratrol could be effective to inhibit the CYP3A mediated metabolism of losartan. The enhanced oral bioavailability of losartan in the presence of resveratrol at 8 mg/kg dose might be due to the inhibition of both CYP3A and P-gp efflux transport by resveratrol. Considering the inhibitory potency of resveratrol against CYP3A, the dose of 0.4 and 2 mg/kg might be too low to inhibit the formation of EXP-3174 [23].

In contrast to the pharmacokinetics of oral losartan, the intravenous pharmacokinetics of losartan was not significantly affected by the concurrent use of resveratrol (Table 3). Therefore, the enhanced oral bioavailability of losartan in the presence of resveratrol might be mainly due to enhanced absorption in the gastrointestinal tract by the inhibition of P-gp efflux transporter and reduced first-

pass metabolism of losartan in the small intestine and/or in the liver by the inhibition of CYP3A4 and CYP2C9 activity in rats.

Taken all together, the pharmacokinetics of losartan was significantly altered by the presence of resveratrol, a dual inhibitor of CYP3A and P-gp. Clinical importance of these findings warrants further investigation in clinical trials.

Conclusion

Resveratrol significantly enhanced the oral bioavailability of losartan in rats. The enhanced bioavailability of losartan might be due to inhibition of the CYP3A and CYP2C9-mediated metabolism of losartan in the liver and the P-gp efflux transporter in the small intestine and/or reduction of the total body clearance of losartan by resveratrol. Therefore, concomitant use of resveratrol with losartan may require close monitoring for potential drug interactions. The clinical importance of these findings should be further investigated in clinical trials.

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Table 1. Mean pharmacokinetic parameters of losartan after an oral (9 mg/kg) administration of losartan to rats in the presence or absence of resveratrol (Mean ± SD, n = 6)

Parameters	Control	Losartan + Resveratrol		
		0.4 mg/kg	2 mg/kg	8 mg/kg
$AUC_{0-\infty}$ (ng·h/mL)	226.0 ± 37.6	249.4 ± 41.5	282.3 ± 49.8*	344.3 ± 59.7**
C_{max} (ng/mL)	18.8 ± 3.4	21.0 ± 3.7	23.5 ± 4.2*	28.2 ± 4.9**
T_{max} (h)	0.92 ± 0.21	0.92 ± 0.21	1.17 ± 0.41	1.17 ± 0.41
CL/F (mL/hr/kg)	664.1 ± 126.0	602.2 ± 112.1	532.2 ± 101.1*	446.8 ± 84.9**
$t_{1/2}$ (h)	10.6 ± 2.1	10.8 ± 2.2	11.2 ± 2.3	11.3 ± 2.4
A.B. (%)	25.4 ± 4.8	28.1 ± 5.1	31.7 ± 5.7*	38.7 ± 7.0**
R.B. (%)	100	110	125	152

* $p < 0.05$, ** $p < 0.01$, significant difference compared to the control group given losartan alone.

$AUC_{0-\infty}$: area under the plasma concentration-time curve from 0 h to infinity; C_{max}: peak plasma concentration; T_{max}: time to reach C_{max}; CL/F, total body clearance; $t_{1/2}$: terminal half-life; A.B.: absolute bioavailability; R.B.: relative bioavailability.

Table 2. Mean pharmacokinetic parameters of EXP-3174 after an oral (9 mg/kg) administration of losartan to rats in the presence or absence of resveratrol (Mean ± SD, n = 6)

Parameters	Control	Losartan + Resveratrol		
		0.4 mg/kg	2 mg/kg	8 mg/kg
$AUC_{0-\infty}$ (ng ·h/mL)	231.0 ± 41.8	244.2 ± 42.2	259.1 ± 44.7	272.3 ± 49.1
C_{max} (ng/mL)	17.3 ± 3.1	18.5 ± 3.2	20.3 ± 3.5	21.7 ± 3.7
T_{max} (h)	1.67 ± 0.52	1.67 ± 0.52	1.83 ± 0.41	2.17 ± 0.41
$t_{1/2}$ (h)	9.5 ± 1.8	9.8 ± 1.9	10.0 ± 1.8	10.4 ± 2.1
M.R. (%)	1.00 ± 0.20	0.98 ± 0.18	0.92 ± 0.16	0.77 ± 0.13*
R.B. (%)	100	106	113	120

* $p < 0.05$, significant difference compared to the control group given losartan alone.

$AUC_{0-\infty}$: area under the plasma concentration-time curve from 0 h to infinity; C_{max}: peak plasma concentration; T_{max}: time to reach C_{max}; R.B.: relative bioavailability; M.R.: metabolite-parent ratio ($AUC_{EXP-3174}$ /$AUC_{losartan}$).

Table 3. Mean pharmacokinetic parameters of losartan after the intravenous administration (3 mg/kg) of losartan to rats in the presence or absence of resveratrol (Mean ± SD, n = 6)

Parameter	Control	Losartan + Resveratrol		
		0.4 mg/kg	2 mg/kg	8 mg/kg
$AUC_{0-\infty}$ (ng·h/mL)	296.1 ± 59.2	311.2 ± 61.7	326.2 ± 65.3	345.1 ± 67.3
CL_t (mL/hr/kg)	168.9 ± 62.8	160.6 ± 28.9	153.4 ± 28.7	144.4 ± 26.1
$t_{1/2}$ (h)	8.2 ± 1.3	8.4 ± 1.4	8.6 ± 1.5	8.7 ± 1.6
R.B. (%)	100	105	110	116

$AUC_{0-\infty}$: total area under the plasma concentration–time curve from time zero to infinity, CL_t: total body clearance, $t_{1/2}$: terminal half-life, R.B.: relative bioavailability.

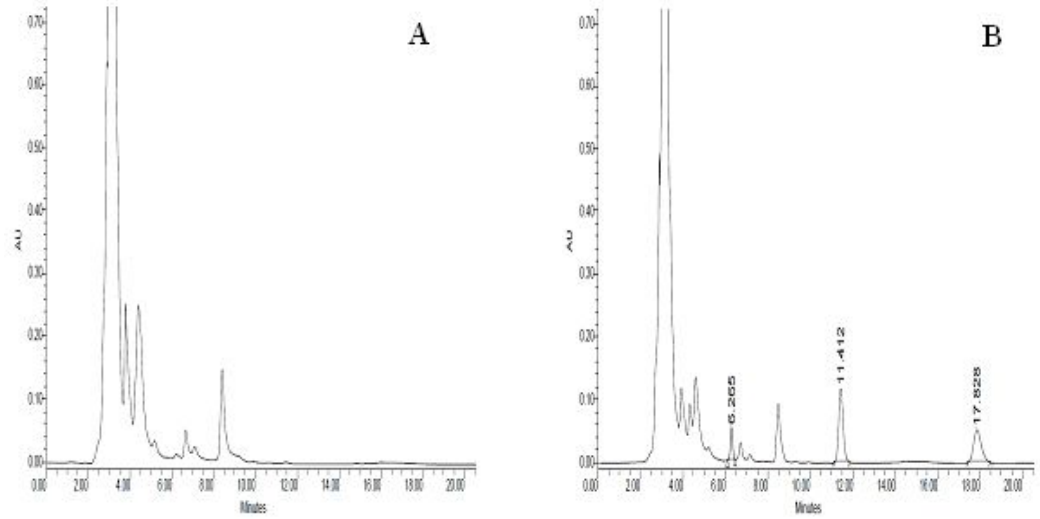

Figure 1. HPLC chromatograms of the rat blank plasma (A), and the plasma spiked with losartan (11.4 min), EXP-3174 (17.9 min), and L-158.809 (internal standard; 6.3 min) (B).

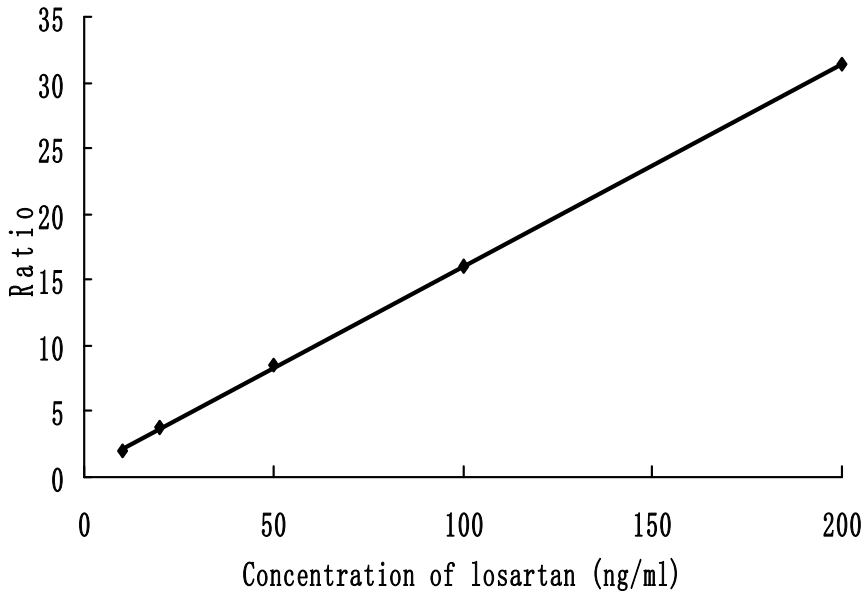

Figure 2. A calibration curve of losartan when spiked into the rat blank plasma. The typical equation describing the calibration curve in rat plasma was y=0.154x+0.6171, where “y” is the peak area ratio of losartan to L-158.809 and “x” is the concentration of losartan.

