Short Communication

Effects of lovastatin on the pharmacokinetics of diltiazem and its main metabolite, desacetyldiltiazem, in rats: possible role of cytochrome P450 3A4 and P-glycoprotein inhibition by lovastatin

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Abstract

Objectives The purpose of this study was to examine the effects of lovastatin on cytochrome P450 (CYP) 3A4 and P-glycoprotein (P-gp) *in vitro* and then to determine the effects of lovastatin on the pharmacokinetics of diltiazem and its main metabolite, desacetyldiltiazem, in rats.

Methods The pharmacokinetic parameters of diltiazem and desacetyldiltiazem were determined after orally administering diltiazem (12 mg/kg) to rats in the presence and absence of lovastatin (0.3 and 1.0 mg/kg). The effect of lovastatin on P-gp as well as CYP3A4 activity was also evaluated.

Key findings Lovastatin inhibited CYP3A4 enzyme activity with a 50% inhibition concentration of 6.06 μM. In addition, lovastatin significantly enhanced the cellular accumulation of rhodamine-123 in MCF-7/ADR cells overexpressing P-gp. Compared with the control (given diltiazem alone), the presence of lovastatin significantly altered the pharmacokinetic parameters of diltiazem. The areas under the plasma concentration–time curve (AUC) and the peak concentration of diltiazem were significantly increased ($P < 0.05$, 1.0 mg/kg) in the presence of lovastatin. Consequently, the absolute bioavailability values of diltiazem in the presence of lovastatin (11.1% at 1.0 mg/kg) were significantly higher ($P < 0.05$) than that of the control group (7.6%). The metabolite–parent AUC ratio in the presence of lovastatin (1.0 mg/kg) was significantly ($P < 0.05$) decreased compared with the control group.

Conclusions It might be considered that lovastatin resulted in reducing the first-pass metabolism in the intestine and/or in the liver via inhibition of CYP3A4 and increasing the absorption of diltiazem in the intestine via inhibition of P-gp by lovastatin.

Keywords CYP3A4; desacetyldiltiazem; diltiazem; lovastatin; P-glycoprotein

Introduction

Diltiazem is a calcium-channel blocker widely used in the treatment of angina, supraventricular arrhythmias and hypertension.[1–3] Diltiazem undergoes complex and extensive phase I metabolism that includes desacetylation, *N*-demethylation and *O*-demethylation. The absolute bioavailability of diltiazem is approximately 40%, with a large intersubject variability.[3,4] In preclinical studies, the estimated hypotensive potency of desacetyldiltiazem appeared to be about half to equivalent compared with diltiazem, whereas the potencies of *N*-demethyldiltiazem and *N*-demethyldesacetyl-diltiazem were about one-third the potency of diltiazem.[5,6] Considering the potential contribution of active metabolites to the therapeutic outcome of diltiazem treatment, it may be important to monitor the levels of active metabolites as well as that of the parent drug in pharmacokinetic studies of diltiazem.

Cytochrome P450 (CYP) 3A4, a key enzyme in the metabolism of diltiazem, is mainly localized in the liver and also in the small intestine.[7–9] Thus, diltiazem could be metabolized in the small intestine as well as the liver.[10–12] Lee *et al*.[13] reported that the extraction ratios of diltiazem in the small intestine and liver after oral administration to rats were about 85 and 63%, respectively. This suggested that diltiazem was highly extracted in the small intestine

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as well as the liver. In addition to extensive metabolism, P-glycoprotein (P-gp) may also account for the low bioavailability of diltiazem and diltiazem could act as a substrate of both CYP3A4 and P-gp[14–16]. Since P-gp is colocalized 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[17–18].

