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박사학위논문

# Effects of cilostazol on the pharmacokinetics of losartan and/or nifedipine in rats

조선대학교 대학원

의 학 과

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흰쥐에서 시로스타졸이 로사르탄과 니페디핀의
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이 논문을 의학박사학위신청 논문으로 제출함.

2014 년 10 월

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# 국 문 초 록

## 흰쥐에서 시로스타졸이 로사르탄과 니페디핀의 약물동태에 미치는 영향

하 성 일
지 도 교 수: 최 동 현
의 학 과
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항혈소판성 혈관확장제인 시로스타졸과 항고혈압제인 로사르탄과 니페디핀의 병용투여가 순환기질환 예방 및 치료를 위해서 처방되는 경우가 많으므로 이에 대한 상호작용을 알아보고자, 흰쥐에 항혈소판제인 시로스타졸 (1.5, 6 mg/kg)과 로사르탄 (경구; 9 mg/kg, 정맥; 3 mg/kg) 및 니페디핀 (경구; 10 mg/kg, 정맥; 2.5 mg/kg)과 병용 경구투여한 후 로살탄 및 니페디핀과 그 활성대사체들의 약물동태학적 변수들을 대조군과 비교 검토하였다.

시로스타졸과 병용 투여시 로사르탄 및 니페디핀의 약물동태학적 변수는 유의성 있게 변화하였다. 대조군에 비해 시로스타졸 (6 mg/kg)과 병용투여군에서 로사르탄 및 니페디핀의 혈장농도곡선하면적 ($AUC_{0-\infty}$)과 최고혈중농도 ($C_{max}$)는 각각 유의성 ($P < 0.05$) 있게 증가되었으며, 전신클리어런스 (CL/F)는 유의성 ($P < 0.05$) 있게 각각 감소되었다.

절대적생체이용률 (AB)도 대조군에 비해 각각 유의성 ($P < 0.05$) 있게 증가되었다. 아울러 시로스타졸 (6 mg/kg)과 로사르탄 및 니페디핀을

병용투여한군에서 대조군에 비해 활성대사체는 혈장농도곡선하면적 ($AUC_{0-\infty}$)이 증가되었으나 유의성은 없었고 로사르탄 및 니페디핀의 대사율 (MR)을 유의성 있게 감소시켰다.

정맥투여군에서는 시로스타졸과 병용투여시 로사르탄 및 니페디핀의 혈장농도곡선하면적 ($AUC_{0-\infty}$)이 유의성 있게 증가되었다. 본 연구에서 항혈소판제인 시로스타졸과 고혈압치료제인 로사르탄 및 니페디핀과 병용투여 하였을 때 경구투여된 로사르탄 및 니페디핀의 생체이용률이 유의성 있게 증가된 것은 소장과 간장에 존재하는 CYP3A4 과 CYP2C9 효소 억제에 의한 로살탄의 대사억제와 CYP3A4 억제에 의한 니페디핀의 대사 억제와 또는 전신 클리어런스 감소에 기인한 것으로 사료된다.

## 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. After oral administration, losartan is rapidly completely absorbed, reaching maximum concentrations 1-2 hours post-dose, but it has alow and highly variable oral bioavailabilty (12.2-66.6%) [4]. Losartan is extensively metabolized to the active metabolite, EXP-3174 which is about 10-fold more than its parent drug [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) 3A4 and CYP2C9 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].

Nifedipine (dimethyl2, 6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate) is a calcium channel-blocking agent that is widely used for the treatment of essential hypertension, coronary artery spasm, and angina pectoris (18). It inhibits the influx of extracellular calcium through myocardial and vascular membrane pores by physically plugging the channel, resulting in decreased intracellular calcium levels, inhibition of the contractile processes of smooth

muscle cells, dilation of the coronary and systemic arteries, increased oxygen delivery to the myocardial tissue, and decreased total peripheral resistance, systemic blood pressure, and afterload (19, 20).

In humans, nifedipine is predominantly metabolized by CYP3A4 to its primary pyridine metabolite, dehydronifedipine (21, 22). CYP enzymes are responsible for the oxidative metabolism of many xenobiotics and play a major role in the phase I metabolism of many drugs (23). CYP3A4 is the most abundant CYP enzyme (30-40%) in adult liver and metabolizes more than 50% of the clinically used drugs including nifedipine, cyclosporine, midazolam, and (23, 24). There are some reports that nifedipine is a substrate of CYP3A4 in human (25-27). P-gp is an adenosine-50-triphosphate (ATP) dependent efflux drug transporter that is constitutively expressed in normal tissues that includes gastrointestinal epithelium, canalicular membrane of the liver, kidney (28, 29) and capillary endothelial cells in the central nervous system (30, 31). Because of such tissue localized and its broad substrate specificity, P-gp appears to play a key role in absorption, distribution, and elimination of many drugs (32, 33). It is generally known that the substrate and/or inhibitors of CYP3A4 and P-gp overlap with each other (34). Dorababu *et al.* (35) reported that nifedipine belonged to a group of P-gp substrate. Since P-gp is co-localized with CYP3A4 in the small intestine, P-gp and CYP3A4 may act synergistically to promote presystemic drug metabolism, resulting in the limited absorption of drugs.

Cilostazol (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2(1H)-quinolinone) is an antiplatelet vasodilator agent that has been used for more than a decade in Japan for the treatment of chronic peripheral arterial occlusive disease (36). Cilostazol was also approved by the US Food and Drug Administration in 1998 for the treatment of symptoms related to intermittent claudication (37). Cilostazol exerts its pharmacologic effects through selective inhibition of phosphodiesterase-3, which is abundant in platelets and vascular smooth muscle cells. These inhibitory effects mediate the antiplatelet properties of cilostazol and

suppress vascular smooth muscle cell proliferation (38). Cilostazol is extensively metabolized by cytochrome P450 (CYP) enzymes, primarily CYP3A4, into the active metabolites 3, 4-dehydrocilostazol and 4'-trans-hydroxy-cilostazol (39, 40). However, the effect of cilostazol on P-gp activity has not been reported. Thus we attempted to evaluate P-gp activity using rhodamine-123 retention assay in P-gp-overexpressed MCF-7/ADR cells, and furthermore, to evaluate CYP3A4 and CYP2C9 activity of cilostazol.

The drug–drug interaction could be possible since cilostazol, antiplatelet vasodilator agent, and losartan and/or nifedipine could be prescribed for the prevention or treatment of cardiovascular diseases as a combination therapy. However, the effect of cilostazol on the pharmacokinetics of losartan and nifedipine *in vivo* has not been reported. Thus, the purpose of this study was to investigate the possible effects of cilostazol on the CYP3A4 and CYP2C9 and P-gp activity and bioavailability or the pharmacokinetics of losartan and/or nifedipine and its active metabolite, EXP-3174 and dehydronifedipine, after oral and intravenous administration of losartanand/or nifedipine with cilostazol in rats.

# Part I. Effects of cilostazol on the pharmacokinetics of losartan and its main metabolite, EXP-3174, in rats

# 국 문 초 록

항혈소판성 혈관확장제인 시로스타졸과 로사르탄과의 병용투여가 순환기 질환 예방 및 치료를 위해서 처방되는 경우가 많으므로 이에 대한 상호작용을 알아보고자, 흰쥐에 항혈소판제인 시로스타졸 (1.5, 6 mg/kg)과 로사르탄 (경구; 9 mg/kg, 정맥; 3 mg/kg)과의 병용 경구투여한 후 로사르탄과 그 활성대사체인, EXP-3174 의 약물동태학적 변수들을 대조군과 비교 검토하였다.

시로스타졸과 병용 투여시 로사르탄의 약물동태학적 변수는 유의성 있게 변화하였다. 대조군에 비해 시로스타졸 (6 mg/kg)과 병용투여군에서 로사르탄의 혈장농도곡선하면적 ($AUC_{0-\infty}$)과 최고혈중농도 ($C_{max}$)는 유의성 ($P < 0.05$) 있게 증가되었으며, 전신클리어런스 (CL/F)는 유의성 ($P < 0.05$) 있게 각각 감소되었다.

절대적생체이용률 (AB)도 대조군에 비해 유의성 ($P < 0.05$) 있게 증가되었다. 아울러 시로스타졸 (6 mg/kg)과 로사르탄을 병용투여한군에서 대조군에 비해 활성대사체는 혈장농도곡선하면적 ($AUC_{0-\infty}$)이 증가되었으나 유의성은 없었으나 로사르탄의 대사율 (MR)을 유의성 있게 감소시켰다.

정맥투여군에서는 시로스타졸과 병용투여시 로사르탄 의 혈장농도곡선하면적 ($AUC_{0-\infty}$)이 유의성 있게 증가되었다. 본 연구에서 항혈소판제인 시로스타졸과 고혈압치료제인 로사르탄을 병용투여 하였을 때 경구투여된 로사르탄의 생체이용률이 유의성 있게 증가된 것은 소장과 간장에 존재하는 CYP3A4 과 CYP2C9 효소의 억제에 의한 로살탄의 대사억제와 또는 전신 클리어런스 감소에 기인한 것으로 사료된다.

## A. Introduction

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cells. These inhibitory effects mediate the antiplatelet properties of cilostazol and suppress vascular smooth muscle cell proliferation (20). Cilostazol is extensively metabolized by cytochrome P450 (CYP) enzymes, primarily CYP3A4, into the active metabolites 3, 4-dehydrocilostazol and 4'-trans-hydroxy-cilostazol (21-22). However, the effect of cilostazol on P-gp activity has not been reported. Thus we attempted to evaluate P-gp activity using rhodamine-123 retention assay in P-gp-overexpressed MCF-7/ADR cells, and furthermore, to evaluate CYP3A4 activity of cilostazol.

The drug–drug interaction could be possible since cilostazol, antiplatelet vasodilator agent, and losartan could be prescribed for the prevention or treatment of cardiovascular diseases as a combination therapy. However, the effect of cilostazol on the pharmacokinetics of losartan *in vivo* has not been reported. Thus, the purpose of this study was to investigate the possible effects of cilostazol on the CYP3A4 and P-gp activity and bioavailability or the pharmacokinetics of losartan and its active metabolite, EXP-3174, after oral and intravenous administration of losartan with cilostazol in rats.

## B. Materials and Methods

### 1. Materials

Losartan, EXP-3174 (a metabolite of losartan) and L-158.809 (internal standard) were obtained from the Merck Co. (NJ, U.S.A.). Cilostazol 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.