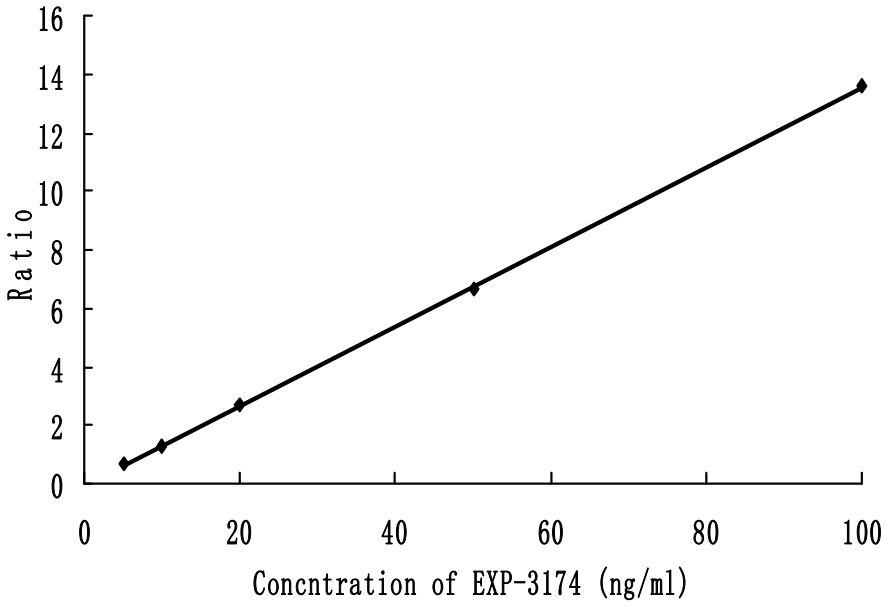

Figure 3. A calibration curve of EXP-3174 when spiked into the rat blank plasma. The typical equation describing the calibration curve in rat plasma was y=0.1359x − 0.0507, where “y” is the peak area ratio of EXP-3174 to L-158.809 and “x” is the concentration of EXP-3174.

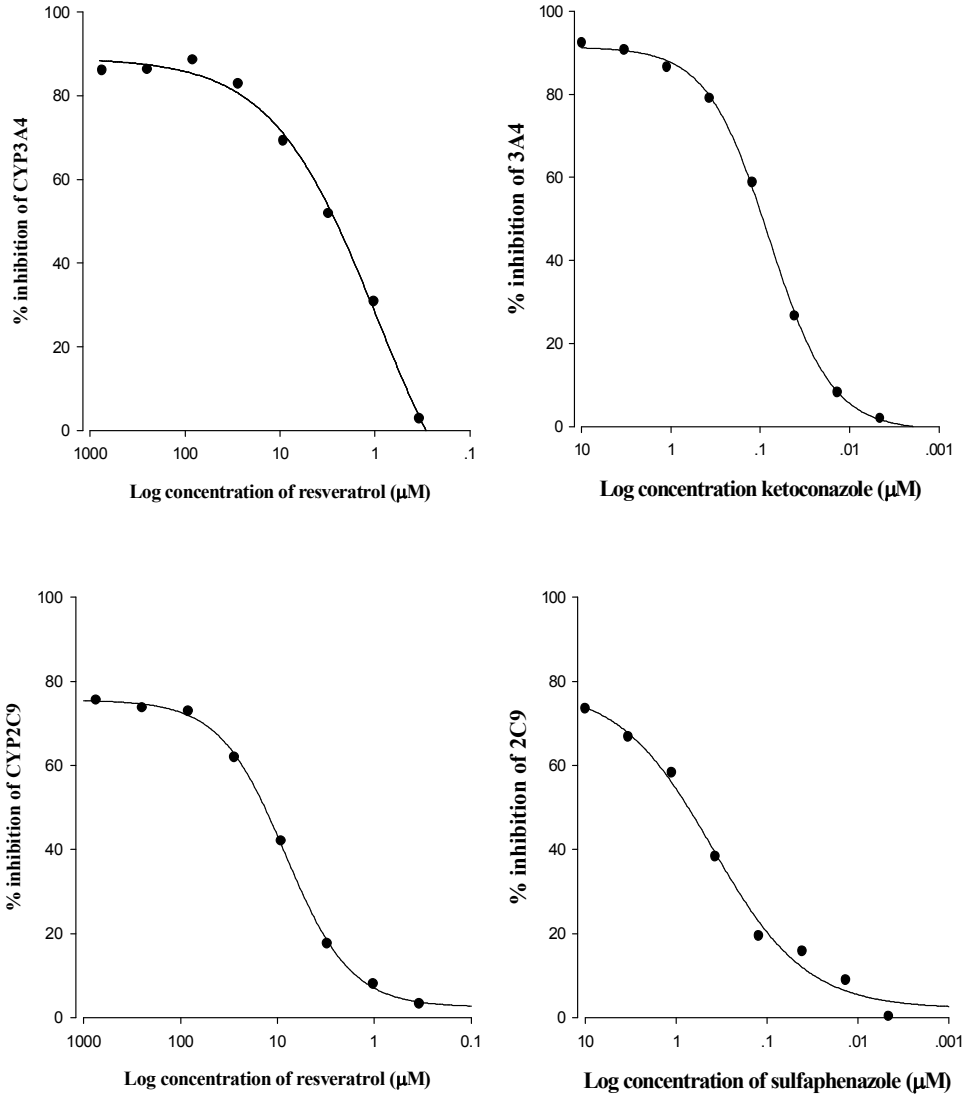

Figure 4. Inhibitory effect of resveratrol on CYP3A4 and 2C9 activity. All experiments were done in duplicate, and results are expressed as the percentage of inhibition.

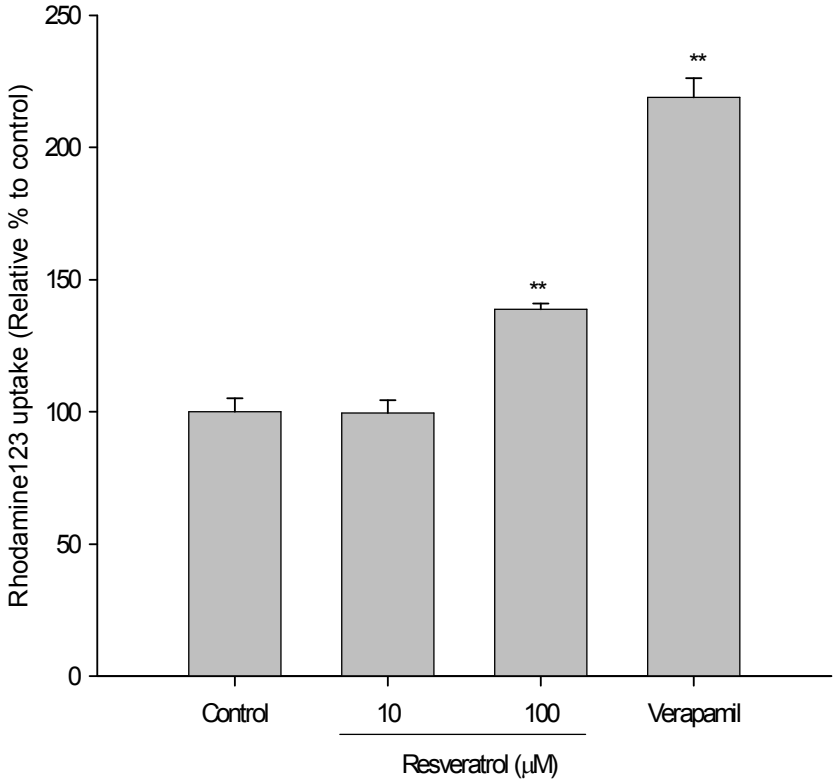

Figure 5. Rhodamine-123 (R-123) retention. After incubation of MCF-7/ADR cells with 20 μM R-123 for 90 min, the R-123 fluorescence values in cell lysates were measured using the excitation and emission wavelengths of 480 and 540 nm, respectively. The values were divided by total protein contents of each sample. Data represents means ± SD of 4 separate samples (significant versus the control MCF-7 cells, $^{**}p < 0.01$). Verapamil (100 μM) was used as a positive control.