Lovastatin, a 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase inhibitor, is widely used in preventing the progression of atherosclerosis by lowering plasma low density lipoprotein levels in patients with hypercholesterolaemia.[19,20] Lovastatin is mainly metabolized by CYP3A4 to a number of active metabolites.[21,22] CYP oxidation is the primary route of phase I metabolism for lovastatin in humans and dogs.[23] Wang et al.[24] reported that HMG-CoA reductase inhibitors (statins) are inhibitors of P-gp in the rodent system, but the effects of lovastatin on CYP3A4 and P-gp inhibition are somewhat ambiguous. We therefore attempted to re-evaluate CYP3A4 and P-gp activity using the rhodamine-123 retention assay in P-gp overexpressed MCF-7/ADR cells, and to further investigate the relationship between diltiazem, CYP3A4 or P-gp substrates and lovastatin.

Antihypertensive agents are commonly co-administered with cholesterol-lowering agents in the clinic. There are some reports on the effects of calcium-channel antagonists on the pharmacokinetics of HMG-CoA reductase inhibitors. Calcium-channel blockers increased plasma concentrations of some statins (lovastatin, pravastatin and simvastatin), possibly through the inhibition of CYP3A4 and P-gp.[25,26] There are fewer reports about the effects of HMG-CoA reductase inhibitors on the bioavailability or pharmacokinetics of calcium-channel antagonists in rats. Moreover, lovastatin and diltiazem could be prescribed for the prevention or treatment of cardiovascular diseases as a combination therapy.

The purpose of this study was to investigate the possible effects of lovastatin on the bioavailability or pharmacokinetics of diltiazem and its active metabolite, desacetyldiltiazem, after oral administration of diltiazem with lovastatin in rats.

Materials and Methods

Materials

Diltiazem hydrochloride, desacetyldiltiazem, imipramine hydrochloride and lovastatin were purchased from Sigma-Aldrich Co. (St Louis, MO, US). Acetonitrile, methanol and tert-butylmethylether were products from Merck Co. (Darmstadt, Germany). Other chemicals were of reagent or high-performance liquid chromatography (HPLC) grade. Dulbecco's modified Eagle's medium was from Hyclone Laboratories (Logan, UT, US), Hank's balanced salt solution was from Invitrogen (Grand Island, NY, US), fetal bovine serum was from PAA Laboratories (Etobiroke, Ontario, Canada), rhodamine was from Calbiochem, (San Diego, CA, US) and the CYP inhibition assay kit was from GENTEST (Woburn, MA, US).

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 $22 \pm 2°C$, 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 anaesthesia.

Intravenous and oral administration of diltiazem

The rats were divided into four groups ($n = 6$ each): oral groups (12 mg/kg of diltiazem dissolved in water; 3.0 ml/kg) without (control) or with 0.3 and 1.0 mg/kg of lovastatin (mixed in distilled water; total oral volume of 3.0 ml/kg), and an intravenous group (4 mg/kg of diltiazem; the same solution used: 0.9% NaCl-injectable solution; total injection volume of 1.5 ml/kg). Lovastatin was administered 30 min prior to oral administration of diltiazem. The drugs were given by gavage using an injector equipped with a long blunt needle (feeding tube) to rats. A blood sample (0.45-ml) was collected into heparinized tubes via the femoral artery at 0 (control), 0.017 (at the end of infusion), 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h for the intravenous study, and 0, 0.1, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h for the oral study. Whole blood (approx. 1 ml) collected from untreated rats was infused via the femoral artery at 0.25, 1, 3 and 8 h, respectively, to replace blood loss due to blood sampling. The blood samples were centrifuged (16 810g, 5 min), and a 200-μl aliquot of plasma samples was stored at −40°C until the HPLC analysis.