### 2. 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 accordiance 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.

### 3. Drug administration

The rats were divided into the following six groups (n = 6, each): an oral control group (9 mg/kg of losartan dissolved in distilled water) with or without (control) oral administration of cilostazol at a dose of 1.5 or 6 mg/kg (mixed in distilled water); 3 mg/kg of losartan dissolved in 0.9% NaCl solution for intravenous administration with or without (control) oral administration of cilostazol at a dose of 1.5 or 6 mg/kg. 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 cilostazol 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.

**4. HPLC assay**

The plasma concentrations of losartan were determined by the HPLC assay modified from Zarghi *et al*. [23]. 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 100-5$C_8$ 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 < 15.2%, respectively.

**5. CYP inhibition assay**

The assays of inhibition on human CYP3A4 and CYP2C9 enzyme activities were performed in a multiwell plate using the CYP inhibition assay kit (GENTEST, Woburn, MA) as described previously [24]. 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 cilostazol 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.

**6. Rhodamine-123 retention assay**

The procedures used for the Rho-123 retention assay were similar to a previously reported method [25]. 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 cilostazol (50 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. Verapamil (100 μM) was used as a positive control.

**7. 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 ($F$) of losartan was calculated by $AUC_{oral}/AUC_{i.v.} \times Dose_{i.v.}/Dose_{oral} \times 100$, and the relative bioavailability ($RB$) of losartan was estimated by $AUC_{with\ cilostazol}/AUC_{control} \times 100$. The metabolite-parent ratio ($MR$) was estimated by ($AUC_{EXP-3174}/AUC_{losartan}$).

**8. 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.

## C. Results

### 1. Inhibition of CYP3A4 and CYP2C9

The inhibitory effect of cilostazol on CYP3A4 and CYP2C9 activity is shown in Figure 1. Cilostazol inhibited CYP3A4 and CYP2C9 enzyme activity and the 50% inhibition concentration ($IC_{50}$) values of cilostazol on CYP3A4 and CYP2C9 activity were 7.7 and 9.8 μM, respectively.

### 2. Rhodamine-123 retention assay

The effect of cilostazol on the cellular accumulation of rhodamine-123 in MCF-7 and MCF-7/ADR cells is shown in Figure 2. Accumulation of rhodamine-123 was reduced in MCF-7/ADR cells overexpressing P-gp compared to that in MCF-7 cells lacking P-gp. The relative cellular uptake of rhodamine-123 was comparable at the concentration range of 10–100 μM cilostazol. This result showed that cilostazol did not significantly inhibited P-gp activity

### 3. Effect of cilostazol on the pharmacokinetics of oral losartan

The mean plasma concentration-time profiles of losartan in the presence or absence of cilostazol were characterized in Figure 3. The mean pharmacokinetic parameters of losartan were also summarized in Table 1.

As shown in Table 1, cilostazol (6 mg/kg) significantly altered the pharmacokinetic parameters of losartan compared to the control (given losartan alone). Cilostazol significantly (6 mg/kg, $p < 0.05$) increased the area under the plasma concentration–time curve ($AUC_{0-\infty}$) of losartan by 52.0% and peak plasma concentration ($C_{max}$) of losartan by 48.4%. The total body clearance ($CL/F$) was significantly (6 mg/kg, $p < 0.05$) decreased by 34.2% in the presence of cilostazol. Consequently, the absolute bioavailability ($F$) of losartan in the presence of

cilostazol was 27.6%, which was enhanced significantly ($p < 0.05$) compared with the oral control group (25.4%). The relative bioavailability (*RB*) of losartan was increased by 1.14- 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 cilostazol.

**4. Effect of cilostazol on the pharmacokinetics of EXP-3174**

The mean plasma concentration-time profiles of EXP-3174 in the presence or absence of cilostazol were characterized in Figure 4. The mean pharmacokinetic parameters of EXP-3174 were also summarized in Table 2.

As summarized in Table 2, cilostazol (6 mg/kg) significantly increased $AUC_{0-\infty}$ of EXP-3174 but it is not significant. Metabolite-parent AUC ratio in the presence of cilostazol (6 mg/kg) significantly decreased by 23.8 % ($p < 0.05$) compared to the control group, suggesting that the presence of cilostazol could be effective to inhibit the CYP3A4- mediated metabolism of losartan.

**5. Effect of cilostazol 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 cilostazol (1.5 and 6 mg/kg) are shown in Figure 5, the corresponding pharmacokinetic parameters are shown in Table 3.

The $AUC_{0-\infty}$ of losartan was significantly increased compared to that in the control. The $t_{1/2}$ of losartan was also prolonged, but this increase was not significant. This suggests that cilostazol inhibited metabolism of losartan via CYP3A4. The enhanced oral bioavailability in the presence of cilostazol may be mainly due to inhibition of the CYP3A4-mediated metabolism of losartan in the small intestine and/or in the liver by cilostazol.

**Table 1.** Mean (± standard deviation) pharmacokinetic parameters of losartan after an oral (9 mg/kg) administration with 1.5 and 6 mg/kg of oral cilostazol

| Parameters | Losartan with cilostazol | | |
|---|---|---|---|
| | control | 1.5 mg/kg | 6 mg/kg |
| $AUC_{0-\infty}$ (ng·h/mL) | 226.0 ± 37.6 | 271.2 ± 49.4 | 343.5 ± 59.1* |
| $C_{max}$ (ng/mL) | 18.8 ± 3.4 | 23.0 ± 4.2 | 27.9 ± 4.6* |
| $T_{max}$ (h) | 0.93 ± 0.21 | 1.17 ± 0.41 | 1.17 ± 0.41 |
| *CL/F* (mL/hr/kg) | 11.1 ± 1.5 | 9.1 ± 1.2[a] | 7.3 ± 0.9* |
| $t_{1/2}$ (h) | 10.8 ± 2.1 | 11.1 ± 2.3 | 11.7 ± 2.4 |
| *F* (%) | 25.4 ± 4.1 | 27.6 ± 4.7 | 32.4 ± 5.3* |
| *RB* (%) | 100 | 120 | 152 |

* $p < 0.05$, significant different compared to the control.

$AUC_{0-\infty}$: 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; *F*: absolute bioavailability; *RB*: relative bioavailability.

**Table 2.** Mean (± standard deiation) pharmacokinetic parameters of EXP-3174 after an oral (9 mg/kg) administration with 1.5 and 6 mg/kg of oral cilostazol

| Parameters | Losartan with cilostazol | | |
|---|---|---|---|
| | control | 1.5 mg/kg | 6 mg/kg |
| $AUC_{0-\infty}$ (ng·h/mL) | 229.0 ± 41.6 | 248.1 ± 44.4 | 264.5 ± 48.1 |
| $C_{max}$ (ng/mL) | 17.3 ± 3.1 | 20.0 ± 3.3 | 21.1 ± 3.5 |
| $T_{max}$ (h) | 1.67 ± 0.53 | 1.84 ± 0.42 | 2.16 ± 0.42 |
| $t_{1/2}$ (h) | 9.5 ± 1.8 | 10.0 ± 1.9 | 10.5 ± 2.2 |
| *MR* (%) | 1.01 ± 0.20 | 0.91 ± 0.16 | 0.77 ± 0.13* |
| *RB* (%) | 100 | 108 | 115 |

* $p < 0.05$, significant different compared to the control group.

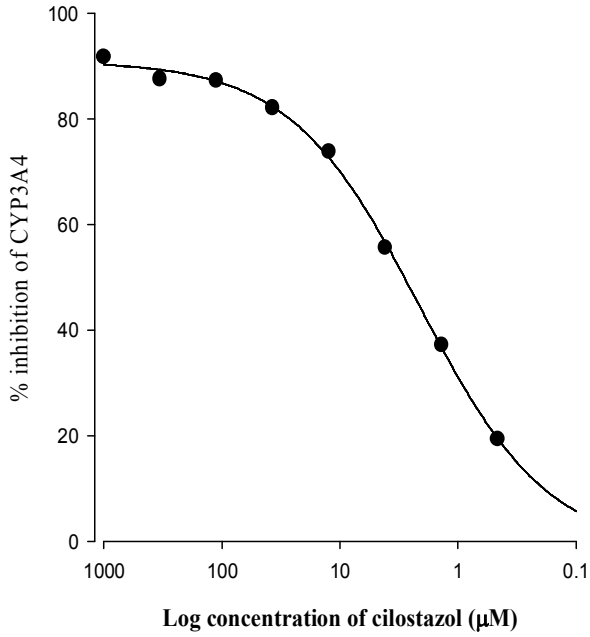
$AUC_{0-\infty}$: area under the plasma concentration-time curve from 0 h to infinity; $C_{max}$: peak plasma concentration; $T_{max}$: time to reach $C_{max}$; $t_{1/2}$: terminal half-life; *RB*: relative bioavailability; *MR*: metabolite-parent ratio ($AUC_{EXP-3174}$ /$AUC_{losartan}$).