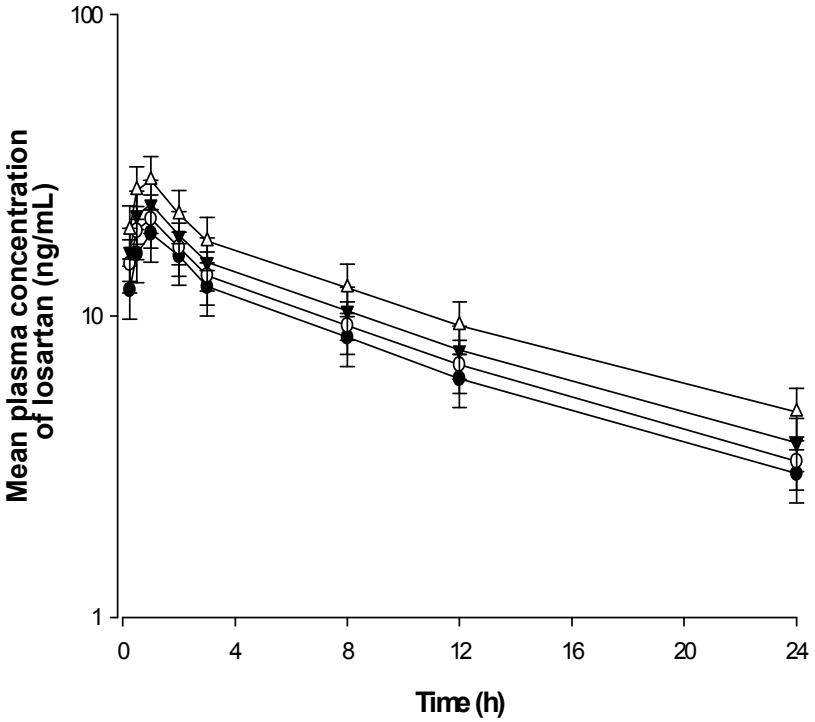

Figure 6. Mean plasma concentration-time profiles of losartan following an oral (9 mg/kg) administration of losartan to rats in the presence and absence of resveratrol (Mean ± SD, n = 6). ●; Control (losartan 9 mg/kg, alone), ○: co-administered with 0.4 mg/kg of resveratrol; ▼: co-administered with 2 mg/kg of resveratrol; △: co-administered with 8 mg/kg of resveratrol.

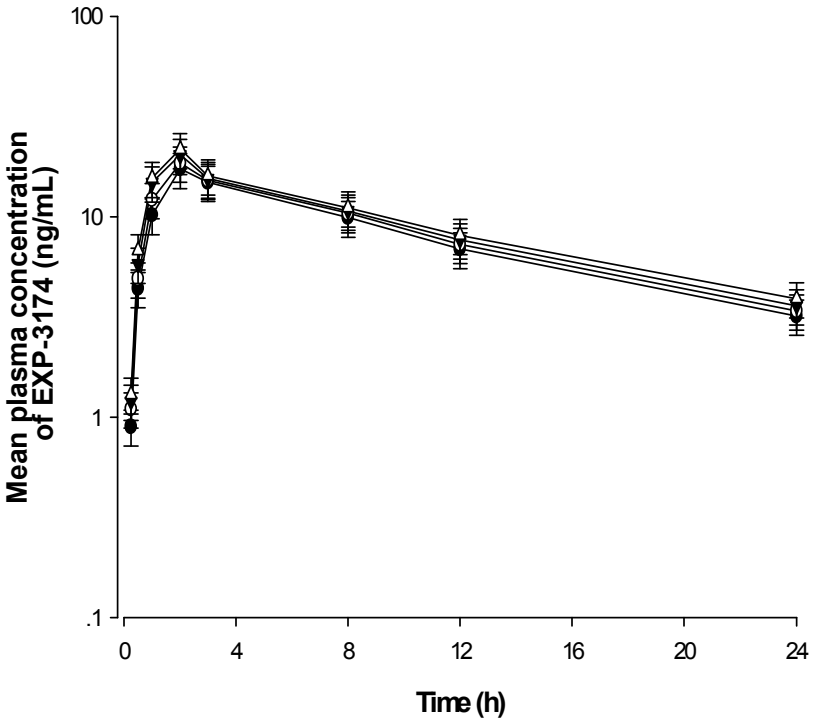

Figure 7. Mean plasma concentration-time profiles of EXP-3174 after an oral administration of losartan (9 mg/kg) to rats in the presence and absence of resveratrol (Mean ± SD, n = 6). ●; Control (losartan 9 mg/kg, alone), ○: co-administered with 0.4 mg/kg of resveratrol; ▼: co-administered with 2 mg/kg of resveratrol; △: co-administered with 8 mg/kg of resveratrol.

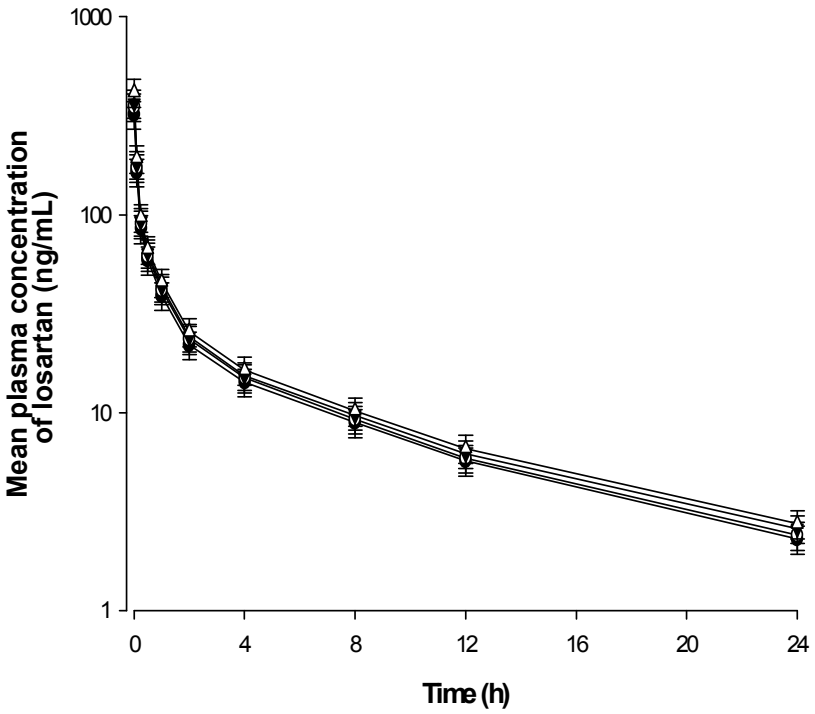

Figure 8. Mean plasma concentration-time profiles of losartan after intravenous of losartan (3 mg/kg) administration to rats in the presence or absence of resveratrol at doses of 2 and 8 mg/kg. (Mean ± SD, n = 6). ●; Control (losartan 3 mg/kg, alone), ○: co-administered with 0.4 mg/kg of resveratrol; ▼: co-administered with 2 mg/kg of resveratrol; △: co-administered with 8 mg/kg of resveratrol.

Part II. Effects of Licocharcon A on the Bioavailability of Losartan in Rats

Abstract

Losartan and licocharcon A interact with CYP enzymes and P-gp, and the increase in the use of health supplements may result in licocharcon A being taken concomitantly with losartan to treat or prevent cardiovascular diseases as a combination therapy. In the present study, the effect of licocharcon A, a natural flavonoid, on the pharmacokinetics of losartan and its active metabolite, EXP-3174, was investigated in rats.

Pharmacokinetic parameters of losartan and EXP-3174 were determined after oral administration of losartan (9 mg/kg) to rats in the presence or absence of licocharcon A (0.4, 2 and 8 mg/kg). The effect of licocharcon A on the P-glycoprotein (P-gp) as well as CYP3A4 and 2C9 activity was also evaluated. Licocharcon A inhibited CYP3A4 and CYP2C9 enzyme activity with 50% inhibition concentration (IC_{50}) of 2.0 and 0.06 μM, respectively. In addition, licocharcon A significantly enhanced the cellular accumulation of rhodamine-123 in MCF-7/ADR cells overexpressing P-gp in a concentration-dependent manner.

The pharmacokinetic parameters of losartan were significantly altered by the licocharcon A compared to those in the control group. The presence of licocharcon A (2 mg/kg or 8 mg/kg) increased $AUC_{0-\infty}$ of losartan by 33.4–63.2% and C_{max} of

losartan by 34.0–62.8%. The total body clearance (CL/F) was significantly decreased (2 mg/kg, $p < 0.05$; 8 mg/kg, $p < 0.01$) by licocharcon A. Consequently, the absolute bioavailability of losartan in the presence of licocharcon A increased significantly (2 mg/kg, $p < 0.05$; 8 mg/kg, $p < 0.01$) compared to that in the control group. The relative bioavailability (R.B.) of losartan was 1.15- to 1.63-fold greater than that of the control group. However, there was no significant change in T_{max}, $t_{1/2}$ of losartan in the presence of licocharcon A. Presence of licocharcon A (8 mg/kg) increased the $AUC_{0-\infty}$ of EXP-3174 but it is not significant. Furthermore, concurrent use of licocharcon A (8 mg/kg) significantly decreased the metabolite-parent AUC ratio (M.R.) by 20%, suggesting that licocharcon A inhibited the CYP-mediated metabolism of losartan to active metabolite, EXP-3174.

In conclusion, the enhanced oral bioavailability of losartan by licocharcon A may result from decreased P-gp-mediated efflux transporter in small intestine and inhibition of CYP 3A- and CYP2C9-mediated metabolism in small intestine and in the liver and/or reduction of total body clearace of losartan.

Based on the results, if the drug interactions are confirmed in the patients with cardiovascular diseases, the dosage of losartan should be readjusted when losartan is used concomitantly with licocharcon A.