HPLC assay

The plasma concentrations of diltiazem were determined using a HPLC assay by a modification of the method reported by Goebel and Kolle.[27] Briefly, 50 μl of imipramine (2 μg/ml), as the internal standard, and 1.2 ml of tert-butylmethylether were added to 0.2 ml of the plasma sample. It was then mixed for 2 min using a vortex mixer and centrifuged at 16 810g for 10 min. The organic layer (1 ml) was transferred to another test tube, to which 0.2 ml of 0.01 N hydrochloride was added and mixed for 2 min. The water layer (50 μl) was injected into the HPLC system. The detector wavelength was set to 237 nm and the column, a μ-bondapack C18 (3.9 × 300 mm, 10 μm; Waters Co., Milford, MA, US), was used at room temperature. A mixture of methanol/acetonitrile/0.04 M ammonium bromide/triethylamine (24 : 31 : 45 : 0.1, v/v/v/v, pH 7.4, adjusted with acetic acid) was used as the mobile phase at a flow rate of 1.5 ml/min. The retention times were: internal standard at 11.1 min, diltiazem at 9.6 min and desacetyldiltiazem at 7.6 min. The detection limits of diltiazem and desacetyldiltiazem in rat plasma were all 5 ng/ml. The coefficients of variation for diltiazem and desacetyldiltiazem were all below 5.0%. Recovery (%) assessed from the replicate analysis ($n = 5$) for 5 days by

adding 20 μg/ml and 100 μg/ml of diltiazem to the rat plasma was shown to be 99.8 ± 4.8 and 98.1 ± 4.6, respectively.

CYP inhibition assay

The inhibition assays on human CYP3A4 enzyme activity were performed in a multiwell plate using a CYP inhibition assay kit (GENTEST) as described previously.[28] Briefly, human CYP enzymes were obtained from baculovirus infected insect cells. CYP substrates (7-BFC for CYP3A4) were incubated with or without test compounds in the enzyme/substrate containing buffer consisting of 1 pmol of P450 enzyme and NADPH generating system (1.3 mM NADP, 3.54 mM 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 a stop solution after 45 min incubation. Metabolite concentrations were measured by spectrofluorometer (Molecular Device, Sunnyvale, CA, US) set at an excitation wavelength of 409 nm and an emission wavelength of 530 nm. A positive control (1 μM ketoconazole for CYP3A4) was run on the same plate producing 99% inhibition. All experiments were done in duplicate and results are expressed as the percent of inhibition.

Rhodamine-123 retention assay

The P-gp overexpressed multidrug resistant human breast carcinoma cell line (MCF-7/ADR cells) was seeded in 24-well plates. At 80% confluence, the cells were incubated in fetal bovine serum free Dulbecco's modified Eagle's medium for 18 h. The culture medium was changed with Hank's 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 lovastatin (1, 3 or 10 μM) or verapamil (100 μM) for 90 min, the medium was completely aspirated. 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 percentage ratio to control.

Pharmacokinetic analysis

The pharmacokinetic parameters were calculated assuming a two-compartment open model by a nonlinear least-square regression analysis using a MULTI program (Oklahoma, Japan).[29] The parameter values were obtained by fitting to the pharmacokinetic model using the simplex algorithm. The area under the plasma concentration–time curve (AUC) was calculated by a trapezoidal rule. The peak concentration (C_{max}) of diltiazem in plasma and time to reach C_{max} were obtained by visual inspection of the data from the concentration–time curve. Total plasma clearance (CL) was calculated by dose/AUC. The apparent volume of distribution at steady state (Vd_{ss}) was estimated by the product of mean residence time$_{i.v.}$ and total body clearance (CL_t) after intravenous dosing. The absolute bioavailability was calculated by $AUC_{oral}/AUC_{i.v.}$ × $dose_{i.v.}/dose_{oral}$, and the relative bioavailability of diltiazem

was calculated by $AUC_{diltiazem\ with\ lovastatin}/AUC_{control}$. The metabolite–parent AUC ratio (MR) was calculated by $AUC_{desacetyldiltiazem}/AUC_{diltiazem}$.

Statistical analysis

All the means are presented with their standard deviation. The pharmacokinetic parameters were compared with a one-way analysis of variance, followed by *a posteriori* testing with the use of the Dunnett correction. A value of $P < 0.05$ was considered statistically significant.