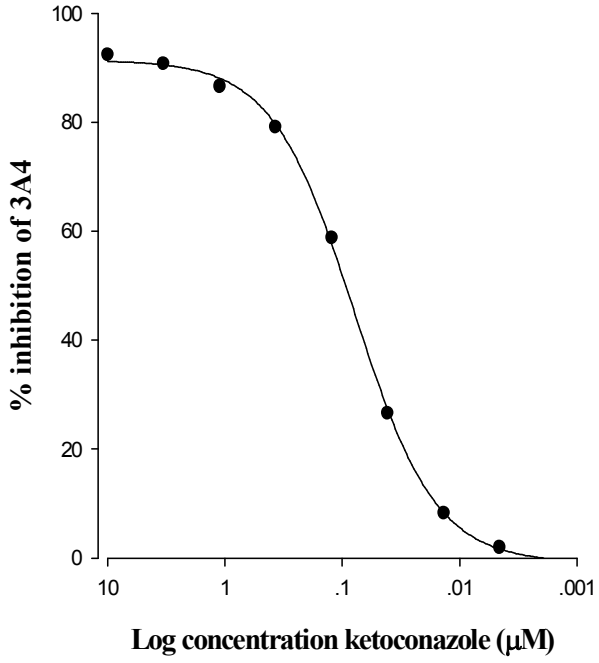
**Table 3.** Mean (± standard deiation) pharmacokinetic parameters of losartan after the intravenous (3 mg/kg) administration with 1.5 and 6 mg/kg of oral cilostazol

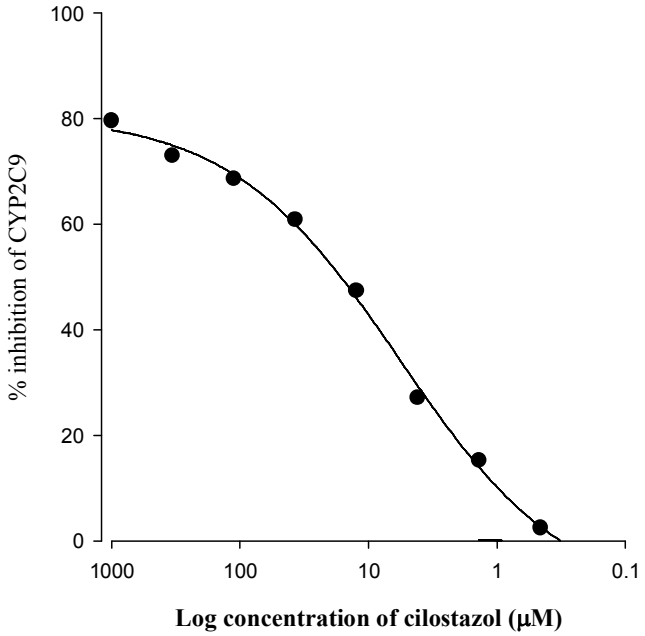
| Parameter | Losartan with cilostazol | | |
|---|---|---|---|
| | 0 mg/kg | 1.5 mg/kg | 6 mg/kg |
| $AUC_{0-\infty}$ (ng·h/mL) | 295.1 ± 59.2 | 327.5 ± 65.0 | 354.1 ± 66.3* |
| $CL_t$ (mL/hr/kg) | 3.78±1.07 | 3.56± 1.02 | 3.33±1.01 |
| $t_{1/2}$ (h) | 8.5 ± 1.3 | 8.6 ± 1.5 | 8.8 ± 1.6 |
| *RB* (%) | 100 | 111 | 120 |

* $p < 0.05$, significant different compared to the control group.

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





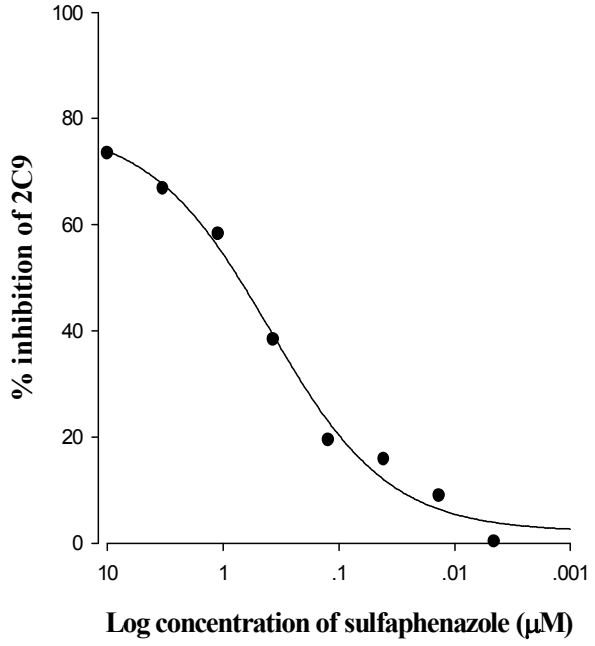




**Fig.1.** Inhibitory effect of cilostazol on CYP3A4 and 2C9 activity. All experiments were done in duplicate, and results are expressed as the percentage of inhibition.

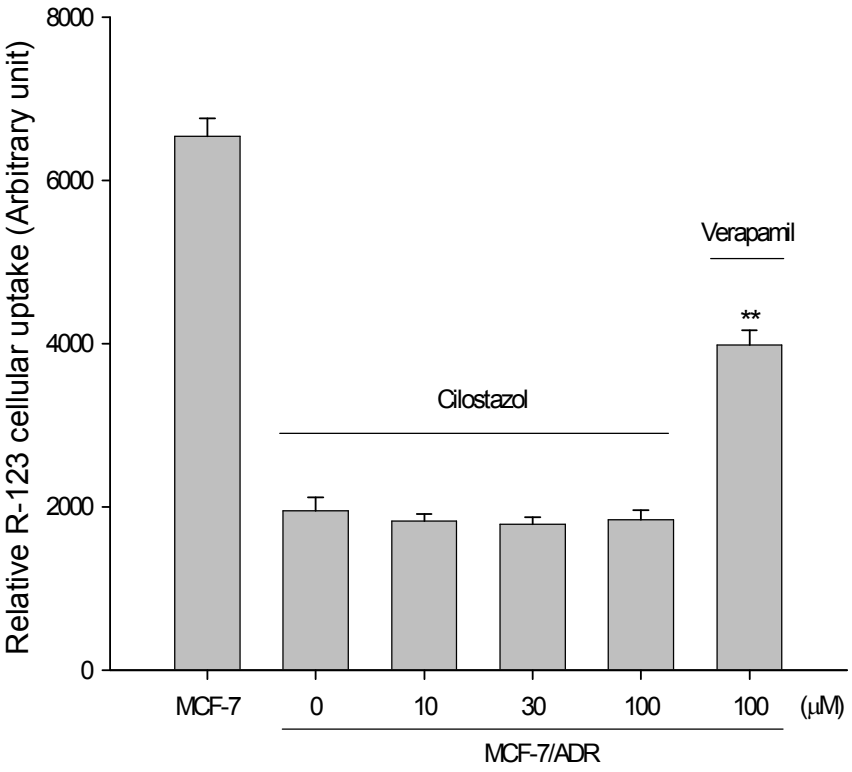



**Fig.2.** Rhodamine-123 (R-123) retention. After incubation of MCF-7/ADR cells with 20 μM R-123 for 90 min, the R-123 fluorescence values in cell lysates were measured using the excitation and emission wavelengths of 480 and 540 nm, respectively. The values were divided by total protein contents of 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.

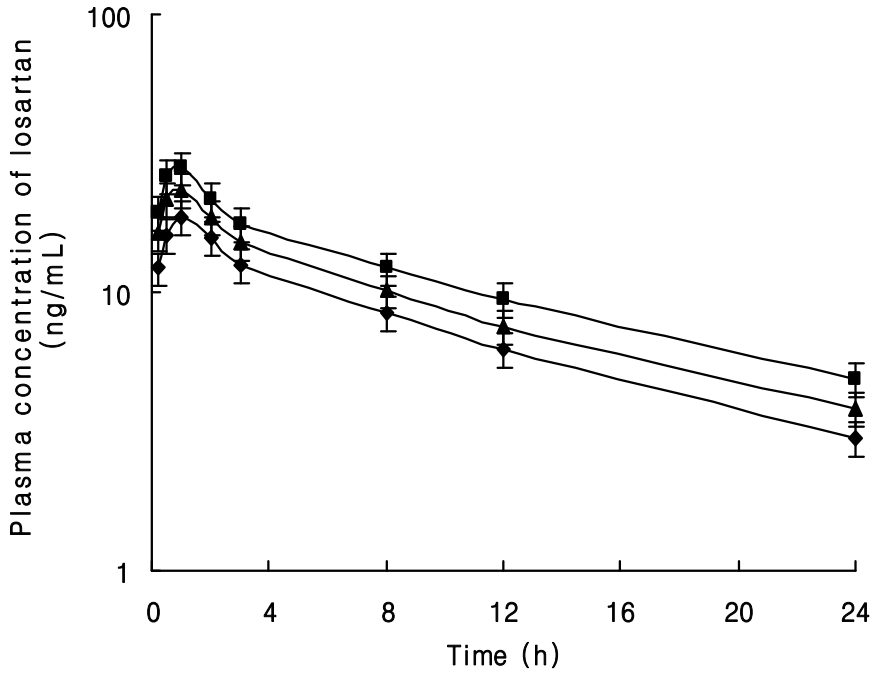



**Fig.3.** Mean plasma concentration-time profiles of losartan following an oral (9 mg/kg) administration of losartan to rats in the presence and absence of cilostazol (Mean ± SD, n = 6). ◆; Control (losartan 9 mg/kg, alone), ▲: with 1.5 mg/kg of cilostazol; ■: with 6 mg/kg of cilostazol

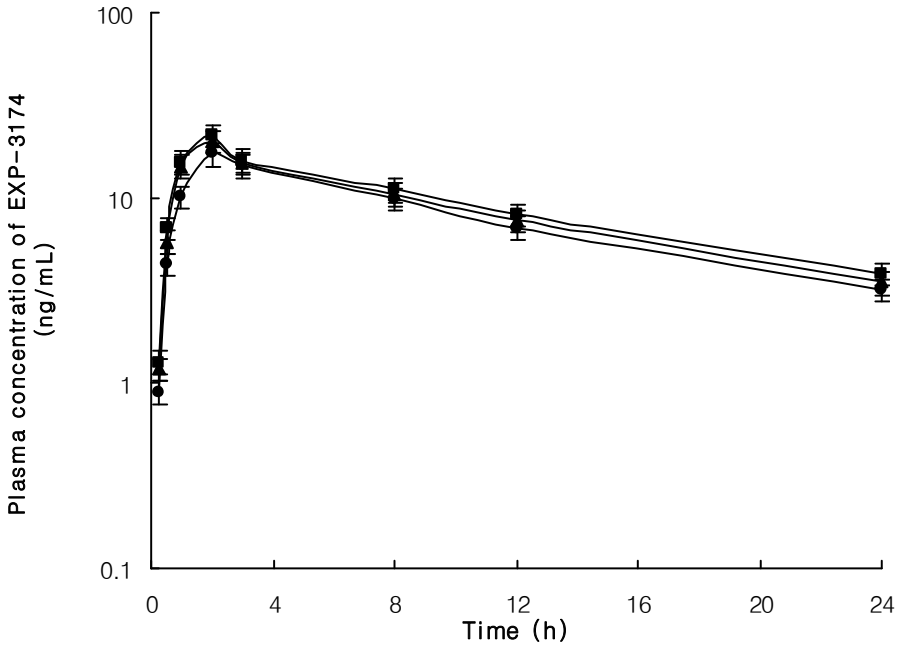



**Fig.4.** Mean plasma concentration-time profiles of EXP-3174 after an oral administration of losartan (9 mg/kg) to rats in the presence and absence of cilostazol (Mean ± SD, n = 6). ●; Control (losartan 9 mg/kg, alone), ▲: with 1.5 mg/kg of cilostazol; ■: with 6 mg/kg of ciostazole