Key words: Losartan · EXP-3174 · Licocharcon A · CYP3A · CYP2C9 · P-gp · Pharmacokinetics · Bioavailability · Rats

Introduction

Losartan is the prototype of a new class of orally active and long-lasting selective antagonists of angiotensin II receptors for the treatment of hypertension [1-3]. Following oral administration, losartan is rapidly absorbed, reaching maximum concentrations 1-2 hours post-dose, but it has low and highly variable oral bioavailabilities (12.1-66.6%) [4]. Losartan is metabolized to active metabolite, EXP-3174 which is about 10-fold more potent than its parent drug. Thus, the clinical hypotensive activity is predominantly mediated by the active metabolite EXP-3174, although losartan itself exhibited good efficacy [5]. In addition to metabolism of losartan to EXP-3174, losartan undergoes glucuronidation on the hydroxyl and tetrazole groups that shows species differences in the extent of oxidation vs. conjugation [6]. Some *in vitro* and *in vivo* studies have indicated that losartan is metabolized to EXP-3174 mainly by cytochrome P450 (CYP) 2C9 and 3A4 enzymes [6-10]. Furthermore, Soldner *et al*. [11] have suggested that losartan is a substrate of P-glycoprotein (P-gp). Since losartan is a substrate of both CYP enzymes and P-gp, the modulation of CYP and P-gp activities may cause the significant changes in the pharmacokinetic profiles of losartan and its active metabolite, EXP-3174. Zaidenstein *et al.* [12] have reported that concomitant intake of grapefruit juice significantly altered some of pharmacokinetic parameters of losartan and its metabolite EXP-3174 in healthy volunteers by the inhibition of CYP3A4 metabolism. Kobayashi *et al.* [13] have demonstrated that bucolome, a

CYP2C9 inhibitor, significantly increased the $AUC_{0-\infty}$ of losartan but decreased the $AUC_{0-\infty}$ of EXP-3174. In addition, flavonoids, epigallocatechin and hesperidin increased $AUC_{0-\infty}$ and C_{max} of verapamil (a substrat of CYP3A4 and P-gp) in rats [14, 15], and morin and hesperidin increased $AUC_{0-\infty}$ and C_{max} of diltiazem (a substrat of CYP3A4 and P-gp) in rats [16, 17]. These previous studies strongly suggested that there should be potential drug interactions should be occurred the inhibition of CYP-mediated metabolism of losartan.

Flavonoids represent a group of phytochemicals that are produced in high quantities by various plants [18]. These compounds exhibit a wide range of beneficial biological activities including antioxidative, radical-scavenging, anti-atherosclerotic, antitumor and antiviral effects [19]. Licochalcone A is an estrogenic flavonoid and the main active compound of the licorice species *Glycyrrhiza inflate* [20]. Licochalcone A also has a wide range of biological and pharmacological activities, including antioxidant, superoxide scavenging [21], anti-leishmanial activity, and effects on the function of parasite mitochondria [22], antimalarial activities both *in vitro* and *in vivo* [23], and antitumor activities in cancer cells [24-26]. Kwon *et al*. demonstrated that licochalcone A exerts anti-inflammatory effects by suppressing nuclear factor-kB (NF-kB) and activator protein-1 (AP-1) signaling [27]. Kim *et al*. provide the first evidence that licochalcone A could inhibit the angiogenesis *in vitro* and *in vivo* and the tumor growth [28].

We evaluated CYP enzymes activities and P-gp activity about licochalcone A

using CYP inhibition assays and rhodamine-123 retention assays in P-gp-over-expressed MCF-7/ADR cells. Choi *et al*. reported the effects of flavonoid (myricetin) on the bioavailability of losartan in rats [29]. Licochalcone A is expected to change the bioavailability and pharmacokinetics of drugs those are substrates of P-gp and/or CYP3A4, if they are used concomitantly.

Losartan and licochalcone A interact with CYP enzymes and P-gp, and the increased use of health supplements may result in licochalcone A being taken concomitantly with losartan to treat or prevent cardiovascular diseases as a combination therapy. It is important to assess the potential pharmacokinetic interactions after the concurrent use of losartan and licochalcone A or licochalcone A containing dietary supplement in order to assure the effectiveness and safety of drug therapy. However, the possible effects of licochalcone A on the bioavailability and pharmacokinetics of losartan have not been reported *in vivo*.

Therefore, in the present study, we investigated the effects of licochalcone A on the pharmacokinetics of losartan and its active metabolite, EXP-3174, in rats.

Materials and Methods

Materials

Losartan, its metabolite EXP-3174 and L-158.809 (internal standard) were kindly provided by Merck Co. (NJ, USA). Licocharcon A was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Acetonitrile, methanol and tert-butylmethylether were purchased from Merck Co. (Darmstadt, Germany). All other chemicals were of reagent grade and all solvents were of HPLC grade.

Animal studies

All animal studies were performed in accordiance with the "Guiding Principles in the Use of Animals in Toxicology" adopted by the Society of Toxicology (USA) and the experimental protocols were approved by the Animal Care Committee of Chosun University. Male Sprague-Dawley rats (280~300 g) were purchased from Dae Han Laboratory Animal Research and Co. (Eumsung, Korea), and had free access to normal standard chow diet (Superfeed Company, Wonju, Korea) and tap water. Throughout the experiment, the animals were housed, four or five per cage, in laminar flow cages maintained at 22 ± 2^{o}C, 50~60% relative humidity, under a 12-h light-dark cycle. The animals were kept in these facilities for at least one week before the experiment. Rats were fasted for 24 h prior to beginning the experiments.

Drug administration

Rats were divided into seven groups (n = 6 per each group) as follows: Group 1: losartan (9 mg/kg, p.o., control), Group 2~4: losartan (9 mg/kg, p.o.) with licocharcon A (0.4, 2 or 8 mg/kg, given orally at 30 min prior to the administration of losartan), and Group 5: intravenous losartan (3 mg/kg) with licocharcon A (0.4, 2 or 8 mg/kg, given orally at 30 min prior to the administration of losartan),. Blood samples were collected from the femoral artery into heparinized tubes at 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h after oral administration of losartan and at 0, 0.1, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h after intravenous administration of losartan. The blood samples were centrifuged at 13,000 rpm for 5 min and the obtained plasma samples were stored at –40℃ until analyzed by HPLC.

HPLC assay

The plasma concentrations of losartan were determined by the HPLC assay reported by Zarghi *et al.* [30] with slight modification. Briefly, 50 μl of L-158.809 (0.2 μg/ml dissolved in methanol; an internal standard) and 0.5 ml of acetonitrile were added to a 0.2-ml aliquot of plasma sample in a polypropylene microtube. The mixture was then stirred for 5 min and centrifuged at 13,000 rpm for 10 min. A 0.5-ml aliquot of the organic layer was transferred into a clean test tube and evaporated under a gentle stream of nitrogen gas at 35°C. The residue was reconstituted with 150 μl of the mobile phase and centrifuged at 13,000 rpm for 5

min and then a 70-μl aliquot of the supernatant was injected into the HPLC system. The HPLC system consisted of two solvent delivery pumps (Model LC-10AD, Shimadzu Co., Japan), a UV-Vis detector (Model SPD-10A), a system controller (Model SCL-10A), a degasser (Model DGU-12A) and an autoinjector (SIL-10AD). The UV detector was set at 215 nm. The stationary phase was a Kromasil KR 100-5C_8 column (5 μm, 4.6 × 250 mm, EKA Chemicals, Sweden) and the mobile phase was acetonitrile:0.01 M phosphate buffer (41:59 v/v, pH 2.5 adjusted with phosphoric acid). The retention times at a flow rate of 0.8 ml/min were as follows: 6.7 min of internal standard, 11.5 min of losartan and 17.1 min of EXP-3174. The lower limit of quantification for losartan and EXP-3174 in the rat plasma were all 5 ng/ml.

CYP3A4 and 2C9 inhibition assay

The assays of inhibition on human CYP3A4 and 2C9 enzyme activities were performed in a multiwell plate using CYP inhibition assay kit (GENTEST, Woburn, MA) as described previously [31]. Briefly, human CYP enzymes were obtained from baculovirus-infected insect cells. CYP substrates [7-benzyloxy-4-trifluoromethylcoumarin (7-BFC) and 7-methoxy-4-(trifluoromethyl) coumarin (7-MFC) for CYP3A4 and 2C9, respectively] were incubated with or without licocharcon A in the enzyme/substrate contained buffer with 1 pmol of P450 enzyme and a NADPH-generating system (1.3 mM NADP, 3.54mM glucose 6-phosphate, 0.4 U/ml glucose 6-phosphate dehydrogenase and 3.3 mM $MgCl_2$) in a

potassium phosphate buffer (pH 7.4). Reactions were terminated by adding stop solution (0.5M Tris-buffer mixed with acetonitrile as a stop solution) after 45-min incubation. Metabolite concentrations were measured by spectrofluorometer (Molecular Device, Sunnyvale, CA) at an excitation wavelength of 409 nm and an emission wavelength of 530 nm. Positive control (1 μM ketoconazole and 2 μM sulfaphenazole for CYP3A4 and 2C9, respectively) was run on the same plate and produced 99% inhibition. All experiments were done in duplicate, and the results are expressed as the percentage of inhibition.