Results

Inhibition of CYP3A4

The inhibitory effect of lovastatin on CYP3A4 activity is shown in Figure 1. The IC50 value of lovastatin on CYP3A4 activity was 6.06 μM. Lovastatin inhibited CYP3A4 enzyme activity in a concentration-dependent manner.

Rhodamine-123 retention assay

The cell-based P-gp activity test using rhodamine-123 showed that lovastatin (10 μM, $P < 0.01$) significantly inhibited P-gp activity (Figure 2). This result suggests that lovastatin significantly inhibits P-gp activity.

Effect of lovastatin on the pharmacokinetics of oral diltiazem

The mean plasma concentration–time profiles of diltiazem in the presence and absence of lovastatin (0.3 and 1.0 mg/kg) are shown in Figure 3. The pharmacokinetic parameters of diltiazem are summarized in Table 1. Lovastatin (1.0 mg/kg) significantly ($P < 0.05$) increased the area under the plasma concentration–time curve from time zero to infinity ($AUC_{0-\infty}$) of diltiazem by 48.5% and peak concentration (C_{max}) of diltiazem by 41.8%. Accordingly, the presence of lovastatin (1.0 mg/kg) significantly ($P < 0.05$) increased the absolute bioavailability of diltiazem in rats. Lovastatin increased the relative bioavailability of diltiazem by 1.21- to 1.48-fold. Lovastatin did not significantly change the Vd_{ss} and CL_t of diltiazem in rats.

Effect of lovastatin on the pharmacokinetics of desacetyldiltiazem

The plasma concentration–time profiles of desacetyldiltiazem are shown in Figure 4. The pharmacokinetic parameters of desacetyldiltiazem are summarized in Table 2. The MRs were significantly ($P < 0.05$) different between cases with and without lovastatin, suggesting that the formation of deacetyldiltiazem was considerably altered by lovastatin. Lovastatin did not significantly change the Vd_{ss} and CL_t of deacetyldiltiazem in rats.

Discussion

Based on the broad overlap in the substrate specificities as well as their colocalization in the small intestine, the primary site of absorption for orally administered drugs, CYP3A4 and P-gp have been recognized as a concerted barrier to drug absorption.[30,31] The prescription of more than one drug as a
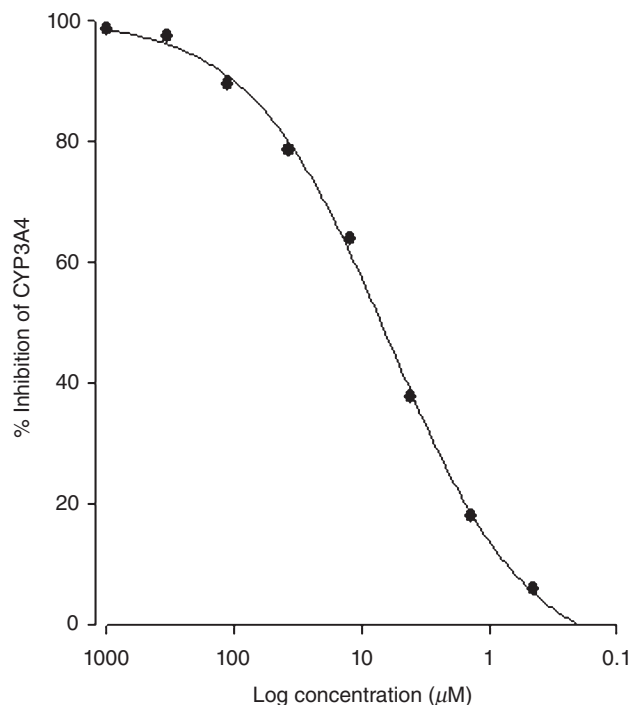
Figure 1 Inhibitory effect of lovastatin on cytochrome P450 3A4 activity. All experiments were done in duplicate and results are expressed as the percent of inhibition.
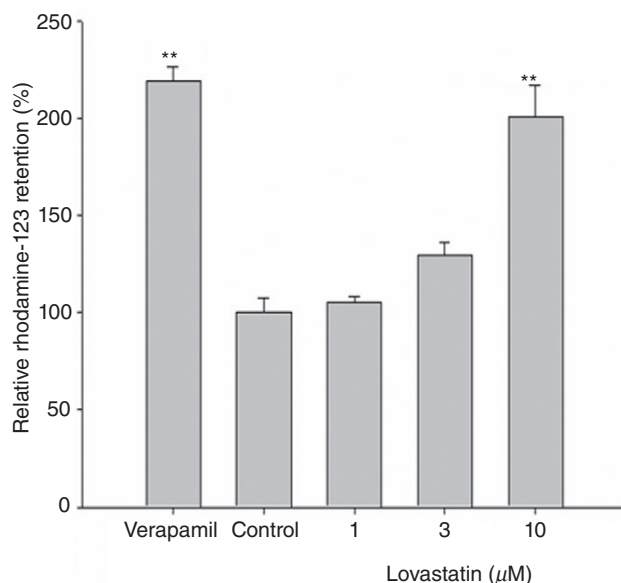