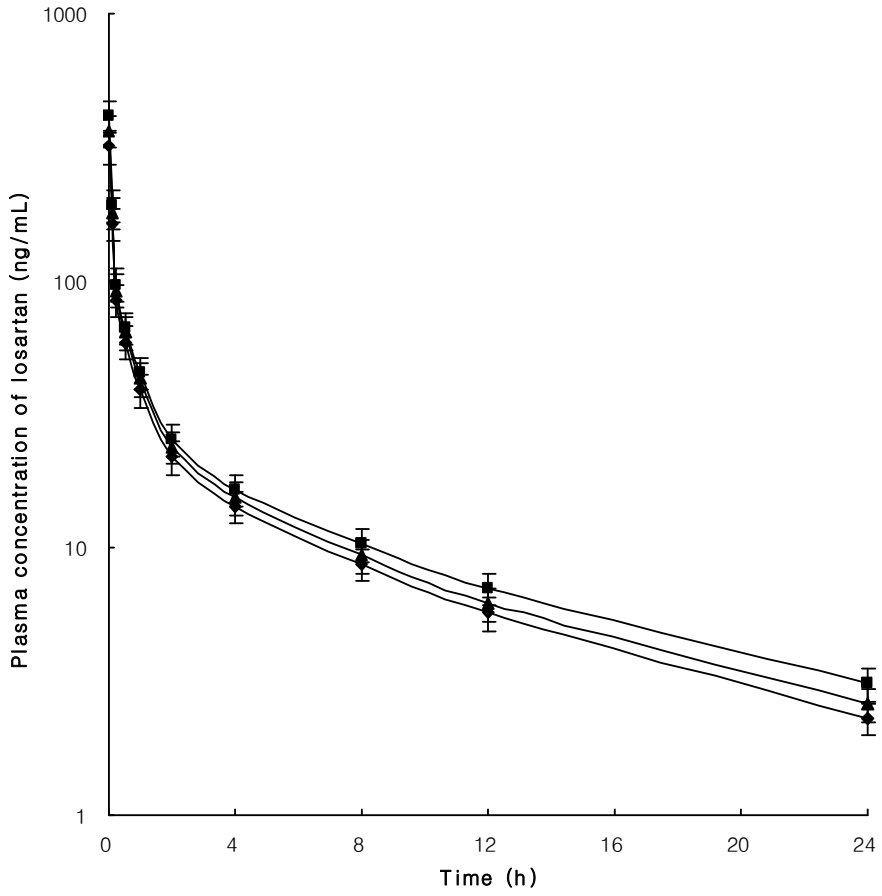



**Fig.5.** Mean plasma concentration-time profiles of losartan after intravenous of losartan (3 mg/kg) administration to rats in the presence or absence of cilostazol at doses of 1.5 and 6 mg/kg. (Mean ± SD, n = 6). ◆: Control (losartan 3 mg/kg, alone), ▲: with 1.5 mg/kg of cilostazol; ■: with 6 mg/kg of cilostazol.

D. **Disscusion**

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 [26-27]. 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, 28].

Total CYP P450 content increased slightly proceeding from the duodenum to the jejunum and then decreased sharply to the ileum (29).

The most abundant CYP isoenzyme in the intestine is 3A4 (30). In vitro metabolism studies in human liver microsomes revealed that hepatic cytochrome P450 enzymes, primarily CYP3A4, are involved in the metabolism of cilostazol (21-22).

Considering that losartan is a substrate of both CYP3A4 and P-gp (6), the effects of cilostazol on the CYP enzyme activity and the cell-based P-gp activity were evaluated.

As shown in Figure 1, cilostazol inhibited CYP3A4 activity with $IC_{50}$ value of 4.1 μM. However, the relative cellular uptake of rhodamine-123 was comparable (Figure 2). This suggested that cilostazol could not inhibit P-gp activity. The inhibitory effect of cilostazol against CYP3A4-mediated metabolism was confirmed by the employment of recombinant CYP enzymes. Therefore, cilostazol, an inhibitor of CYP3A4 may significantly impact the bioavailability of losartan, a substrate of CYP3A4. CYP3A4 expressed in rat is similar to the function of CYP3A4 in human (31-34). This study evaluated the influence of cilostazol on the

pharmacokinetics of losartan in rats in order to assess the potential drug interactions between cilostazol and losartan.

As shown in Table 1, the presence of cilostazol significantly enhanced the $AUC_{0-\infty}$ and $C_{max}$ of losartan in rats. Subsequently, the relative bioavailability (RB) of losartan was increased by 113 to 138% in the presence of cilostazol (1.5 and 6.0 $mg \cdot kg^{-1}$). Those results were similar to reports by Bramer *et al.* (21) in that cilostazol significantly enhanced the $AUC_{0-\infty}$ and $C_{max}$ of lovastatin, a substrate of CYP enzymes and by Yang et al.(32,34) in that simvastatin and ticlopidine significantly increased the $AUC_{0-\infty}$ and $C_{max}$ of losartan in rats.

The pharmacokinetic profiles of EXP-3174 were also evaluated in the presence or absence of cilostazol (Table 2). The metabolite-parent ratio (MR)in the presence of 6 mg/kg of cilostazol was decreased significantly ($p < 0.05$) compared to the control, suggesting that the presence of cilostazol could be effective to inhibit the CYP3A mediated metabolism of losartan. Those results were similar to reports by Yang et al.(32,34) in that simvastatin and ticlopidine significantly decreased the metabolite-parent ratio (MR) of losartan in rats.

The enhanced oral bioavailability of losartan in the presence of cilostazol at 6 mg/kg dose might be due to the inhibition of both CYP3A4 and CYP2C9.

Since the present study raised awareness of potential drug interactions by concomitant use of cilostazol with losartan, this finding has to be further evaluated in clinical studies. The increase in the oral bioavailability of losartan might be mainly attributed to reduced first-pass metabolism of losartan via the inhibition of the CYP3A4 in the small intestine and/or in the liver rather than to inhibition of P-gp in the intestine by cilostazol.

## E. Conclusion

The enhanced oral bioavailability of losartan in the presence of cilostazol might be due to the inhibition of both CYP3A4 and CYP2C9 in the small intestine and/or in the liver and reduction of total body clearance of losartan by cilostazol.

Since the present study raised awareness of potential drug interactions by concomitant use of cilostazol with losartan, this finding has to be further evaluated in clinical studies. The increase in the oral bioavailability of losartan might be mainly attributed to reduced first-pass metabolism of losartan via the inhibition of the CYP3A4 in the small intestine and/or in the liver rather than to inhibition of P-gp in the intestine by cilostazol.

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## Abstract

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 cilostazol (1.5 and 6 mg/kg). Cilostazol inhibited CYP3A4 and CYP2C9 enzyme activity with 50% inhibition concentration ($IC_{50}$) of 7.7 and 9.8 μM, respectively. The pharmacokinetic parameters of losartan were significantly altered by the presence of cilostazol compared with the control group. Cilostazol significantly increased the area under the plasma concentration–time curve ($AUC_{0-\infty}$) of losartan by 52.0% and peak plasma concentration ($C_{max}$) of losartan by 48.4%. The total body clearance ($CL/F$) was significantly decreased by cilostazol by 34.2%. Consequently, the absolute bioavailability ($F$) of losartan in the presence of cilostazol was 27.6%, which was enhanced significantly ($p < 0.05$) compared with the oral control group (25.4%). Metabolite-parent AUC ratio in the presence of cilostazol (6 mg/kg) significantly ($p < 0.05$) decreased by 23.8% compared to the control group. In iv administration, cilostazol significantly increased the area under the plasma concentration–time curve ($AUC_{0-\infty}$) of losartan by 20.0%, implying that coadministration of cilostazol could be effective to inhibit the CYP3A4-and CYP2C9-mediated metabolism of losartan. In conclusion, the enhanced oral bioavailability of losartan by cilostazol may result from inhibition of CYP3A4-and CYP2C9-mediated metabolism in small intestine and in the liver and/or reduction of total body clearace of losartan by cilostazol.


**Key words:** losartan; EXP-3174; cilostazol; CYP3A4; CYP2C9; P-gp; pharmacokinetics; bioavailability; rats

# Part II. Effects of cilostazol on the pharmacokinetics of nifedipine and its main metabolite, dehydronifedipine, in rats

# 국 문 초 록

항혈소판제인 시로스타졸과 니페디핀과의 병용투여가 순환기 질환 예방 및 치료를 위해서 처방되는 경우가 많으므로 이에 대한 상호작용을 알아보고자, 흰쥐에 항혈소판제인 시로스타졸 (1.5, 6 mg/kg)과 니페디핀 (경구; 10 mg/kg, 정맥; 2.5 mg/kg)과의 병용 경구투여한 후 니페디핀과 그 활성대사체인, 디히드로니페디핀의 약물동태학적 변수들을 대조군과 비교 검토하였다.

시로스타졸과 병용 투여시 니페디핀의 약물동태학적 변수는 유의성 있게 변화하였다. 대조군에 비해 시로스타졸 (6 mg/kg)과 병용투여군에서 니페디핀의 혈장농도곡선하면적 ($AUC_{0-\infty}$)과 최고혈중농도 ($C_{max}$)는 유의성 ($P < 0.05$) 있게 증가되었으며, 전신클리어런스 (CL/F)는 유의성 ($P < 0.05$) 있게 각각 감소되었다.

절대적생체이용률 (AB)도 대조군에 비해 유의성 ($P < 0.05$) 있게 증가되었다. 시로스타졸 (6 mg/kg)과 니페디핀을 병용투여한군에서 대조군에 비해 활성대사체는 혈장농도곡선하면적 ($AUC_{0-\infty}$)이 증가되었으나 유의성은 없었으나 니페디핀의 대사율 (MR)을 유의성 있게 감소시켰다.

정맥투여군에서는 시로스타졸과 병용투여시 니페디핀의 혈장농도곡선하면적 ($AUC_{0-\infty}$)이 유의성 있게 증가되었다. 본 연구에서 항혈소판제인 시로스타졸과 고혈압치료제인 니페디핀을 병용투여 하였을 때 경구투여된 니페디핀의 생체이용률이 유의성 있게 증가된 것은 소장과 간장에 존재하는 CYP3A4 효소의 억제에 의한 니페디핀의 대사억제와 또는 전신 클리어런스 감소에 기인한 것으로 사료된다.