Rhodamine-123 retention assay

The procedures used for the Rho-123 retention assay were similar to a previously reported method [32]. MCF-7/ADR cells were seeded in 24-well plates. At 80% confluence, the cells were incubated in FBS-free DMEM for 18 h. The culture medium was changed to Hanks' balanced salt solution and the cells were incubated at 37°C for 30 min. After incubation of the cells with 20 μM rhodamine-123 in the presence of licocharcon A (1, 3 and 10 μM) for 90 min, the medium was completely removed. The cells were then washed three times with an ice-cold phosphate buffer (pH 7.0) and lysed in lysis buffer. The rhodamine-123 fluorescence in the cell lysates was measured using excitation and emission wavelengths of 480 and 540 nm, respectively. Fluorescence values were normalized to the total protein content of each sample and presented as the ratio to control.

Pharmacokinetic analysis

Non-compartmental pharmacokinetic analysis was performed using WinNonlin software version 5.2.1 (Pharsight Co., Mountain View, CA, USA). The area under the plasma concentration-time curve (AUC_{0-t}) from time zero to the time of last measured concentration (C_{last}) was calculated by the linear trapezoidal rule. The AUC zero to infinite ($AUC_{0-\infty}$) was obtained by the addition of AUC_{0-t} and the extrapolated area determined by C_{last}/K_{el}. The peak plasma concentration (C_{max}) and the time to reach the peak plasma concentration (T_{max}) were observed values from the experimental data. The elimination rate constant (K_{el}) was calculated by log-linear regression of losartan or EXP-3174 concentration data during the elimination phase. The terminal half-life ($t_{1/2}$) was calculated by $0.693/K_{el}$. The total body clearance for i.v. route (CL_t) was calculated from D/ AUC, where D is the dose of losartan. The mean residence time (MRT) was calculated by dividing the first moment of AUC ($AUMC_{0-\infty}$) by $AUC_{0-\infty}$. The apparent volume of distribution at steady state (V_{dss}) was estimated by the product of $MRT_{i.v.}$ and CL_t after i.v. dosing. The bioavailability (A.B.) of losartan was calculated by $AUC_{oral}/AUC_{i.v.} \times Dose_{i.v.}/Dose_{oral} \times 100$, the relative bioavailability (R.B.) was calculated by $(AUC_{with\ losartan}/AUC_{control}) \times 100$ and the metabolite-parent ratio (M.R.) was estimated by $(AUC_{EXP-3174}/AUC_{losartan})$.

Statistical analysis

All data were expressed as the mean ± SD. The pharmacokinetic parameters were compared by one-way ANOVA, followed by a posteriori testing with the Dunnett correction. A P value < 0.05 was considered statistically significant.

Results

Inhibition of CYP3A4 and 2C9

The inhibitory effect of licocharcon A on CYP3A4 and CYP2C9 activity is shown in Fig. 12. Licocharcon A inhibited CYP3A4 and CYP2C9 enzyme activity and the 50 % inhibition concentration (IC_{50}) values of licocharcon A on CYP3A4 and CYP2C9 activity were 2.0 and 0.06 μM, respectively.

Rhodamine-123 retention assay

As shown in Fig. 13, accumulation of rhodamine-123, a P-gp substrate, was reduced in MCF-7/ADR cells overexpressing P-gp compared to that in MCF-7 cells lacking P-gp. The concurrent use of licocharcon A enhanced the cellular uptake of rhodamine-123 in a concentration dependent manner and showed statistically significant ($p < 0.01$) increase at the concentration range of 3-10 μM. This result suggests that licocharcon A significantly inhibits P-gp activity.

Effects of licocharcon A on the pharmacokinetics of oral losartan

The plasma concentration-time profiles of losartan in the presence or absence of licocharcon A were characterized in Fig.14. The mean pharmacokinetic parameters of losartan were also summarized in Table 4.

As shown in Table 4, the presence of licocharcon A (2 or 8 mg/kg) significantly altered the pharmacokinetic parameters of losartan compared to those in the control

group (given losartan alone). Licocharcon A significantly (2 mg/kg, $p < 0.05$; 8 mg/kg, $p < 0.01$) increased $AUC_{0-\infty}$ of losartan by 33.4–63.2% and C_{max} of losartan by 34.0–62.8%. The total body clearance (CL/F) was significantly decreased (2 mg/kg, $p < 0.05$; 8 mg/kg, $p < 0.01$) by licocharcon A. Consequently, the absolute bioavailability (A.B.) of losartan in the presence of licocharcon A was significantly (2 mg/kg, $p < 0.05$; 8 mg/kg, $p < 0.01$) higher than that in the control group. The relative bioavailability (R.B.) of losartan was 1.15- to 1.63-fold greater than that of the control group. However, there was no significant change in T_{max}, $t_{1/2}$ of losartan with licocharcon A. Given that losartan is a substrate of P-gp, and CYP3A4 and 2C9, the enhanced bioavailability of losartan might be due to the inhibition of the CYP3A and CYP2C9-mediated metabolism of losartan in the liver and the P-gp efflux pump in the small intestine by licocharcon A.

Effects of licocharcon A on the pharmacokinetics of EXP-3174

The plasma concentration-time profiles of EXP-3174, an active metabolite, in the presence of licocharcon A were increased compared to those in the control group in Fig. 15. As shown in Table 5, licocharcon A significantly (8 mg/kg, $p < 0.05$) increased $AUC_{0-\infty}$ of losartan but it is not significant. The metabolite-parent AUC ratios decreased by 23% in the presence of licocharcon A compared to that in the control group, suggesting that licocharcon A inhibit the CYP-mediated metabolism of losartan. However, there was no significant change in C_{max}, T_{max}, $t_{1/2}$ of EXP-3174 with licocharcon A.

Effects of lococharcon A on the pharmacokinetics of intravenous losartan

Mean arterial plasma concentration-time profiles of losartan following an intravenous administration of losartan (3 mg/kg) to rats in the presence or absence of licocharcon A (0.4, 2 and 8 mg/kg) are shown in Figure 16, the corresponding pharmacokinetic parameters are shown in Table 6.

The $AUC_{0-\infty}$ of losartan was increased, but was not statistically significant compared to that in the control. The $t_{1/2}$ of losartan was also prolonged, but this increase was not significant. The pharmacokinetics of intravenous losartan was not affected by the concurrent use of licocharcon A in contrast to those of oral losartan. Accordingly, the enhanced oral bioavailability in the presence of licocharcon A, while there was no significant change in the pharmacokinetics of intravenous losartan, may be mainly due to inhibition of the CYP3A mediated metabolism of losartan in the small intestine and/or in the liver rather than to reduction of renal elimination of losartan by licocharcon A.

Discussion

Some *in vitro* and *in vivo* studies have indicated that losartan is metabolized to EXP-3174 mainly by CYP2C9 and 3A4 enzymes [6-10]. Furthermore, Soldner *et al*. [11] have suggested that losartan is a substrate of P-glycoprotein (P-gp). Considering that losartan is a substrate of both CYP enzymes and P-gp, the modulation of CYP and P-gp activities may cause the significant changes in the pharmacokinetic profiles of losartan and its active metabolite, EXP-3174.

P-gp is colocalized with CYP3A4 in the apical membrane of the intestine [33, 34], and they act synergistically in regulating the first-pass metabolism and bioavailability of many orally drugs. The inhibitory effect of licocharcon A against CYP3A4-mediated metabolism was confirmed by the employment of recombinant CYP3A4 enzyme. As shown in Fig. 12, licocharcon A exhibited inhibitory effect against CYP3A4-mediated metabolism with IC_{50} of 2.0 μM. Furthermore, the cell-based assay using rhodamine-123 indicated that licocharcon A (3-10 μM) significantly ($p < 0.01$) inhibited P-gp-mediated drug efflux (Fig. 13). Those results appeared to be consistent with the findings of previous studies [35, 36].

Those results suggest that licocharcon A might be effective to improve the bioavailability of losartan, a substrate of CYP3A4 and P-gp. Therefore, the pharmacokinetic characteristics of losartan were evaluated in the absence and the presence of licocharcon A in rats. As CYP3A9 expressed in rat is corresponding to the ortholog of CYP3A4 in human [37], rats were selected as an animal model in

this study to evaluate the potential pharmacokinetic interactions mediated by CYP3A4, although there should be some extent of difference in enzyme activity between rat and human [38].

As summarized in Table 4, licocharcon A significantly increased $AUC_{0-\infty}$ of losartan by 33.4–63.2% and C_{max} of losartan by 34.0–62.8%. The total body clearance (CL/F) was significantly decreased (2 mg/kg, $p < 0.05$; 8 mg/kg, $p < 0.01$) by licocharcon A. The absolute bioavailability (A.B.) of losartan in the presence of licocharcon A was significantly ($p < 0.05$) higher than that in the control group. Licocharcon A inhibited CYP3A4 and CYP2C9 isozymes and P-gp activity in present study. These results appeared to be consistent with the finding that myricetin, a flavonoid, is a CYP3A4, 2C9 and P-gp inhibitor and significantly increased the $AUC_{0-\infty}$ and C_{max} of losartan [29]. These results were also consistent with the findings of some previous studies [14-17], in which epigallocatechin and hesperidin increased bioavailability of verapamil (a substrat of CYP3A4 and P-gp) in rats, and in which morin and hesperidin increased $AUC_{0-\infty}$ and C_{max} of diltiazem (a substrat of CYP3A4 and P-gp) in rats. Piao *et al.* [39] reported that morin, a flavonoid, significantly enhanced the bioavailability of nicardipine that nicardipine might be due to the inhibition of P-gp and intestinal metabolism by morin.