Figure 2 Rhodamine-123 retention in lovastatin-treated MCF-7/ADR cells. Cells were incubated with 20 μM rhodamine-123 for 90 min. Data represent means ± SEM of four separate samples. **$P < 0.01$, significantly different compared with control MCF-7 cells. Verapamil (100 μM) was used as a positive control.

Figure 3 Mean plasma concentration–time profiles of diltiazem. Rats were administered intravenous (4 mg/kg) or oral (12 mg/kg) diltiazem in the presence and absence of lovastatin (mean ± SD, $n = 6$). ●, Control (diltiazem 12 mg/kg, p.o.); ○, presence of 0.3 mg/kg lovastatin; ▼, presence of 1.0 mg/kg lovastatin; ■, injection of diltiazem (4 mg/kg, i.v.).

combination therapy is increasingly common in current medical practice. Cholesterol-lowering agents such as HMG-CoA reductase inhibitors could be co-administered with calcium-channel blockers in the treatment of hypertension.[32]
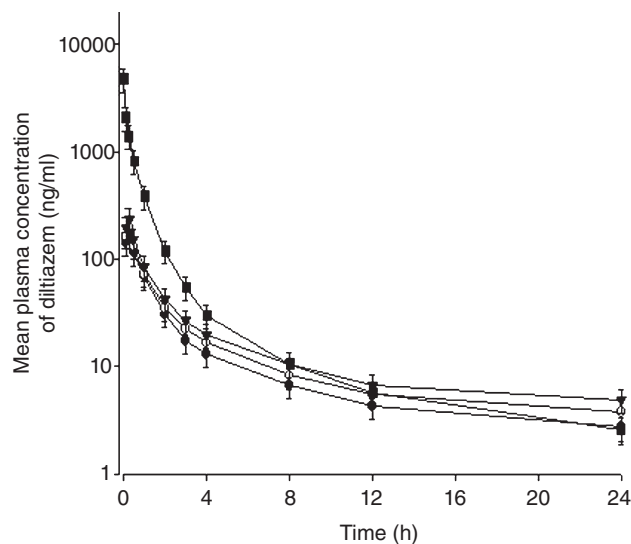
Considering that the drugs used in combination therapy often share the same metabolic pathways or cellular transport pathways, there exists a high potential for pharmacokinetic as well as pharmacodynamic drug interactions between calcium-channel antagonists and HMG-CoA reductase inhibitors. Indeed, some studies have reported that calcium-channel