## A. Introduction

Nifedipine (dimethyl2, 6-dimethyl-4-(2-nitrophenyl)-1, 4-dihydropyridine-3,5-dicarboxylate) is a calcium channel-blocking agent that is widely used for the treatment of essential hypertension, coronary artery spasm, and angina pectoris (1). It inhibits the influx of extracellular calcium through myocardial and vascular membrane pores by physically plugging the channel, resulting in decreased intracellular calcium levels, inhibition of the contractile processes of smooth muscle cells, dilation of the coronary and systemic arteries, increased oxygen delivery to the myocardial tissue, and decreased total peripheral resistance, systemic blood pressure, and afterload (2, 3).

In humans, nifedipine is predominantly metabolized by CYP3A4 to its primary pyridine metabolite, dehydronifedipine (4, 5). CYP enzymes are responsible for the oxidative metabolism of many xenobiotics and play a major role in the phase I metabolism of many drugs (6). CYP3A4 is the most abundant CYP enzyme (30-40%) in adult liver and metabolizes more than 50% of the clinically used drugs including nifedipine, cyclosporine, midazolam, and (7,8). There are some reports that nifedipine is a substrate of CYP3A4 in human (9-11). P-gp is an adenosine-50-triphosphate (ATP) dependent efflux drug transporter that is constitutively expressed in normal tissues that includes gastrointestinal epithelium, canalicular membrane of the liver, kidney (12,13) and capillary endothelial cells in the central nervous system (14,15). Because of such tissue localized and its broad substrate specificity, P-gp appears to play a key role in absorption, distribution, and elimination of many drugs (16, 17). It is generally known that the substrate and/or inhibitors of CYP3A4 and P-gp overlap with each other (18). Dorababu *et al.* (19) reported that nifedipine belonged to a group of P-gp substrate. Since P-gp is co-localized with CYP3A4 in the small intestine, P-gp and CYP3A4 may act synergistically to promote presystemic drug metabolism, resulting in the limited

absorption of drugs.

Cilostazol (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2(1H)-quinolinone) is an antiplatelet vasodilator agent that has been used for more than a decade in Japan for the treatment of chronic peripheral arterial occlusive disease (20). Cilostazol was also approved by the US Food and Drug Administration in 1998 for the treatment of symptoms related to intermittent claudication (21). Cilostazol exerts its pharmacologic effects through selective inhibition of phosphodiesterase-3, which is abundant in platelets and vascular smooth muscle cells. These inhibitory effects mediate the antiplatelet properties of cilostazol and suppress vascular smooth muscle cell proliferation (22). Cilostazol is extensively metabolized by cytochrome P450 (CYP) enzymes, primarily CYP3A4, into the active metabolites 3,4-dehydrocilostazol and 4'-trans-hydroxy-cilostazol (23,24). However, the effect of cilostazol on P-gp activity has not been reported. Thus we attempted to evaluate P-gp activity using rhodamine-123 retention assay in P-gp-overexpressed MCF-7/ADR cells, and furthermore, to evaluate CYP3A4 activity of cilostazol.

The drug–drug interaction could be possible since cilostazol and nifedipine could be prescribed for the prevention or treatment of cardiovascular diseases as a combination therapy. However, the effect of cilostazol on the pharmacokinetics of nifedipine in vivo has not been reported. Thus, the purpose of this study was to investigate the possible effects of cilostazol on the CYP3A4 and P-gp activity and bioavailability or the pharmacokinetics of nifedipine and its active metabolite, dehydronifedipine, after oral and intravenous administration of nifedipine with cilostazol in rats.

## B. Materials and Methods

### 1. Materials

Nifedipine, dehydronifedipine, cilostazol and amlodipine [internal standard for the high-performance liquid chromatographic (HPLC) analysis of nifedipine] were purchased from the Sigma-Aldrich Co. (St. Louis, MO, USA). Methanol, isooctane, methyl-*tert*-butyl ether (MTBE), analytical grade acetic acid and triethylamine (TEA) were products from Merck Co. (Darmstadt, Germany). Rhodamine was from Calbiochem (USA), the CYP inhibition assay kit was from GENTEST (Woburn, MA, US). Other chemicals were of reagent or HPLC grade.

Apparatus used in this study included an HPLC equipped with a Waters 1515 isocratic HPLC Pump, a Waters 717 plus auto sampler and a Waters$^{TM}$ 2487 scanning UV detector (Waters Co., Milford, MA, USA), an HPLC column temperature controller (Phenomenex Inc., CA, USA), a Bransonic® Ultrasonic Cleaner (Branson Ultrasonic Co., Danbury, CT, USA), a vortex-mixer (Scientific Industries Co., NY, USA), and a high-speed microcentrifuge (Hitachi Co., Tokyo, Japan).

### 2. Animal studies

All animal study protocols were approved by the Animal Care Committee of Chosun University (Gwangju, Republic of Korea). Male Sprague-Dawley rats (270-300 g) were purchased from Dae Han Laboratory Animal Research Co. (Eumsung, Republic of Korea), and were given free access to a normal standard chow diet (No. 322-7-1; Superfeed Co., Wonju, Republic of Korea) and tap water. Throughout the experiments, 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 rats were acclimated under these conditions for at least 1 week. Each rat was fasted for at least 24 h prior to the experiment. The left femoral artery (for blood sampling) and left femoral vein (for drug administration in the intravenous study) were cannulated using a polyethylene tube (SP45; i.d., 0.58 mm,

o.d., 0.96 mm; Natsume Seisakusho Company, Tokyo, Japan) while each rat was under light ether anesthesia.

**3. Intravenous and oral administration of nifedipine**

The rats were divided into six groups ($n$=6, each): oral groups [10 mg · $kg^{-1}$ of nifedipine dissolved in distilled water (1.0 ml · $kg^{-1}$)] without (control) or with 1.5 and 6.0 mg · $kg^{-1}$ of cilostazol (mixed in distilled water; total oral volume of 1.0 ml · $kg^{-1}$), and intravenous groups (2.5 mg · $kg^{-1}$ of nifedipine; the same solution used: 0.9% NaCl-injectable solution; total injection volume of 1.0 ml · $kg^{-1}$) without (control) or with 1.5 and 6.0 mg · $kg^{-1}$ of cilostazol. A feeding tube was used to administer nifedipine and cilostazol intragastrically. Cilostazol was administered 30 min prior to oral administration of nifedipine. A blood sample (0.5-ml aliquot) was collected into heparinized tubes via the femoral artery at 0.017 (at the end of infusion), 0.1, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h for the intravenous study, and 0.25, 0.5, 0.75, 1, 2, 4, 8, 12, and 24 h for the oral study. Whole blood (approximately 1.2 ml) collected from untreated rats was infused via the femoral artery at 0.75, 4, and 8 h, respectively, to replace blood loss due to blood sampling. The blood samples were centrifuged (13,000 rpm, 3 min), and a 200-µl aliquot of plasma samples was stored in the deep freezer at -40$^{o}$C until the HPLC analysis.

**4. HPLC assay**

The plasma concentrations of nifedipine were determined using an HPLC assay by a modification of the method reported by Grundy *et al.* (25). Briefly, 50-µL of amlodipine (3 µg · $ml^{-1}$), as the internal standard and 50-µl of 1.0 M sodium hydroxide were added to 0.2-ml of the plasma sample. It was then mixed for 3 s and 1-ml MTBE-isooctane (75:25, v/v) was added. The resultant mixure was vortex-mixed for 1 min and centrifuged at 3,000 rpm for 5 min. The organic layer (0.8 ml) was transferred into a clean test tube and evaporated under a gentle stream of nitrogen gas (no heat applied). The dried extract was reconstituted with 200 µl of

mobile phase vortex-mixed for 1 min and aliquots of 160 μl were transferred to a clean autosampler vial. A 70-μl aliquot of the supernatant was injected into the HPLC system. The UV detector wavelength was set to 350 nm; and the column, a Nova-pack $C_8$ (100mm×8 mm I.D., 4 μm; Waters Co., Milford, MA, USA), was used at room temperature. A mixture of methanol : water (62:38, v/v, pH 4.5, adjusted with acetic acid, 320 μl TEA/1000 ml mixture was added) was used as the mobile phase at a flow rate of 1.0 ml · $min^{-1}$. The retention times were: internal standard at 16.8 min, nifedipine at 8.2 min, and dehydronifedipine at 6.5 min. The detection limits of nifedipine and dehydronifedipine in rat plasma were all 5 ng · $ml^{-1}$. The coefficients of variation for nifedipine and dehydronifedipine were all below 5.0%.

**5. CYP3A4 inhibition assay**

The assay of inhibition on human CYP3A4 enzyme activity was performed in a multiwell plate using CYP inhibition assay kit (GENTEST, Woburn, MA) as described previously (26). Briefly, human CYP enzyme was obtained from baculovirus-infected insect cells. CYP substrate (7-BFC for CYP3A4) was incubated with or without test compounds in the enzyme/substrate buffer with 1 pmol of P450 enzyme and an NADPH-generating system (1.3 mM NADP, 3.54mM glucose 6-phosphate, 0.4 U · $ml^{-1}$ 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 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 for CYP3A4) was run on the same plate and produced 99% inhibition. All experiments were done in duplicate, and the results were expressed as the percent of inhibition.

**6. Rhodamine-123 retention assay**

The procedures used for the Rho-123 retention assay were similar to a reported method (27). 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 or absence of cilostazol (10, 30 and 100 μM) and verapamil (positive control) for 90 min, the medium was completely removed. The cells were then washed three times with ice-cold phosphate buffer (pH 7.0) and lysed in EBC lysis buffer. 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 were presented as the ratio to control.

**7. Pharmacokinetic analysis**

The plasma concentration data were analyzed by the non-compartmental method using Thermo Kinetica Software Version 5.0 (Thermo Fisher Scientific Inc., Miami, OK, USA). The parameter values were obtained by fitting to the pharmacokinetic model using the simplex algorithm. The area under the plasma concentration–time curve ($AUC_{0-\infty}$) was calculated by a trapezoidal rule. The peak concentration ($C_{max}$) of nifedipine in plasma and time to reach $C_{max}$ ($T_{max}$) were obtained by visual inspection of the data from the concentration–time curve. The terminal half-life ($t_{1/2}$) was calculated by $0.693/K_{el}$. Total body clearance (CL/F) was calculated by dose/AUC. The extent of absolute bioavailability (F) was calculated by $AUC_{oral}/AUC_{i.v.} \times dose_{i.v.}/dose_{oral}$, and the relative bioavailability (RB) of nifedipine was calculated by $AUC_{nifedipine\ with\ cilostazol}/AUC_{control}$. The metabolite–parent AUC ratio (MR) was calculated by $AUC_{dehydronifedipine}/AUC_{nifedipine}$.