Also those results appeared to be consistent with the findings of some previous studies [12, 13]. Zaidenstein *et al.* [12] reported significant differences in the pharmacokinetic parameters of losartan and its metabolite EXP-3174 as a result of concomitant intake of grapefruit juice, a CYP3A4 inhibitor.

As summarized in Table 5, licocharcon A significantly decreased metabolite-parent AUC ratios (M.R.). This result appeared to be consistent with the findings of some previous studies [12, 13]. The M.R., a characteristic of the magnitude of metabolic conversion, was significantly changed by the inhibition of CYP3A4-mediated metabolism of losartan after co-administration of grapefruit juice [12, 13]. Concurrent use of bucolome, a CYP2C9 inhibitor, significantly increased the AUC of losartan by the inhibition of metabolic conversion of losartan to EXP-3174. Epigallocatechin and morin significantly decreased M.R. of verapamil and diltiazem, respectively [14, 16]. Those studies in conjunction with our present findings, suggest that the combination of losartan and CYP (CYP2C9, CYP3A4) inhibitors could result in a significant pharmacokinetic drug interaction.

Althrough many CYP enzymes are in the intestine, there is no evidence of significant oxidation of losartan by the enterocytes using either *in vitro* or in situ absorption models [40]. In contrast, some previous studies have suggested that the active metabolite EXP-3174 detected in rats is most likely of hepatic origin [44, 41]. In the present study, therefore, the decrease in the metabolite-parent AUC ratios (M.R.) of losartan might be mainly due to the inhibitory effect of licocharcon A on the first-pass effects (metabolism) in the liver and/or in the small intestine. Licocharcon A did not significantly change the pharmacokinetic parameters of intravenous administration of losartan (Table 6), suggesting that licocharcon A may improve the oral bioavailability of losartan by more increasing the absorption or reducing intestinal metabolism of losartan by the inhibition of CYP3A and

CYP2C9 rather than renal eliminatin by licocharcon A.

Therefore, the enhanced bioavailability of losartan might be mainly due to inhibition of the CYP3A and CYP2C9-mediated metabolism of losartan in the liver and the P-gp efflux transporter in the small intestine by licocharcon A.

Although potential adverse effects, this interaction may provide a therapeutic benefit whereby it enhances bioavailability and lowers the dose administered. Since the present study raised the awareness about the potential drug interactions by concomitant use of licocharcon A, a natural flavonoid, with losartan, the clinical significance of this finding need to be further evaluated in the clinical studies.

Conclusion

Licocharcon A significantly enhanced the oral bioavailability of losartan in rats. The enhanced bioavailability of losartan might be due to inhibition of the CYP3A and CYP2C9-mediated metabolism of losartan in the liver and the P-gp efflux transporter in the small intestine by licocharcon A. Therefore, concomitant use of licocharcon A with losartan may require close monitoring for potential drug interactions. The clinical importance of these findings should be further investigated in clinical trials.

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Table 4. Mean pharmacokinetic parameters of losartan after oral administration of losartan (9 mg/kg) to rats in the presence or absence of licocharcon A (Mean ± SD, n = 6)

Parameters	Control	Losartan + Licocharcon A		
		0.4 mg/kg	2 mg/kg	8 mg/kg
$AUC_{0-\infty}$ (ng·h/mL)	225.0 ± 38.3	260.2 ± 44.2	300.1 ± 51.0*	367.3 ± 62.4**
C_{max} (ng/mL)	18.8 ± 3.4	21.9 ± 3.9	25.2 ± 4.5*	30.6 ± 5.5**
T_{max} (h)	0.92 ± 0.21	0.92 ± 0.21	1.17 ± 0.41	1.17 ± 0.41
CL/F (mL/hr/kg)	664.8 ± 126.3	577.3 ± 109.7	499.7 ± 94.9*	411.3 ± 78.1**
$t_{1/2}$ (h)	10.6 ± 2.1	10.8 ± 2.2	10.8 ± 2.2	11.0 ± 2.3
A.B. (%)	25.4 ± 4.8	33.3 ± 6.3	38.5 ± 7.3*	46.8 ± 8.9**
R.B. (%)	100	115	133	163

* $p < 0.05$, ** $p < 0.01$, significant difference compared to the control group given losartan alone.

$AUC_{0-\infty}$: area under the plasma concentration-time curve from 0 h to infinity; C_{max}: peak plasma concentration; T_{max}: time to reach C_{max}; CL/F, total body clearance; $t_{1/2}$: terminal half-life; A.B.: absolute bioavailability; R.B.: relative bioavailability.

Table 5. Mean pharmacokinetic parameters of EXP-3174 after oral administration of losartan (9 mg/kg) to rats in the presence or absence of licocharcon A (Mean ± SD, n = 6)

Parameters	Control	Losartan + Licocharcon A		
		0.4 mg/kg	2 mg/kg	8 mg/kg
$AUC_{0-\infty}$ (ng ·h/mL)	231.1 ± 39.3	245.0 ± 41.7	264.1 ± 44.9	282.3 ± 48.1
C_{max} (ng/mL)	17.3 ± 3.1	18.5 ± 3.3	20.5 ± 3.7	21.9 ± 3.9
T_{max} (h)	1.67 ± 0.52	1.67 ± 0.52	1.83 ± 0.41	2.17 ± 0.41
$t_{1/2}$ (h)	9.5 ± 1.8	9.8 ± 1.9	10.2 ± 1.9	10.5 ± 2.1
R.B. (%)	100	106	114	122
M.R. (%)	1.00 ± 0.20	0.94 ± 0.19	0.88 ± 0.17	0.77 ± 0.14*

* $p < 0.05$, significant difference compared to the control group given losartan alone.

$AUC_{0-\infty}$: area under the plasma concentration-time curve from 0 h to infinity; C_{max}: peak plasma concentration; T_{max}: time to reach C_{max}; R.B.: relative bioavailability; M.R.: metabolite-parent ratio ($AUC_{EXP-3174}/AUC_{losartan}$).

Table 6. Mean pharmacokinetic parameters of losartan after intavenous (3 mg/kg) administration to rats in the presence or absence of licocharcon A (Mean ± SD, n = 6)

Parameter	Control	Losartan + Licocharcon A		
		0.4 mg/kg	2 mg/kg	8 mg/kg
$AUC_{0-\infty}$ (ng·h/mL)	296.1 ± 59.2	314.0 ± 62.8	331.3 ± 66.3	349.1 ± 69.8
CL_t (mL/hr/kg)	168.9 ± 62.8	159.3 ± 28.7	150.8 ± 27.1	142.4 ± 25.6
$t_{1/2}$ (h)	8.2 ± 1.3	8.4 ± 1.4	8.6 ± 1.5	8.7 ± 1.6
R.B. (%)	100	106	112	118

$AUC_{0-\infty}$: total area under the plasma concentration–time curve from time zero to infinity, CL_t: total body clearance, $t_{1/2}$: terminal half-life, R.B.: relative bioavailability.

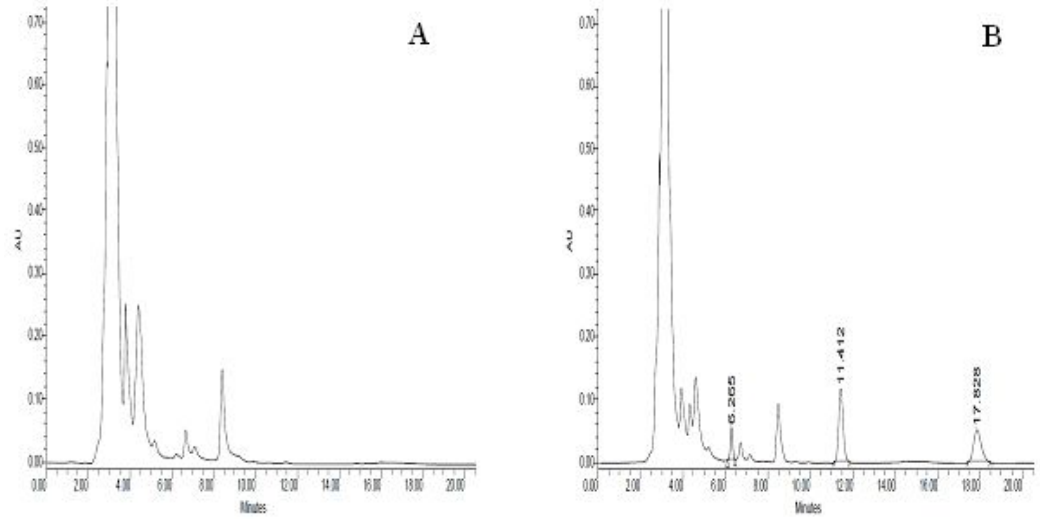

Figure 9. HPLC chromatograms of the rat blank plasma (A), and the plasma spiked with losartan (11.4 min), EXP-3174 (17.9 min), and L-158.809 (internal standard; 6.3 min) (B)