Table 1 Mean pharmacokinetic parameters of diltiazem

Parameters	Diltiazem (control)	Diltiazem + lovastatin		Diltiazem (i.v.)
		0.3 mg/kg	1.0 mg/kg	
$AUC_{0-\infty}$ (ng h/ml)	342 ± 69	413 ± 89	508 ± 107*	1512 ± 399
C_{max} (ng/ml)	165 ± 35	197 ± 44	234 ± 53*	
T_{max} (h)	0.33 ± 0.13	0.29 ± 0.10	0.29 ± 0.10	
V_{dss} (ml/kg)	52.2 ± 14.9	47.3 ± 13.4	42.4 ± 12.2	
CL_t (ml/min per kg)	45.2 ± 13.8	41.9 ± 10.5	38.0 ± 9.9	4.5 ± 1.3
AB (%)	7.6 ± 1.7	9.1 ± 2.0	11.1 ± 2.6*	
RB (%)	100	121	148	

Rats were administered intravenous (4 mg/kg) or oral (12 mg/kg) diltiazem in the presence and absence of lovastatin (mean $\pm$ SD, $n = 6$). AB, absolute bioavailability; AUC, area under the plasma concentration–time curve; CL_t, total body clearance; C_{max}, peak plasma concentration; RB, relative bioavailability; T_{max}, time to peak concentration; Vd_{ss}, volume of distribution at the steady state. *$P < 0.05$, significantly different compared with the control (given diltiazem alone).
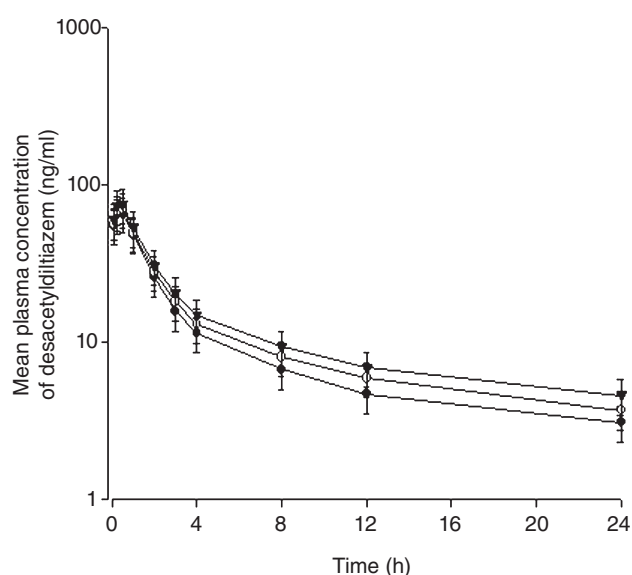

Figure 4 Mean plasma concentration–time profiles of desacetyldiltiazem. Rats were administered oral diltiazem (12 mg/kg) in the presence and absence of lovastatin (mean $\pm$ SD, $n = 6$). ●, Control (diltiazem 12 mg/kg, p.o.); ○, presence of 0.3 mg/kg lovastatin; ▼, presence of 1.0 mg/kg lovastatin.

Table 2 Mean pharmacokinetic parameters of desacetyldiltiazem

Parameters	Diltiazem (control)	Diltiazem + lovastatin	
		0.3 mg/kg	1.0 mg/kg
$AUC_{0-\infty}$ (ng h/ml)	284 ± 84	320 ± 91	370 ± 99
C_{max} (ng/ml)	66.0 ± 12.4	70.0 ± 18.0	75.0 ± 15.0
T_{max} (h)	0.46 ± 0.10	0.46 ± 0.10	0.46 ± 0.10
V_{dss} (ml/kg)	133 ± 36	129 ± 34	125 ± 31
CL_t (ml/min per kg)	57.4 ± 14.6	52.9 ± 12.7	48.2 ± 12.1
MR	0.84 ± 0.12	0.77 ± 0.11	0.72 ± 0.09*