**8. Statistical analysis**

All the means were presented with their standard deviation. 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.

## C. Results

### 1. Inhibition of CYP3A4 activity

The inhibitory effect of cilostazol on CYP3A4 activity is shown in Figure 6. Cilostazol inhibited CYP3A4 activity in a concentration-dependent manner. Cilostazol inhibited CYP3A4 activity with an $IC_{50}$ value of 4.1 μM.

### 2. Rhodamine-123 retention assay

Accumulation of rhodamine-123, a P-glycoprotein substrate, was increased in MCF-7/ADR cells overexpressing P-glycoprotein compared to that in MCF-7 cells lacking P-glycoprotein, as shown in Figure 7. The concurrent use of cilostazol did not enhance the cellular uptake of rhodamine-123 in a concentration-dependent manner. This result suggests that cilostazol did not inhibit P-gp activity.

### 3. Effect of cilostazol on the pharmacokinetics of oral nifedipine

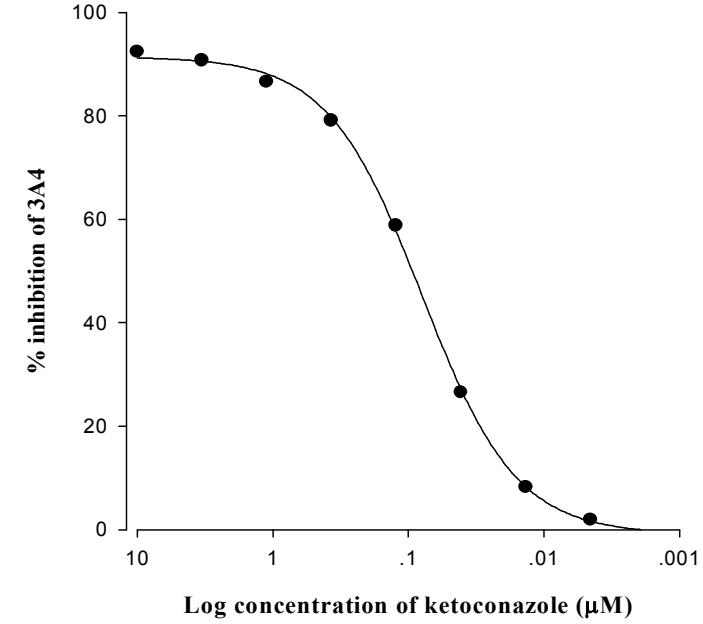
The mean plasma concentration–time profiles of nifedipine in the presence and absence of cilostazol (1.5 and 6.0 mg · $kg^{-1}$) are shown in Figure 8. The pharmacokinetic parameters of nifedipine are summarized in Table 4. Cilostazol (6.0 mg · $kg^{-1}$) significantly ($p < 0.05$) increased the area under the plasma concentration–time curve from time zero to time infinity ($AUC_{0-\infty}$) of nifedipine by 41.0%, and peak concentration ($C_{max}$) of nifedipine by 35.3%. The total body clearance (CL/F) was significantly decreased (6.0 mg · $kg^{-1}$, $p < 0.05$) by cilostazol (31.0%). Accordingly, the extent of absolute bioavailability ($F$) values of nifedipine in the presence of cilostazol (6.0 mg · $kg^{-1}$) were significantly ($p < 0.05$) higher (20.2%) than that of the control group. Cilostazol increased the relative bioavailability (RB) of nifedipine by 1.14- to 1.41-fold. There were no significant differences in the time to reach peak plasma concentration ($T_{max}$), terminal half-life ($t_{1/2}$) of nifedipine in the presence of cilostazol.

**4. Effect of cilostazol on the pharmacokinetics of dehydronifedipine**

The plasma concentration–time profiles of dehydronifedipine are shown in Figure 9. The pharmacokinetic parameters of dehydronifedipine are summarized in Table 5. The $AUC_{0-\infty}$ of dehydronifedipine was not significantly increased (6.0 mg · $kg^{-1}$, $p < 0.05$) by cilostazol. The MR ratios were significantly ($p < 0.05$, 6.0 mg · $kg^{-1}$) decreased (20.0%) by cilostazol, suggesting that the formation of dehydronifedipine was considerably altered by cilostazol. Thus, the increased bioavailability of nifedipine by cilostazol may be mainly due to the inhibition of CYP3A4 activity in the small intestine and/or in the liver by cilostazol.

**5. Effect of cilostazol on the pharmacokinetics of intravenous nifedipine**

Mean arterial plasma concentration-time profiles of nifedipine following an intravenous administration of nifedipine (2.5 mg · $kg^{-1}$) to rats in the presence or absence of cilostazol (1.5 and 6.0 mg · $kg^{-1}$) are shown in Figure 10, while the corresponding pharmacokinetic parameters are shown in Table 6. The $AUC_{0-\infty}$ and $CL_t$ of nifedipine were significantly changed by 19.0 and 22.2% compared to those in the control group. The $t_{1/2}$ of nifedipine was also prolonged, but this increase was not statistically significant. Accordingly, the enhanced bioavailability in the presence of cilostazol may be mainly due to inhibition of the CYP3A-mediated metabolism of nifedipine in the small intestine and/or in the liver by cilostazol.



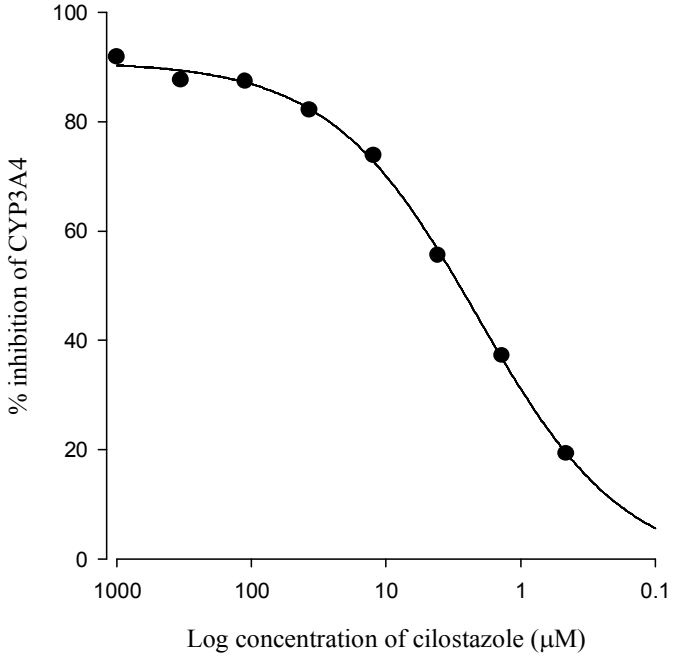




**Fig. 6.** Inhibitory effect of ketokonazole and cilostazol on CYP3A4 activity. All experiments were done in duplicate, and the results were expressed as the percent of inhibition.

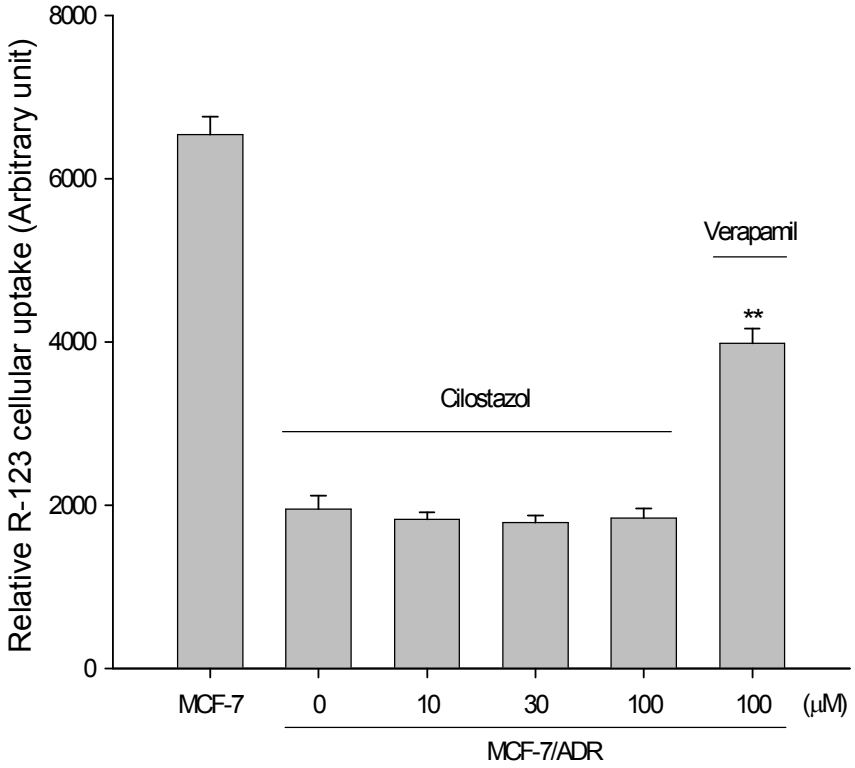



**Fig.7.** Effects of cilostazol on the cellular accumulation of rhodamine-123 in MCF-7 and MCF-7/ADR cells. Mean±SD ($n$=6). **$p < 0.01$ *vs* control group.