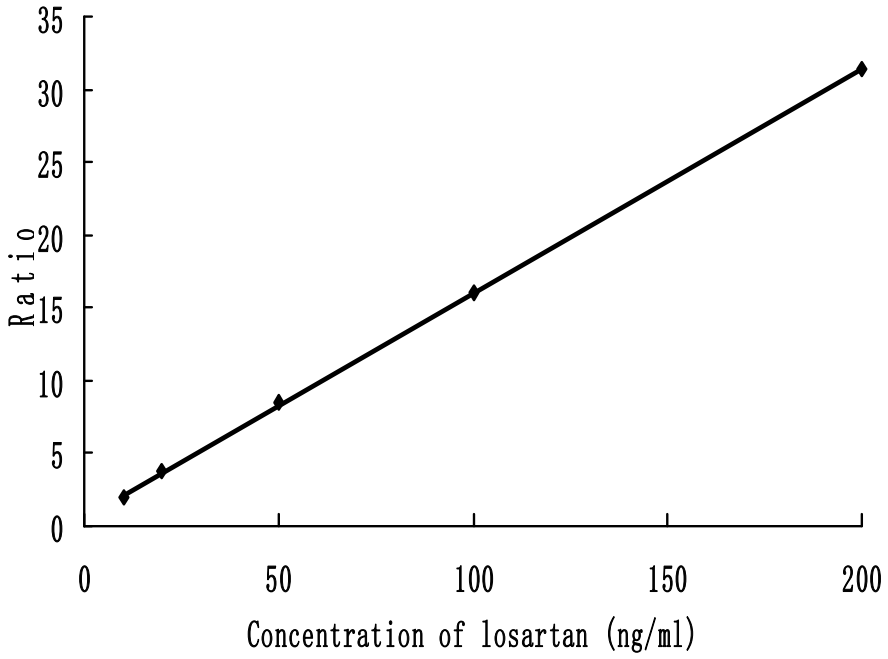

Figure 10. A calibration curve of losartan when spiked into the rat blank plasma. The typical equation describing the calibration curve in rat plasma was y=0.154x+0.6171, where “y” is the peak area ratio of losartan to L-158.809 and “x” is the concentration of losartan.

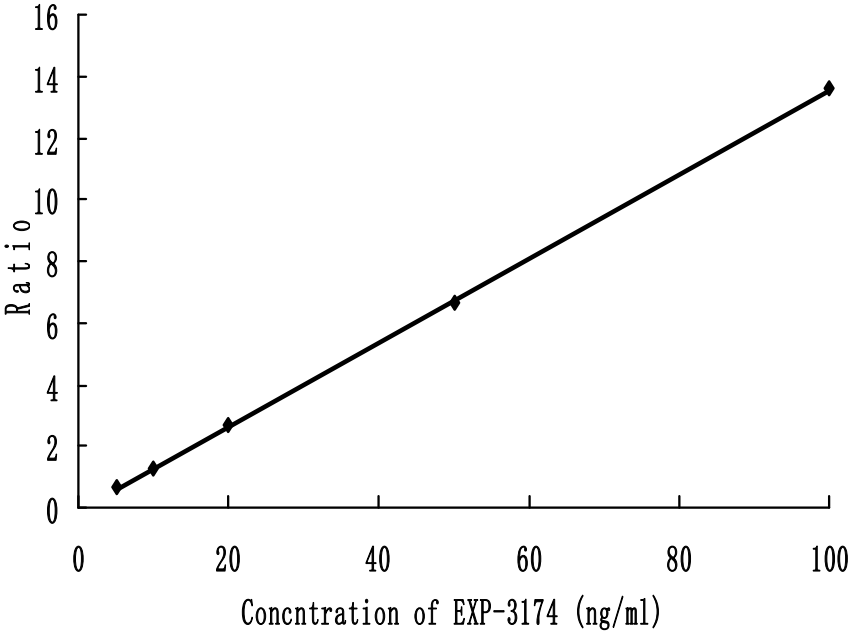

Figure 11. A calibration curve of EXP-3174 when spiked into the rat blank plasma. The typical equation describing the calibration curve in rat plasma was $y=0.1359x-0.0507$, where "y" is the peak area ratio of EXP-3174 to L-158.809 and "x" is the concentration of EXP-3174.

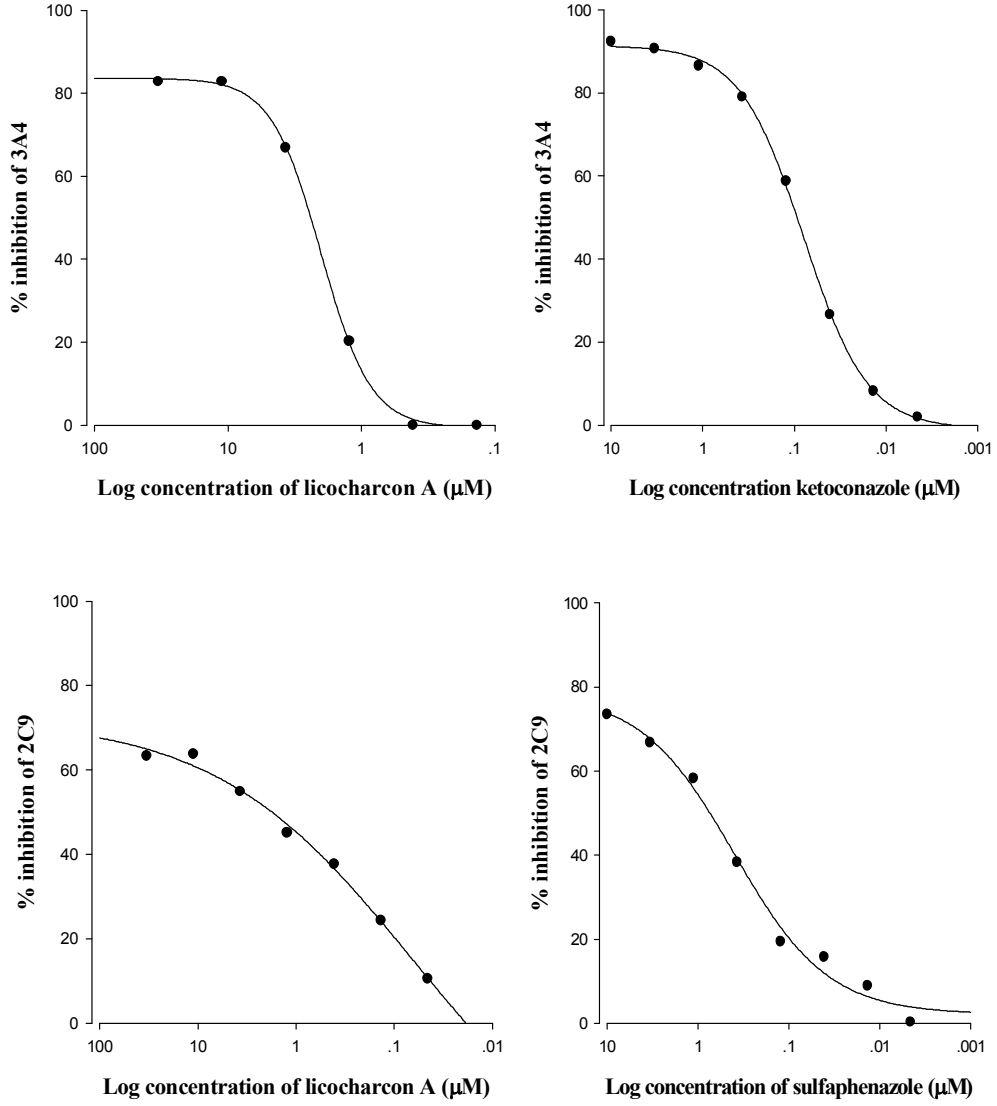

Figure 12. Inhibitory effect of licocharcon A on CYP3A4 and 2C9 activity. All experiments were duplicate, and results are expressed as the percent of inhibition.

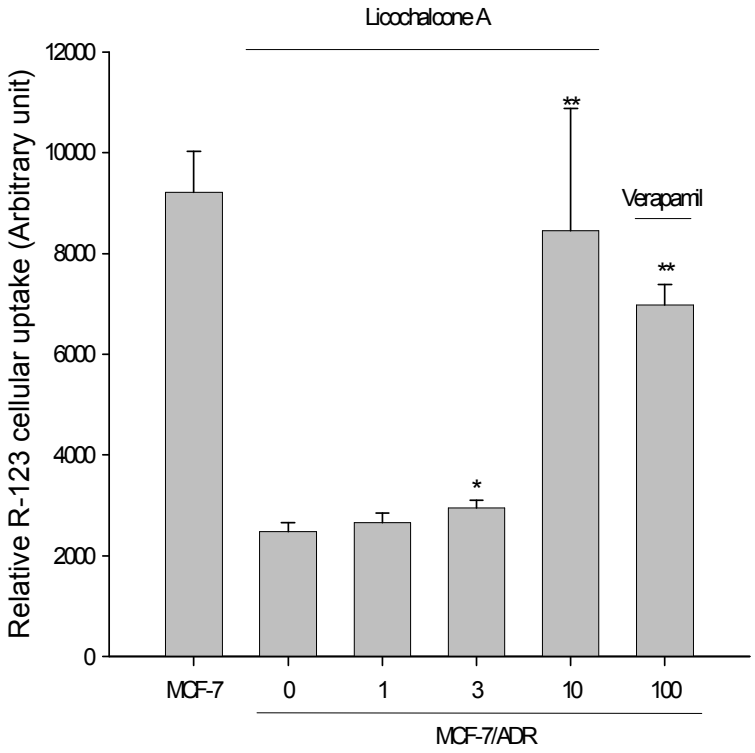

Figure 13. Rhodamine-123 retention. MCF-7/ADR cells were preincubated with licocharcon A for 24 h. After incubation of MCF-7/ADR cells with 20 μM R-123 for 90 min. Data represents mean ± SD of 6 separate samples (significant versus control MCF-7 cells, * $p < 0.05$, ** $p < 0.01$).