Rats were administered oral diltiazem (12 mg/kg) in the presence and absence of lovastatin (mean $\pm$ SD, $n = 6$). AUC, area under the plasma concentration–time curve; CL_t, total body clearance; C_{max}, peak plasma concentration; MR, metabolite–parent AUC ratio; T_{max}, time to peak concentration; Vd_{ss}, volume of distribution at the steady state. *$P < 0.05$, significantly different compared with the control (given diltiazem alone).

blockers increased the plasma concentrations of lovastatin or simvastatin.[25,26] Similarly, as the dual substrates of CYP3A4 and P-gp, diltiazem and lovastatin may undergo the same metabolic pathways and/or cellular transport pathways after co-administration. Therefore, lovastatin could affect the bioavailability or pharmacokinetics of diltiazem in rats.

Lovastatin exhibited an inhibitory effect against CYP3A4-mediated metabolism, with an IC50 of 6.06 μM. Furthermore, the cell-based assay using rhodamine-123 indicated that lovastatin (10 μM) significantly ($P < 0.01$) inhibited P-gp-mediated drug efflux (Figure 2). These results suggest that lovastatin might be effective to improve the bioavailability of diltiazem, a dual substrate of CYP3A4 and P-gp. This result is consistent with a report by Wang *et al.* showing that lovastatin inhibited the activity of P-gp.[24] Therefore, the pharmacokinetic characteristics of diltiazem were evaluated in the absence and the presence of lovastatin in rats. CYP3A9

expressed in rats corresponds to the ortholog of CYP3A4 in humans.[33] Human CYP2C9 and 3A4 and rat CYP2C11 and 3A1 have 77 and 73% protein homology, respectively.[34] Rats were selected as the 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.[35]

The presence of lovastatin significantly enhanced the $AUC_{0-\infty}$ and C_{max} of diltiazem in rats. Subsequently, the relative bioavailability of diltiazem was increased by 122 to 149% in the presence of lovastatin (0.3 and 1.0 mg/kg). These results were consistent with reports by Zhang *et al.*[36] showing that diltiazem is a substrate of P-gp as well as CYP3A4, and that lovastatin is an effective inhibitor of P-gp and CYP3A4 transport. These results were similar to the report by Hong *et al.*[37] demonstrating that oral atorvastatin significantly increased the bioavailability of diltiazem by inhibition of CYP3A4 and P-gp in rats. This suggested that the extraction ratio of diltiazem across the rat intestinal tissue was significantly reduced by P-gp and/or CYP3A4; these results were similar to the reports by Cho *et al.* and Hong *et al.*[38,39] showing that hesperidin or resveratrol significantly increased the $AUC_{0-\infty}$ and C_{max} of diltiazem by inhibition of CYP3A4 and P-gp in rats.

The MR in the presence of lovastatin (1.0 mg/kg) was significantly ($P < 0.05$) decreased compared with that of the control group. This result was similar to reports by Hong et al.[37] in that atorvastatin significantly decreased the MR in rats, but was not similar to the reports by Cho et al. and Hong et al.[38,39] in that hesperidin or resveratrol significantly increased the $AUC_{0-\infty}$ of desacetyldiltiazem but they could not decrease the MR in rats. Taken together, the pharmacokinetic parameters of diltiazem were significantly altered by the presence of lovastatin in rats. The present study raises awareness of potential drug interactions by concomitant use of lovastatin with diltiazem, a finding that should be further evaluated in clinical studies.

The increased bioavailability of diltiazem in the presence of lovastatin might be due to an inhibition of the P-gp-mediated efflux pump in intestine and inhibition of CYP3A4-mediated metabolism in intestine and/or liver by lovastatin.

Conclusions

The presence of lovastatin enhanced the systemic bioavailability of diltiazem. Therefore, concomitant use of diltiazem with lovastatin may require close monitoring for potential drug interactions. Furthermore, human trials are warranted to address the potential drugs interaction in clinical practice.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

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