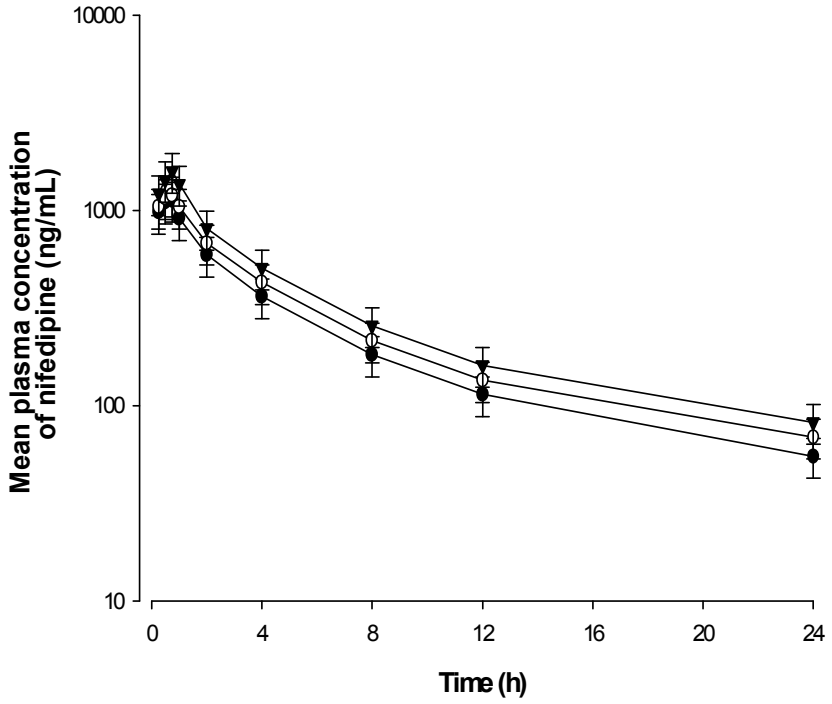



**Fig.8.** Mean plasma concentration-time profiles of nifedipine after oral (10 mg · $kg^{-1}$) administration of nifedipine in the presence and absence of cilostazol to rats. Mean±SD (*n*=6). -●-: Control (only nifedipine 10 mg · $kg^{-1}$ alone); -○-: with cilostazol 1.5 mg · $kg^{-1}$; -▼- with cilostazol 6.0 mg · $kg^{-1}$.

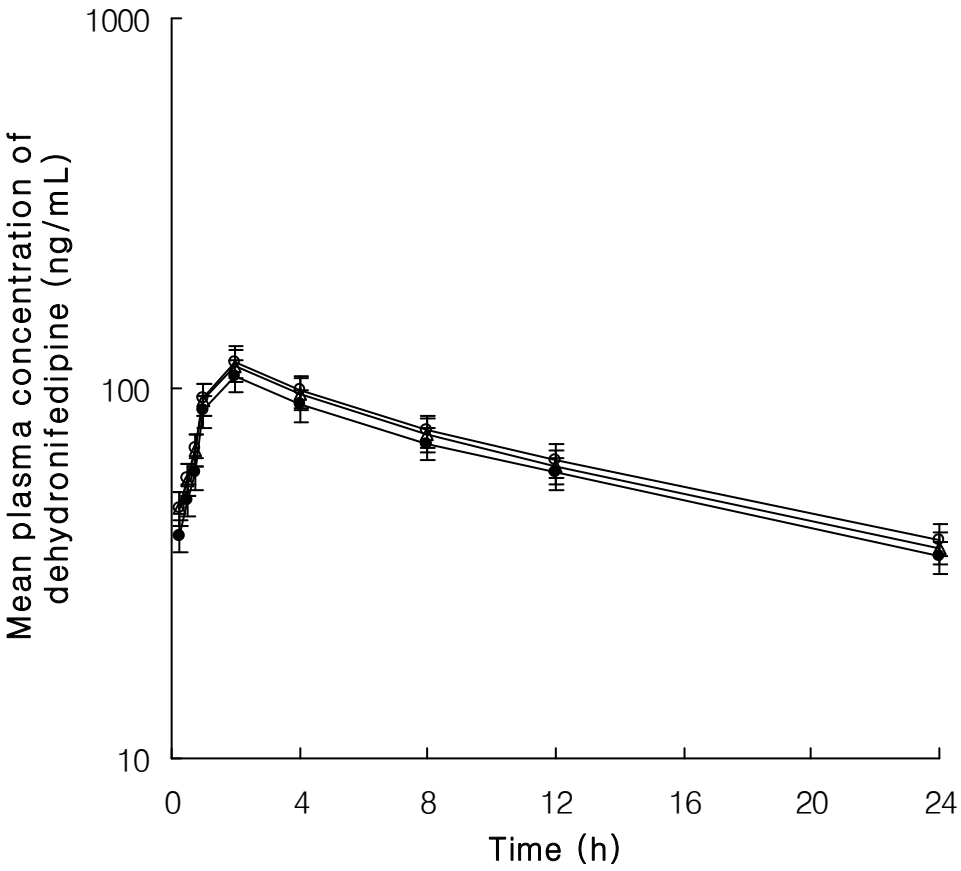



**Fig. 9.** Mean plasma concentration-time profiles of dehydronifedipine after oral administration of nifedipine (10 mg · $kg^{-1}$) in the presence and absence of cilostazol to rats. Mean±SD (*n*=6). -●-: Control (only nifedipine 10 mg · $kg^{-1}$ alone); -△-: with cilostazol 1.5 mg · $kg^{-1}$; -○- with cilostazol 6.0 mg · $kg^{-1}$.

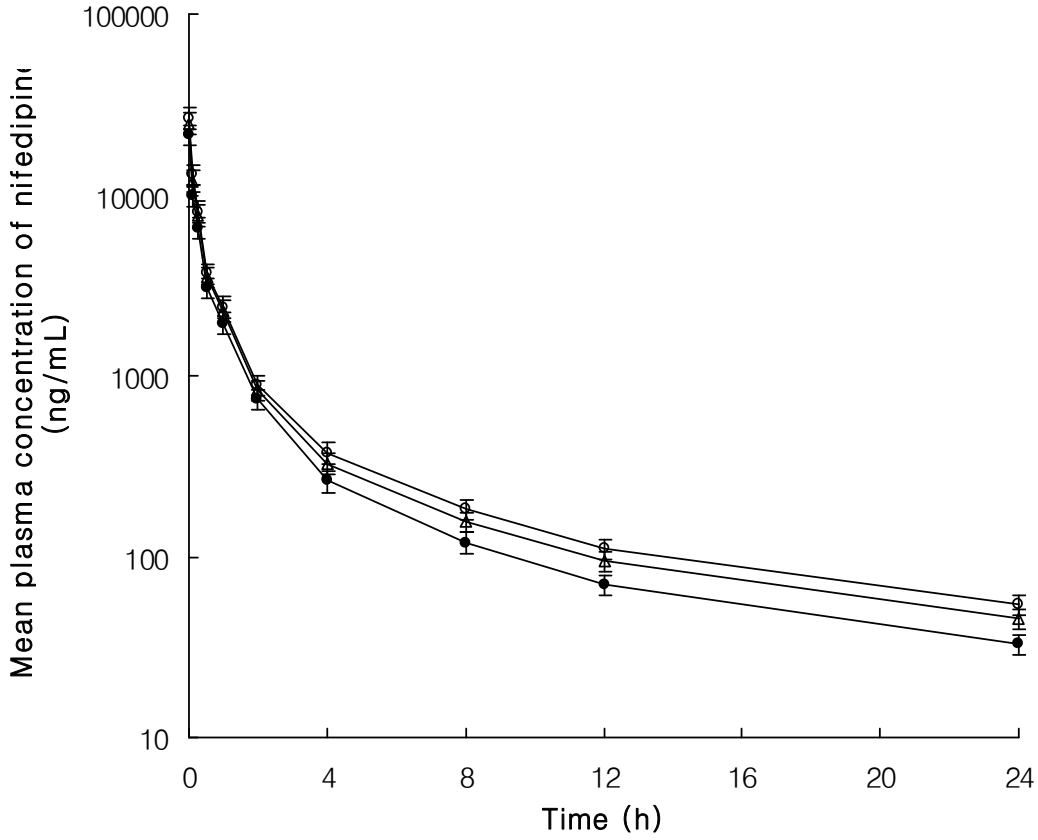



**Fig. 10.** Mean plasma concentration-time profiles of nifedipine after intravenous (2.5 mg · $kg^{-1}$) administration of nifedipine in the presence and absence of cilostazol to rats. Mean±SD (*n*=6). -●-: Control (only nifedipine 2.5 mg · $kg^{-1}$ alone); -Δ-: with cilostazol 1.5 mg · $kg^{-1}$; -○- with cilostazol 6.0 mg · $kg^{-1}$.

**Table 4.** Pharmacokinetic parameters of nifedipine after oral (10 mg · $kg^{-1}$) administration in the presence or absence of cilostazol at doses of 1.5 and 6.0 mg · $kg^{-1}$. *n*=6. * $P < 0.05$ *vs* control group (only nifedipine alone).

| Parameter | Control | Nifedipine+Cilostazol | |
|---|---|---|---|
| | | 1.5 mg · $kg^{-1}$ | 6.0 mg · $kg^{-1}$ |
| $AUC_{0-\infty}$ (ng · $ml^{-1}$· h) | 5931±1067 | 6761±1258 | 8370±1524* |
| $C_{max}$ (ng · $ml^{-1}$) | 1130±223 | 1159±233 | 1529±310* |
| $T_{max}$ (h) | 0.76±0.10 | 0.78±0.10 | 0.81±0.13 |
| $t_{1/2}$ (h) | 9.5±1.8 | 10.1±2.1 | 10.3±2.1 |
| CL/F (ml · $min^{-1}$) | 28.1±3.1 | 24.4±2.6 | 19.4±2.2* |
| *F* (%) | 15.8±2.6 | 16.3±2.8 | 19.0±3.0* |
| RB (%) | 100 | 114 | 141 |

* $p < 0.05$, significant difference compared to control. $AUC_{0-\infty}$, area under the plasma concentration-time curve from 0 h to infinity; $C_{max}$, peak plasma concentration; $T_{max}$, time to reach peak plasma concentration; $t_{1/2}$, terminal half-life; CL/F, total body clearance; *F*, extent of absolute oral bioavailability; RB, relative bioavailability.

**Table 5.** Pharmacokinetic parameters of dehydronifedipine after oral (10 mg · $kg^{-1}$) administration in the presence or absence of cilostazol at doses of 1.5 and 6.0 mg · $kg^{-1}$. $n$=6. [a]$P$<0.05 *vs* control group (only nifedipine alone).

| Parameter | Control | Nifedipine+Cilostazol | |
|---|---|---|---|
| | | 1.5 mg · $kg^{-1}$ | 6.0 mg · $kg^{-1}$ |
| $AUC_{0-\infty}$ (ng · $ml^{-1}$· h) | 2206±380 | 2360±424 | 2510±441 |
| $C_{max}$ (ng · $ml^{-1}$) | 110±19.2 | 114±20.9 | 120±22.1 |
| $T_{max}$ (h) | 1.7±0.5 | 1.8±0.4 | 1.8±0.4 |
| $t_{1/2}$ (h) | 15.1±2.6 | 16.4±2.7 | 17.2±2.9 |
| RB (%) | 100 | 107 | 114 |
| MR | 37.2±3.3 | 34.9±2.9 | 29.8±2.6* |

* $p$ < 0.05, significant difference compared to control. $AUC_{0-\infty}$, area under the plasma concentration-time curve from 0 h to infinity; $C_{max}$, peak plasma concentration; $T_{max}$, time to reach peak plasma concentration; $t_{1/2}$, terminal half-life; RB, relative bioavailability; MR, metabolite–parent AUC ratio.