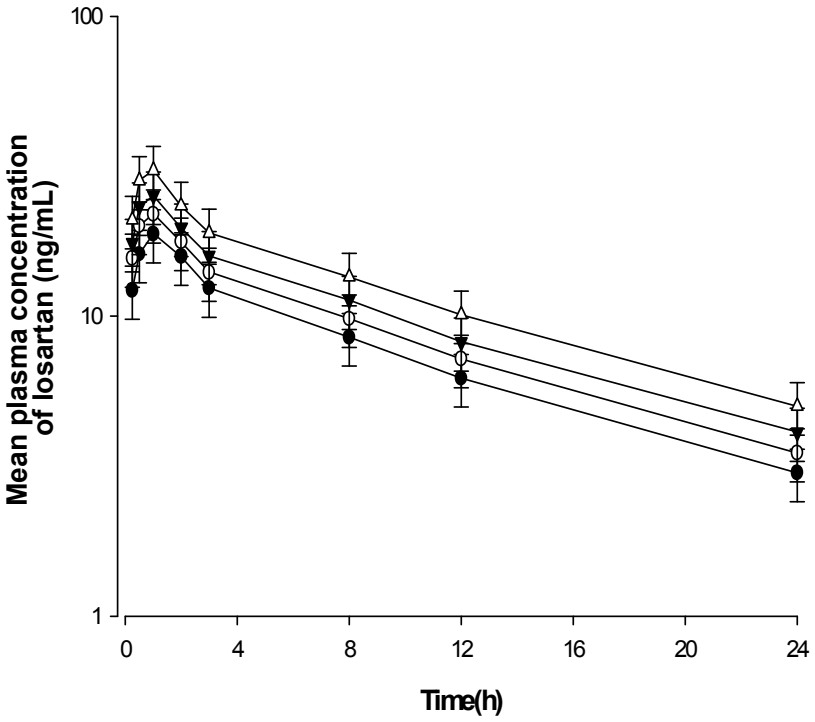

Figure 14. Mean plasma concentration-time profiles of losartan after oral administration of losartan (9 mg/kg) to rats in the presence or absence of licocharcon A at doses of 0.4, 2 and 8 mg/kg. (Mean ± SD, n = 6). ●: Control (losartan 9 mg/kg, alone); ○: co-administered with 0.4 mg/kg of licocharcon A; ▼: co-administered with 2 mg/kg of licocharcon A, △: co-administered with 8 mg/kg of licocharcon A.

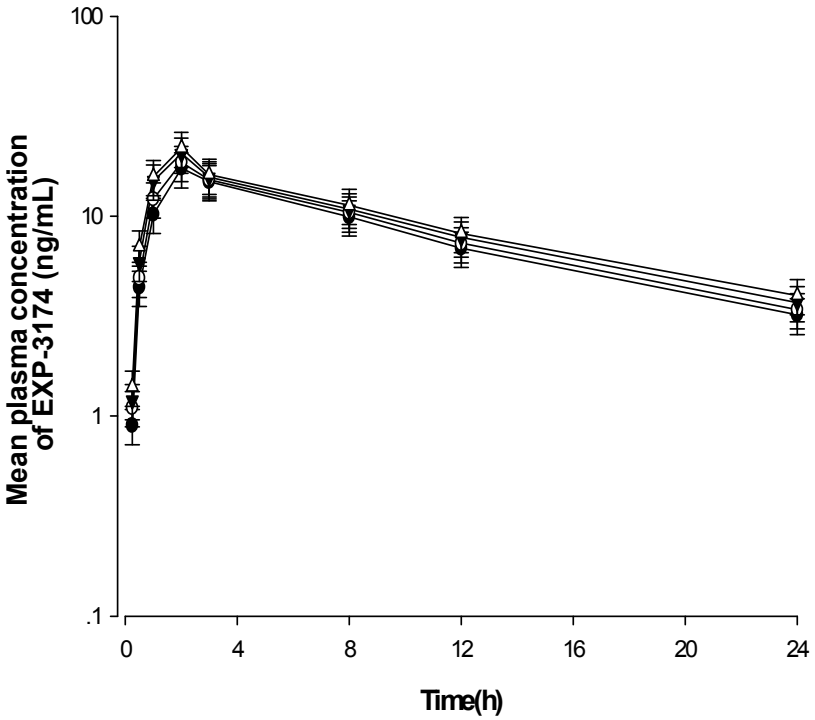

Figure 15. Mean plasma concentration-time profiles of EXP-3174 after oral administration of losartan (9 mg/kg) to rats in the presence or absence of licocharcon A at doses of 0.4, 2 and 8 mg/kg. (Mean ± SD, n = 6). ●: Control (losartan 9 mg/kg, alone); ○: co-administered with 0.4 mg/kg of licocharcon A; ▼: co-administered with 2 mg/kg of licocharcon A, △: co-administered with 8 mg/kg of licocharcon A.

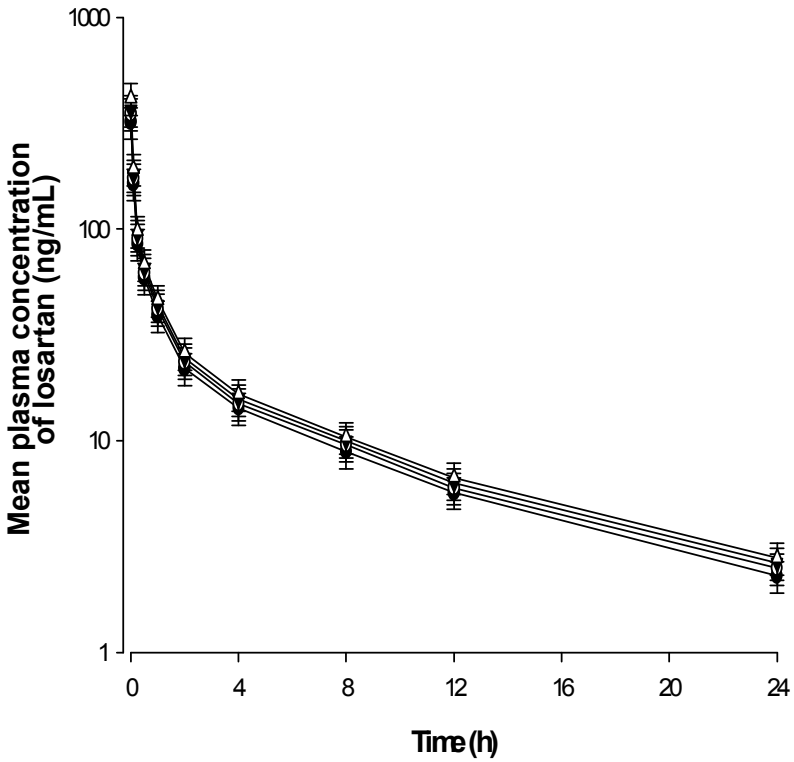

Figure 16. Mean plasma concentration-time profiles of losartan after intravenous (3 mg/kg) administration to rats in the presence or absence of licocharcon A at doses of 0.4, 2 and 8 mg/kg. (Mean ± SD, n = 6). ●: Control (losartan 3 mg/kg, alone); ○: co-administered with 0.4 mg/kg of licocharcon A; ▼: co-administered with 2 mg/kg of licocharcon A, △: co-administered with 8 mg/kg of licocharcon A.

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새로운 배움과 학위를 취득하며 무언가를 이루었다는 성취감에 마음이 뿌듯하며 이 모든 것을 이루는데 밑거름이 되게 하신 모든 분들께 감사의 말을 전하고자 합니다.

좋은 연구 테마를 주시고 물심양면으로 격려와 조언으로 학위의 꿈을 주신 최준식 교수님께 머리숙여 감사를 드립니다.

부족한 저의 학위논문을 지적 및 보완하여 심사하여 주신 서울대학교 심창구 교수님과 전남대학교 신상철 교수님께도 감사를 드립니다.

학위논문을 실험할 때 도와주신 이 성 선생님께도 진심으로 감사를 드립니다.

항상 기도해 주시고 염려해 주신 양가 부모님께 한없는 감사를 드립니다.

그리고 항상 저의 옆에서 고생을 아끼지 않은 사랑하는 아내와 아이들에게 고마움을 전하고 싶습니다.

박사학위를 가진 약사로써 이웃과 환자들에게 봉사하고, 더욱 더 매진하여 세상의 빛과 소금이 되어 하나님께 영광을 돌리는 약사가 되고자 노력 하겠습니다.

저작물 이용 허락서

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논문제목	한글: 흰쥐에서 레스베라트롤 및 리코찰콘이 로살탄의 생체이용률에 미치는 영향. 영문: Effects of resveratrol and licocharcon A on the bioavailability of losartan in rats				

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

- 다 음 -

1. 저작물의 DB 구축 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 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 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 (√) 반대 ( )

2011 년 06 월

저작자: 손홍묵 (서명 또는 인)

조선대학교 총장 귀하