**Table 6.** Pharmacokinetic parameters of nifedipine after intravenous (2.5 mg · $kg^{-1}$) administration in the presence or absence of cilostazol at doses of 1.5 and 6.0 mg · $kg^{-1}$. *n*=6.

| Parameter | Control | Nifedipine+Cilostazol | |
|---|---|---|---|
| | | 1.5 mg · $kg^{-1}$ | 6.0 mg · $kg^{-1}$ |
| $AUC_{0-\infty}$ (ng · $ml^{-1}$ · h) | 9343±1401 | 10370±1439 | 11121±1652* |
| $CL_t$ (ml · $min^{-1}$) | 4.5±0.5 | 3.9±0.4 | 3.5±0.3* |
| $t_{1/2}$ (h) | 8.8±1.5 | 8.9±1.6 | 9.3±1.8 |
| RB (%) | 100 | 111 | 119 |

* $p < 0.05$, significant difference compared to control.

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

## D. Discussion

The importance of first-pass metabolism for limiting systemic drug availability is well established; however, intestinal drug metabolism can further diminish systemic availability. Through functional enzyme activity studies and immunoblot analyses, CYP3A expression in mature enterocytes, located mainly in the villi tips, of jejunal mucosa was shown to be comparable to or even exceed the expression of CYP3A in hepatocytes (28). Total CYP P450 content increased slightly proceeding from the duodenum to the jejunum and then decreased sharply to the ileum (29). Using in situ hybridization with a probe specific for CYP3A4, McKinnon confirmed CYP3A expression throughout the entire small intestine, with highest levels in the proximal regions (30). The most abundant CYP isoenzyme in the intestine is 3A4 (31). In vitro metabolism studies in human liver microsomes revealed that hepatic cytochrome P450 enzymes, primarily CYP3A4, are involved in the metabolism of cilostazol (23,24).

Considering that nifedipine is a substrate of both CYP3A4 and P-gp (9, 10, 11,19), the effects of cilostazol on the CYP enzyme activity and the cell-based P-gp activity were evaluated. As shown in Figure 6, cilostazol inhibited CYP3A4 activity with $IC_{50}$ value of 4.1 μM. However, the relative cellular uptake of rhodamine-123 was comparable (Figure 7). This suggested that cilostazol could not inhibit P-gp activity. The inhibitory effect of cilostazol against CYP3A4-mediated metabolism was confirmed by the employment of recombinant CYP enzymes. Therefore, cilostazol, an inhibitor of CYP3A4 may significantly impact the bioavailability of nifedipine, a substrat of CYP3A4. As CYP3A9 expressed in rat is corresponding to the ortholog of CYP3A4 in human (32), rats' CYP3A2 are similar to human's CYP3A4 (33,34). Human 3A4 and rat 3A1 have 73% protein homology (35). 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 (36).

This study evaluated the influence of cilostazol on the pharmacokinetics of nifedipine in rats in order to assess the potential drug interactions between cilostazol and nifedipine.

As shown in Table 4, the presence of cilostazol significantly enhanced the $AUC_{0-\infty}$ and $C_{max}$ of nifedipine in rats. Subsequently, the relative bioavailability (RB) of nifedipine was increased by 114 to 141% in the presence of cilostazol (1.5 and 6.0 mg · $kg^{-1}$). Those results were similar to reports by Bramer *et al.* (24) in that cilostazol significantly enhanced the $AUC_{0-\infty}$ and $C_{max}$ of lovastatin, a substrate of CYP enzymes. This study's results were also consistent with the report that oral diallyl trisulfide (major organosulfur compounds derived from garlic) significantly increased the bioavailability of nifedipine by inhibition of CYP3A4 in rats (37).

Kuroha *et al.* and Nishimura et al (38, 39)reported that ketoconazole and Coenzyme Q10 significantly increased the $AUC_{0-\infty}$ and $C_{max}$ of nifedipine in rats.

The $AUC_{0-\infty}$ of dehydronifedipine was not significantly increased by the presence of 6.0 mg · $kg^{-1}$ of oral cilostazol (Table 5). The metabolite-parent ratio (MR) in the presence of cilostazol (6.0 mg · $kg^{-1}$) was significantly ($p < 0.05$) decreased compared to that of the control group (20.0%). This result suggests that cilostazol inhibited presystemic metabolism of nifedipine, resulting in the enhanced oral bioavailability of nifedipine.

After intravenous administration of nifedipine with cilostazol, the AUC of nifedipine was significantly increased by 19.0% (Table 6). This suggests that the effects of oral cilostazol on the inhibition of hepatic metabolism of nifedipine via CYP3A4 was effective. Since the present study raised awareness of potential drug interactions by concomitant use of cilostazol with nifedipine, this finding has to be further evaluated in clinical studies. The increase in the oral bioavailability of nifedipine might be mainly attributed to reduced first-pass metabolism of nifedipine via the inhibition of the CYP3A4 in the small intestine and/or in the liver rather than both to inhibition of P-gp in the intestine and to reduction of renal elimination of nifedipine by cilostazol.

## E. Conclusion

The increased bioavailability of nifedipine in rats might be mainly due to the inhibition of CYP3A4-mediated metabolism in the small intestine and/or liver rather than the inhibition of P-gp activity by cilostazol. Therefore, concomitant use of cilostazol with nifedipine will require close monitoring of potential drug interactions for the safe therapy of cardiovascular diseases. The clinical importance of these findings should be further investigated in clinical trials.

## F. **References**

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**Abstract**

The purpose of this study was to investigate the possible effects of cilostazol on the pharmacokinetics of nifedipine and its main metabolite, dehydronifedipine, in rats. The effect of cilostazol on P-glycoprotein (P-gp) and cytochrome P450 (CYP) 3A4 activity was evaluated.

The pharmacokinetic parameters of nifedipine and dehydronifedipine were determined after oral and intravenous administration of nifedipine to rats in the presence and absence of cilostazol (1.5 and 6.0 mg · $kg^{-1}$).

Cilostazol inhibited CYP3A4 enzyme activity in a concentration-dependent manner with a 50% inhibition concentration ($IC_{50}$) of 4.1 μmol. In addition, cilostazol did not significantly enhance the cellular accumulation of rhodamine-123 in MCF-7/ADR cells overexpressing P-glycoprotein. The areas under the plasma concentration-time curve ($AUC_{0-\infty}$) and the peak concentration ($C_{max}$) of nifedipine were significantly (6.0 mg · $kg^{-1}$, $p < 0.05$) increased by 41.0% and 35.3%, repectively, in the presence of cilostazol compare to those of control. The total body clearance (CL/F) was significantly (6.0 mg · $kg^{-1}$, $p < 0.05$) decreased by cilostazol (31.0%). Consequently, the absolute bioavailability (AB) of nifedipine in the presence of cilostazol (6.0 mg · $kg^{-1}$) was significantly ($p < 0.05$) higher (20.2%) than that of the control group. Moreover, the relative bioavailability (RB) of nifedipine was 1.14- to 1.41-fold greater than that in the control group. The metabolite-parent AUC ratio (MR) in the presence of cilostazol (6.0 mg · $kg^{-1}$) significantly decreased (20.0%) compared to the control group. In iv, $AUC_{0-\infty}$) of nifedipine was significantly (6.0 mg · $kg^{-1}$, $p < 0.05$) increased by 19.0% in the presence of cilostazol compare to that of control group.

The enhanced bioavailability of nifedipine in rats might be mainly due to the inhibition of CYP3A4-mediated metabolism in the small intestine and/or liver and reduction of CL/F of nifedipine by cilostazol.

**Keywords:** nifedipine, dehydronifedipine, cilostazol, CYP3A4, P-gp, pharmacokinetics, bioavailability, rats.

# Abstract

## Effects of cilostazol on the pharmacokinetics of losartan and/or nifedipine in rats

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The purpose of this study was to investigate the possible effects of cilostazol, antiplatelet vasodilator agent, on the pharmacokinetics of losartan and/or nifedipine and their main metabolite in rats.

The pharmacokinetic parameters of losartan and nifedipine were determined after oral and intravenous administration of losartan (9 and 3 mg/kg) and nifedipine (10 and 2.5 mg/kg) to rats in the presence and absence of cilostazol (1.5 and 6 mg/kg). The effect of cilostazol on the P-glycoprotein (P-gp) as well as CYP2C9 and CYP3A4 activity was also evaluated. Cilostazol significantly inhibited CYP2C9 and CYP3A4 enzyme. However cilostazol did not significantly enhance the cellular accumulation of rhodamine-123 in MCF-7/ADR cells overexpressing P-gp.

Compared to the control group, cilostazol significantly altered the pharmacokinetic parameters of losartan and nifedipine. The areas under the plasma

concentration-time curve ($AUC_{0-\infty}$) and the peak concentration ($C_{max}$) of losartan and nifedipine were significantly ($P < 0.05$) increased in the presence of cilostazol, respectively. The total body clearance (CL/F) was significantly decreased ($P < 0.05$) by cilostazol, respectively. Consequently, the absolute bioavailability (AB) values of losartan and nifedipine in the presence of cilostazol (6 mg/kg) were significantly higher than that of the control group, respectively. In intravenous administration, the areas under the plasma concentration-time curve ($AUC_{0-\infty}$) of losartan and nifedipine were significantly ($P < 0.05$) increased in the presence of cilostazol, repectively. The metabolite-to-parent AUC ratio (MR) in the presence of cilostazol (6 mg/kg) significantly decreased compared to the control group.

The increased bioavailability of losartan and nifedipine in the presence of cilostazol might be due to an inhibition of CYP2C9- and CYP3A4-mediated metabolism in the small intestine and/or in the liver or to a reduction of total body clearance.

Key words: Nifedipine, losartan, CYP2C9, CYP3A4, P-gp, pharmacokinetics, bioavailability, rats

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